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Shao-Yao Ying *Editor*

MicroRNA Protocols

Second Edition

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MicroRNA Protocols

Second Edition

Edited by

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Humana Press

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Preface

Since the first microRNA (miRNA), the product of *lin-4*, a heterochronic gene of *Caenorhabditis elegans*, was discovered by Ambros and his coworkers, the role of miRNAs in biomedical research has been examined as "fine-tuning modulators of gene expression," and research articles on miRNAs have been exploding exponentially. Extensive studies have been conducted on the identification, biogenesis, and processing of these small molecules as well as research on the exact mechanism by which miRNAs bring about translational silencing of their targets, using bioinformatic, genetic, and biochemical approaches. In addition, numerous publications point to an important role of miRNAs in development, reprogramming, epigenetics, pathogenesis of cancer, oncogenes and tumor suppressors, biomarkers of various disease onset, and regulation of adipogenesis and obesity. On the other hand, there are still many questions related to miRNA properties and miRNA-mediated networks in the biomedical field which need to be examined.

The specific chapters of this edition are related to the analysis of miRNA, targets and expression profiling, various methods to determine its regulation of gene expression, the preparation and isolation of miRNAs in specific tissues, its detection in the saliva, and potential application in cosmetics, wound healing, and prostate cancer. The first several chapters deal with the length variety generated by recombinant human Dicer, miRNA deep sequencing data, and the retroelement-miRNA entangling, as well as the setting up and identification of intronic miRNAs. Many chapters provide target validation, expression profiling, and regulation of gene expression by miRNAs; the emphasis here is to provide various methods that are readily reproducible for the analysis of the functional significance of miRNAs. Additional chapters describe techniques for studying miRNAs in synaptoneurosomes, culture adipocytes and adipose tissues, and saliva. Recently, miRNAs are involved in the reprogramming of somatic and/or cancer cells; several chapters outline approaches to study neuronal differentiation, embryonic stem cells, and generation of tumor-free induced pluripotent stem (iPS) cells. The use of miRNAs in cosmetics, wound healing, and prostate cancer may have immense practical benefit in developing these small RNA molecules as drug agents.

miRNA has opened a new avenue for our understanding of gene expression and will become one of the most widely applied techniques in biomedical research and other currently underrepresented disciplines. It is our hope that this volume will stimulate the reader to explore diverse ways to understand the mechanisms in which miRNAs facilitate the molecular aspects of not only the biomedical research but also other research fields.

Los Angeles, CA, USA *Shao-Yao Ying*

Contents

Preface . . . . . *v*
Contributors . . . . . *ix*

1 The MicroRNA. 1
Shao-Yao Ying, Donald C. Chang, and Shi-Lung Lin

2 Analysis of MicroRNA Length Variety Generated by Recombinant Human Dicer 21
Julia Starega-Roslan and Wlodzimierz J. Krzyzosiak

3 A User-Friendly Computational Workflow for the Analysis of MicroRNA Deep Sequencing Data 35
Anna Majer, Kyle A. Caligiuri, and Stephanie A. Booth

4 The RNA Gene Information: Retroelement-MicroRNA Entangling as the RNA Quantum Code 47
Yoichi Robertus Fujii

5 Setting Up an Intronic miRNA Database 69
Ludwig Christian Hinske, Jens Heyn, Pedro A.F. Galante, Lucila Ohno-Machado, and Simone Kreth

6 Identify Intronic MicroRNA with Bioinformatics 77
Chih-Hao Kuo, Mark D. Goldberg, Shi-Lung Lin, Shao-Yao Ying, and Jiang F. Zhong

7 Experimental miRNA Target Validation 83
Jens Heyn, Ludwig Christian Hinske, Carola Ledderose, Elisabeth Limbeck, and Simone Kreth

8 Enrichment Analysis of miRNA Targets. 91
Jianzhen Xu and Chi-Wai Wong

9 MicroRNA Expression Profiling During Neural Differentiation of Mouse Embryonic Carcinoma P19 Cells 105
Botao Zhao, Bing Huang, Wei Li, and Youxin Jin

10 Evaluating the MicroRNA Targeting Sites by Luciferase Reporter Gene Assay. 117
Yi Jin, Zujian Chen, Xiqiang Liu, and Xiaofeng Zhou

11 Expression Pattern Analysis of MicroRNAs in *Caenorhabditis elegans*. 129
Meltem Isik and Eugene Berezikov

12 Use of Viral Systems to Study miRNA-Mediated Regulation of Gene Expression in Human Cells 143
Eleonora Forte and Micah A. Luftig

13 Cloning, Expression, and Functional Analysis of Genomic miRNA Using Retroviral System in Cancer Cells 157
Abdol Hossein Rezaeian, Yuan Gao, and Hui-Kuan Lin

14 Preparing Synaptoneurosomes from Adult Mouse Forebrain 173
Giovanni Lugli and Neil R. Smalheiser

15 Isolation of Total RNA and Detection Procedures for miRNA Present in Bovine-Cultured Adipocytes and Adipose Tissues 181
Scott L. Pratt, T. Ashley Burns, Marcy D. Owens, and Susan K. Duckett

16 miRNA and shRNA Expression Vectors Based on mRNA and miRNA Processing 195
Ping Wu, Melissa A. Wilmarth, Feng Zhang, and Guangwei Du

17 Gene Silencing In Vitro and In Vivo Using Intronic MicroRNAs 209
Shi-Lung Lin and Shao-Yao Ying

18 Vector-Free Methods for Manipulating miRNA Activity In Vitro and In Vivo 231
Angeliki Pantazi and Athanasios Zovoilis

19 MicroRNA Expression Profiling of Human-Induced Pluripotent and Embryonic Stem Cells 247
Amit Sharma and Joseph C. Wu

20 MicroRNA Expression During Neuronal Differentiation of Human Teratocarcinoma NTera2D1 and Mouse Embryonic Carcinoma P19 Cells. . . . 257
Hirohiko Hohjoh

21 Isolation and Identification of Gene-Specific MicroRNAs 271
Shi-Lung Lin, Donald C. Chang, and Shao-Yao Ying

22 Transgene-Like Animal Models Using Intronic MicroRNAs 279
Shi-Lung Lin, Shin-Ju E. Chang, and Shao-Yao Ying

23 Mechanism and Method for Generating Tumor-Free iPS Cells Using Intronic MicroRNA miR-302 Induction 295
Shi-Lung Lin and Shao-Yao Ying

24 Salivary MicroRNAs and Oral Cancer Detection 313
Janice M. Yoshizawa and David T.W. Wong

25 Application of Intronic MicroRNA Agents in Cosmetics 325
Jack S.K. Chen and David T.S. Wu

26 MicroRNAs in Skin and Wound Healing 343
Jaideep Banerjee and Chandan K. Sen

27 MiRNA Targets of Prostate Cancer 357
Jia Han Deng, Qinggao Deng, Chih-Hao Kuo, Sean W. Delaney, and Shao-Yao Ying

Index *371*

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Chapter 1

The MicroRNA

Shao-Yao Ying, Donald C. Chang, and Shi-Lung Lin

Abstract

MicroRNAs (miRNAs), widely distributed, small regulatory RNA genes, target both messenger RNA (mRNA) degradation and suppression of protein translation based on sequence complementarity between the miRNA and its targeted mRNA. Different names have been used to describe various types of miRNA. During evolution, RNA retroviruses or transgenes invaded the eukaryotic genome and inserted itself in the noncoding regions of DNA, conceivably acting as transposon-like jumping genes, providing defense from viral invasion and fine-tuning of gene expression as a secondary level of gene modulation in eukaryotes. When a transposon is inserted in the intron, it becomes an intronic miRNA, taking advantage of the protein synthesis machinery, i.e., mRNA transcription and splicing, as a means for processing and maturation. Recently, miRNAs have been found to play an important, but not life-threatening, role in embryonic development. They might play a pivotal role in diverse biological systems in various organisms, facilitating a quick response and accurate plotting of body physiology and structures. Based on these unique properties, manufactured intronic miRNAs have been developed for in vitro evaluation of gene function, in vivo gene therapy, and generation of transgenic animal models. The biogenesis and identification of miRNAs, potential applications, and future directions for research are presented in this chapter, hopefully providing a guideline for further miRNA and gene function studies.

Key words: Small RNA, Noncoding RNAs, Small interfering RNA, MicroRNA, Intronic miRNA, Transposons, Biogenesis, Mechanism, Identification, Targeting, Fine-tuning, Gene function, Gene therapy, Antiviral vaccine, Drug development, Future directions

1. Introduction

The microRNA (miRNA) is a form of small, single-stranded RNA, 18- to 25-nucleotides (nt) long. It is transcribed from DNA, and instead of being translated into protein, it regulates the functions of other genes in protein synthesis. Therefore, miRNAs are genes that modulate other protein-coding genes. Even after considering the thousands of new putative genes identified from sequencing of

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the human genome, as well as the genes encoding transfer RNAs (tRNAs), ribosomal RNA (rRNAs), and small nucleolar RNA (snoRNAs), nearly 95% of the genome is noncoding DNA, a percentage that varies from species to species. Changes in these sequences are frequently associated with clinical and circumstantial malfunction. Some of these noncoding sequences are responsible for RNA-mediated gene silencing through an RNA interference (RNAi)-like mechanism. One potentially important class of genes corresponding to RNAs that lack significant open reading frames and seem to encode RNA as their final product is the miRNAs. These miRNAs can play critical roles in development, protein secretion, and gene regulation. Some of them are naturally occurring antisense RNAs, whereas others have structures that are more complex. To understand the diseases caused by dysregulation of these miRNAs, a tissue-specific expression system is needed to recreate the function and mechanism of individual miRNAs in vitro and in vivo.

This chapter provides a simple and general view of the concept that RNAs can directly regulate gene functions, with particular attention to a step-by-step approach to the study of miRNA. Hopefully, this information will help researchers who are new to this field to overcome problems encountered in the functional analysis of miRNA.

1.1. Small RNAs or Noncoding RNAs

A noncoding RNA (ncRNA) is any RNA molecule that functions without being translated into a protein. An ncRNA is also called a small RNA (sRNA). Less frequently, it is called nonmessenger RNA, small nonmessenger RNA, tiny ncRNA, small modulatory RNA, or small regulatory RNA. Broadly speaking, the DNA sequence from which an ncRNA is transcribed can be considered an RNA gene. In this chapter, we confine our discussion to sRNAs; that is, transcripts of fewer than 300 nt that participate directly in RNA processing and degradation, but indirectly in protein synthesis and gene regulation. Because type II RNA polymerases (Pol-II) are inefficient in generating sRNAs of this size, the sRNAs are either directly transcribed by type III RNA polymerases or indirectly processed from a large transcript of Pol-II.

1.1.1. Transfer RNA

The most prominent example of ncRNA is tRNA, which is involved in the process of translation and is the first type of sRNA that was identified and characterized (1). tRNA is RNA that transfers a specific amino acid to a growing polypeptide chain at the ribosomal site of protein synthesis during translation. The tRNA is an sRNA, 74- to 93-nt long, consisting of amino acid attachment and codon recognition sites, allowing translation of specific amino acids into a polypeptide. The secondary and tertiary structure of

tRNAs are cloverleafs with four to five domains and an L-shaped three-dimensional structure, respectively.

1.1.2. Nucleolar RNA

Another example of ncRNA is rRNA. rRNA is the primary constituent of ribosomes.

rRNA is transcribed from DNA and, in eukaryotes, it is processed in the nucleolus before being transported through the nuclear membrane. rRNA may produce snoRNAs, the second type of sRNA. Many of the newly discovered snoRNAs are synthesized in an intron-processing pathway. Several snoRNAs and sno-ribonucleoproteins (RNPs) are known to be needed for processing of rRNA, but precise functions remain to be defined. In principle, snoRNAs could have several roles in ribosome synthesis including: folding of pre-rRNA, formation of rRNP substrates, catalyzing RNA cleavages, base modification, assembly of pre-ribosomal subunits, and export of product rRNP particles from the nucleus to the cytoplasm.

The snoRNA acts as a guide to direct pseudouridylation and 2′-*O*-ribose methylation of rRNA in the nucleolus. Consequently, the snoRNA guides the snoRNP complex to the modification site of the target rRNA via sequence hybridization. The proteins then catalyze the modification of bases in the rRNA. Therefore, this type of RNA is also called guided RNA.

The snoRNA is also associated with proteins forming part of the mammalian telomerase, as well as with proteins involved in imprinting on the paternal chromosomes. It is encoded in introns of genes transcribed by Pol-II, even when some of the host genes do not code for proteins. As a result, the intron, but not the exon, of these genes is evolutionarily conserved in vertebrates. In this way, some of the introns of the genes employed in plants or invertebrates are still functioning in vertebrates.

The structure of snoRNAs consists of conserved sequences base-paired to their target

RNAs. Nearly, all vertebrate guide snoRNAs originate from introns of either protein-coding or ncRNAs transcribed by Pol-II, whereas only a few yeast guide snoRNAs derive from introns, suggesting that introns accumulated during evolution reflect the conservation of transgenes incorporated into the introns, as mentioned above (2–4). These introns are processed through pathways involving endonucleolytic cleavage by ribonuclease (RNase) III-related enzymes, exonucleolytic trimming, and possibly RNA-mediated cleavage, which occur in large complexes called exosomes (5, 6).

1.1.3. Nuclear RNA

Small nuclear RNA (snRNA) is a class of sRNA molecules that are found within the nuclei of eukaryotic cells. They are involved in a variety of important processes, such as RNA splicing (removal

of introns from heteronuclear RNA) and maintaining the telomeres. snRNA are always associated with specific proteins, and the complexes are referred to as snRNP. Some examples of snRNA are U2 snRNAs, pre-5S rRNAs, and U6 snRNAs. U2 snRNAs in embryonic stem cells and pre-5S rRNAs in *Xenopus* oocytes facilitate cell survival after ultraviolet irradiation by binding to the conserved protein, R0. Eukaryotic U6 snRNAs are the five types of spliceosomal RNA involved in messenger RNA (mRNA) splicing (U1–U6). These snRNAs have a secondary structure consisting of a stem-loop, an internal loop, a stem-closing internal loop, and the conserved protein-binding site (7).

1.1.4. Phage and Viral RNA

Another form of sRNAs is 30 ribonucleotides in length and functions as a priming initiator for bacteriophage F1 DNA replication (8, 9). This function is solely to initiate a given site on the phage DNA, suggesting a primitive defense against foreign pathogen invasion. The phage T4-derived intron is involved in an RNA–RNA interaction in the inhibition of protein synthesis (10).

1.1.5. Small Interfering RNA

The small interfering RNAs (siRNAs) are small double-stranded RNA (dsRNA) molecules, 20- to 25-nt in length, that interfere with the expression of genes via a part ofRNAi involving the enzyme Dicer. The siRNA story began with the observation of pigment expression in the *Petunia* plant. van der Krol et al. (11) tried to intensify flower pigmentation by introducing additional genes, but unexpectedly observed reduced floral pigmentation in some plants, suggesting that gene silencing may be involved in naturally occurring regulation of gene function. This introduction of multiple transgenic copies of a gene into the *Petunia* plant resulted in gene silencing of not only the transgenic, but also the endogenous gene copy, as has been observed by others (12). This suggests that cosuppression of homologous genes (the transfer gene and the endogenous gene) and possibly methylation are involved (12, 13). This phenomenon is termed RNAi. Note that the transgene introduced to the *Petunia* plant is a dsRNA, which is perfectly complementary to the target gene.

When dsRNA was injected into *Caenorhabditis elegans*, Fire and his coworkers noticed gene silencing and RNAi (14). RNAi is a mechanism by which small regulatory RNAs possessing a sequence complementary to that of a portion of a target gene interfere with the expression of that gene. It is thought that the dsRNA, once it enters the cells, is cut up by an RNase-III familial endonuclease, known as Dicer. Dicer consists of an amino-terminal helicase domain, a PAZ domain, two RNase III motifs, and a dsRNA-binding motif. Therefore, Dicer binds to the dsRNA and excises the dsRNA into siRNAs. These siRNAs locate other single-stranded RNA molecules that are completely complementary to either

strand of the siRNA duplex. Then, the RNA-degrading enzymes (RNases) destroy the RNAs complementary to the siRNAs. This phenomenon is also called post-transcriptional gene silencing (PTGS) or transgene quelling. In other words, gene silencing can be activated by introducing transgenes, RNA viruses, or dsRNA sequences that are completely complementary to the targeted gene transcripts.

In mammals, dsRNAs longer than 30 nt will activate an antiviral response, which will lead to the nonspecific degradation of RNA transcripts, the production of interferon, and the overall shutdown of host cell protein synthesis (15). As a result, long dsRNA will not produce gene-specific RNAi activity in mammalian cells (16).

Several terms have been used to describe the same or similar phenomenon in different biological systems of different species, including siRNAs (17), small temporal Ranks (18), heterochromatic siRNAs (19), and small modulatory dsRNAs (20).

1.1.6. MicroRNA

miRNAs are small, single-stranded RNA genes possessing the reverse complement of the mRNA transcript of another protein-coding gene. These miRNAs can inhibit the expression of the target protein-coding gene. miRNA was first observed in *C. elegans* as RNA molecules of 18- to 23-nt that are complementary to the 3′ untranslated regions (UTR) of the target transcripts, including the *lin-4* (21) and *let-7* (22) genes. As a result, the development of the worm was regulated by these RNA genes. Subsequently, miRNAs were found to occur in diverse organisms, ranging from worms, to flies, to humans (23), suggesting that these molecules represent a gene family that has evolved from an ancient ancestral sRNA gene.

The miRNA is thought to be transcribed from DNA that is not translated, but regulates the expression of other genes. Primary transcripts of the miRNA genes (pri-miRNAs) are long RNA transcripts consisting of at least a hairpin-like miRNA precursor. Pri-miRNAs are processed in the nucleus to precursor (pre)-miRNAs by the RNase Drosha, with the help of microprocessor (24) and exported from the nucleus by Exportin-5 (25). The 60- to 90-nt miRNA precursors form the stem and loop structures, and the cytoplasmic RNase III enzyme, Dicer, excises the miRNA from the pre-miRNA hairpin stem region. miRNAs and siRNAs seem to be closely related, especially taking the dsRNA and hairpin structures into account. The siRNA can be considered a duplex form of miRNA in which the RNA molecule contains both miRNA and its reverse complement. Therefore, one can consider siRNAs a type of miRNA precursor.

miRNAs suppress gene expression based on their complementarity to a part of one or more mRNAs, usually at a site in the

3′-UTR. The annealing of the miRNA to the target mRNA inhibits its protein translation. In some cases, the formation of dsRNA through the binding of miRNA triggers the degradation of the mRNA transcript through a process similar to RNAi, although, in other cases, it is thought that the miRNA complex blocks the protein translation machinery or otherwise prevents protein translation without causing the mRNA to be degraded.

Because most of the miRNA suppresses gene function based on partial complementarity, conceivably, one miRNA may target more than one mRNA, and many miRNAs may act on one mRNA, coordinately modulating the intensity of gene expression in various tissues and cells. Therefore, miRNAs may have a broad function in fine-tuning the protein-coding genes. Indeed, the discovery of miRNAs has revolutionized our understanding of gene regulation in the postgenome era.

1.1.7. Intronic miRNA

Some small regulatory RNAs are produced from intronic RNA fragments. For example, snoRNAs are produced from intronic segments from genes encoding ribosomal proteins and nucleolar proteins. In addition, some sRNAs are produced from genes in which exons no longer have the capacity to encode proteins. This type of intron processing involves RNase III-related enzymes, exonucleolytical trimming, and, possibly, RNA-mediated cleavage. Therefore, intronic miRNA is a new class of miRNA derived from the processing of introns of a protein-coding gene.

The major difference between the intronic miRNAs and the previously described intergenic miRNAs, such as *lin-4* and *let-7*, is the requirement of Pol-II and spliceosomal components for the biogenesis of intronic miRNAs (26). Both intronic and intergenic-miRNAs may share the same assembly process, namely the RNA-induced silencing complex (RISC), the effector of RNAi-related gene silencing. Although siRNA-associated RISC assembly has been used to predict miRISC assembly, the link between final miRNA maturation and RISC assembly remains to be determined. The characteristics of Dicer and RISC in siRNA vs. miRNA mechanisms are distinctly different (27, 28).

The intronic miRNAs need to fulfill the following requirements. First, they share the same promoter with their encoding gene transcripts. Second, they are located in the nonprotein-coding region of a primary gene transcript (the pre-mRNA). Third, they are coexpressed with the gene transcripts. Last, they are removed from the transcript of their coding genes by nuclear RNA splicing and excision processes to form mature miRNAs.

Certain of the currently identified miRNAs are encoded in the genomic intron region of a gene, but they are of an orientation opposite to that of the protein-coding gene transcript. Therefore, these miRNAs are not considered to be intronic miRNAs because

they do not share the same promoter with the gene and they are not released from the protein-coding gene transcript by RNA splicing. The promoters of these miRNAs are located in the antisense direction to the gene, probably using the gene transcript as a potential target for the antisense miRNAs. A good example of this type of miRNA is *let-7c*, which is an intergenic miRNA located in the antisense region of the intron of a gene.

Transposon and Intronic Mirna

The intronic and other ncRNAs may have evolved to provide a second level of gene expression in eukaryotes, enabling fine-tuning of the complex network of gene activity.

In bacterial and organellar genomes, group II introns contain both catalytic Ranks and retrotransposable elements. The retrotransposable elements make this type of intron mobile. Therefore, these introns are reversely spliced directly into a DNA target site and subsequently reverse-transcribed by the intron-encoded gene. After insertion into the DNA, the introns are spliced out of the gene transcript to minimize the damage to the host.

There is a potential evolutionary relationship between group II introns and both eukaryotic spliceosomal introns and non-LTR-retrotransposons. Taking advantage of this feature, it is feasible to design mobile group II introns to be incorporated into gene-targeting vectors as "targetrons," to specifically target various genes (29). There is evidence that introns in *Caenorhabditis* genes are recently gained and some of them are actually derived from "donor" introns present in the same genome. Further, a few of these new introns apparently derive from other introns in the same gene (30). Perhaps the splicing machinery determines where introns are added to genes. On the other hand, some newly discovered brain-specific snoRNAs of unknown function are encoded in introns of tandem repeats, and the expression of these introns is paternally imprinted.

From an evolutionary vantage, transposons are probably very old and may exist in the common ancestor genome. They may enter the host multiple times for selfish parasitical reasons. This feature of transposons is similar to that of retroviruses. Too much transposon activity can destroy a genome. To counterattack the activity of transposons and viruses, some organisms developed a mechanism to remove and/or silence the activity of transposons and viruses. For example, bacteria frequently delete their genes so that transposons and retroviruses incorporated in the genome are removed. In eukaryotes, miRNA is a way of reducing transposon activity. Conceivably, miRNA may be involved in resistance against viruses, similar to the diversity of antibody production in an immune system, or in a to-be-identified mechanism for fighting disease.

Identical twins derived from the same zygote have the same genetic information in their nuclear DNA. Any differences

between monozygotic twins later in life are mostly the result of environmental influences rather than genetic inheritance. However, monozygotic twins may not share all of their DNA sequences. Female monozygotic twins can differ because of differences in X-chromosome inactivation. Consequently, one female twin can have an X-linked condition, such as muscular dystrophy, and the other twin can be free of the condition. Monozygotic twins frequently demonstrate slightly different (but definitely distinguishing) disease susceptibility and, more generally, different physiology. For example, myotonic dystrophy is a dominantly inherited, multisystemic disease with a consistent constellation of seemingly unrelated and rare clinical features, including myotonia, muscular dystrophy, cardiac conduction defects, posterior iridescent cataracts, and endocrine disorders (31). Type 2 myotonic dystrophy is caused by a CCTG expansion (mean, ~5,000 repeats) located in intron 1 of the zinc finger protein 9 gene (32). It is possible that monozygotic twins with this disorder display symptom heterogeneity because of miRNAs or different levels of insertion of intronic genes.

Class II transposons can cut and paste. The enzyme transposase binds to the ends of the transposon, which are repeats, and the target site on the genome, which is cut to leave sticky ends. These two components are joined together by ligases. In this way, transposons increase the size of the genome because they leave multiple copies of themselves in the genome. It is highly possible that transposons are selectively advantageous for the genome to modulate gene regulation via miRNAs. It is not be too far-fetched to suggest that when transposons are inserted in the introns of the protein-coding gene, under appropriate conditions, they, a part of them, or their secondary structures, may become intronic miRNAs.

1.1.8. PIWI-Interacting RNA

PIWI-interacting RNAs, or piRNAs, are a class of sRNAs, first discovered in mammalian testes that are coupled with PIWI proteins, such as MILI, and control transposon activity (33). piRNAs are predominantly expressed in the germlines of various species. PIWI proteins, germline-specific Argonaute (AGO) proteins such as Argonaute 3, Aubergine, and PiWi, are a part of the AGO family of proteins which can bind to miRNA or siRNA to form RISCs (34). Loss-of-function of PIWI proteins and/or the piRNA loci on the genome lead to derepression of transposons and causes severe defects in gametogenesis and fertility (Citation piRNA). There are primary and secondary piRNAs that serve different purposes in the control of gametogenesis. The primary piRNA guides the PIWI proteins to possible transposon mRNA targets and aids in the cleavage of the transposon, promoting the

creation of a secondary piRNA from the cleaved mRNA which would target the antisense transposons (35). A second cleavage event would lead to the original primary antisense piRNA that can target other transposons. This process is known as the Ping Pong cycle, which optimizes the existing piRNA to target active transposons (35).

1.1.9. Repeat-Associated siRNA

Repeat-associated siRNA (rasiRNA) are a subcategory of piRNAs. Like piRNAs, it interacts with the Piwi-argonaute protein family and utilizes the Ping Pong cycle for its production (36). It is longer than all the other small noncoding RNAs in RNAi (37). For its production, RasiRNA does not require the dicer enzyme, which is essential in miRNA and siRNA production, but instead requires the AGO proteins to direct the cleavage process. RasiRNA are produced from antisense strands of transposable elements (TE) and affects the antisense strands of TE and repeated sequences (37). RasiRNAs hold an important role in transcriptional silencing and regulating cell structure. The loss of rasiRNAs may lead to the loss of germ cells or important developmental elements in organisms (37).

1.1.10. Trans-Acting siRNA

Trans-acting siRNAs (tasiRNAs) are a subcategory of siRNAs in plants. Its production is guided by various plant miRNAs, for example miR-390, 160, and 167 targets tasiRNA precursors like TAS3 to cleave them with dicer-like proteins DCL4 and prepare them into tasiRNA (38). Various miRNA pathways in tasiRNA production have been found to be highly conserved between monocot and dicot plants, indicating a long history of tasiRNA production. TasiRNA has been shown to act, like other siRNA, in RNAi. In experiments with Arabidopsis thaliana, TAS3 tasiRNAs and their corresponding miRNAs have been linked to various ARF genes, which are instrumental in the growth and development of the plant (39). The precise signaling pathways of tasiRNAs are suggested to be similar to other siRNAs, though there have not been studies verifying it as such.

1.1.11. RNA Activation

Recently, emerging evidence shows that small dsRNAs targeting gene promoters are potent in inducing prolonged gene activation at the transcriptional level (40). This phenomenon is termed as RNA activation (RNAa) and is evolutionarily conservative. The sRNAs are referred to small activating RNAs (saRNA). Also, saRNAs induce epigenetic changes on the target promoter, resulting in alterations of gene expression (41). Altogether, it is feasible that saRNAs with optimal properties can be used as therapeutic agents to induce de novo activation or re-expression of silenced tumor (or metastasis) suppressor genes.

2. Biogenesis and Mechanism of miRNAs

The investigation of the biogenesis and mechanism of miRNAs still is in its early stage. siRNA seems to be a form of miRNA duplex predominantly occurring in plants and lower animals. The biogenesis and mechanism of siRNAs are very similar to those of miRNA. However, there are some differences between these two pathways.

Five steps are involved in miRNA biogenesis in vertebrates. First, miRNA is generated as a long pri-miRNA, most likely mediated by Pol-II (42, 43). The pri-miRNA is transcribed from the genome. Second, the long pri-miRNA is excised by Drosha-like RNase III endonucleases and/or spliceosomal components to form the approx 60- to 70-nt pre-miRNA. The pre-miRNA exhibits considerable secondary structure, including regions of imperfectly paired dsRNA, which are sequentially cleaved to one or more miRNAs. This step depends on the origin of the pri-miRNA, whether located in an exon or an intron, respectively (24, 42). Third, the pre-miRNA is exported out of the nucleus by Ran-GTP and a receptor, Exportin-5 (25, 44). Fourth, in the cytoplasm, Dicer-like endonucleases cleave the pre-miRNA to form mature 18- to 25-nt-long miRNA. Last, the mature miRNA is incorporated into an RNP to form the RISC, which executesRNAi-related gene silencing (45, 46). Only one of the two strands is the miRNA; the other counterpart is named miRNA*. The mature miRNA can block mRNA translation based on partial complementarity between the miRNA and the targeted mRNA, particularly via base pairing with the 3′ UTR of the mRNA. If there is a perfect complementarity between the miRNA and the targeted mRNA, mRNA degradation occurs similarly to that mediated by siRNA. Autoregulatory negative feedback via miRNAs regulates some genes, including those involved in the RNA silencing mechanism itself.

Although the assembly of RISC for siRNA has been reported in an in vitro system, and a similar assembly probably also occurs for miRNA, the link between final miRNA maturation and RISC assembly remains unknown. However, there is evidence that the actions of Dicer and RISC in siRNA and miRNA processing are distinct (27). In recent studies using zebrafish, it was demonstrated that the stem-loop structure of the pre-miRNAs is involved in strand selection for mature miRNA during RISC assembly. These findings further suggest that the duplex structure of siRNA may not be strictly required for the assembly of miRNA-associated RISC in vivo. Proposed pathways for the biogenesis of miRNA are based on the in vitro model developed for siRNA. For these reasons, future work needs to focus on distinguishing the individual

properties and differences in action of Dicer and RISC in siRNA and miRNA processing. Conceivably,siRNA is a defense mechanism against immediate insertion of viral genes or transposons in plants and lower animals. In contrast, over evolutionary time, miRNA selects segments of transposons for incorporation into the genome for fine-tuning of gene regulation in vertebrates, including human beings. This hypothesis for the differences between siRNA and miRNA gene silencing may provide a clue toward explaining the prevalence of native siRNAs in invertebrates and their relative scarcity in mammals.

In plants, siRNAs and their dsRNA precursors trigger DNA methylation, as well as RNAi (47–49). Another functional type of siRNAs is a specialized ncRNA molecule that is X-chromosome encoded, Xist. Xist is preferentially expressed from only one of the two female X chromosomes and builds up in *cis* along the chromosome from which it was transcribed. That X chromosome is tightly packaged in transcriptionally inactive heterochromatin; therefore, only one female X chromosome is active. This phenomenon is associated with DNA methylation. Similarly, the viruses, transgenes, and transposons that have been incorporated into the introns of the mammalian genome during evolution may take advantage of these characteristics by splicing the pri-miRNAs and incorporating them into Dicer-like proteins for gene silencing and mRNA degradation.

Argonaute I is a key protein that is required for both the siRNA and miRNA pathways, and it is likely to be the endonuclease that cleaves the mRNA targeted by the RISC (50, 51). Elucidating the roles of the full complement of the AGO protein family will reveal further modulation of the RISC, and more generally, of sRNA regulation. Additional proteins involved in the RISC at the convergence of the PTGS and miRNA pathways have been reported (52, 53).

Introns account for the largest proportion of noncoding sequences in the protein-coding DNA of the genome. The transcription of the genomic protein-coding DNA generates pre-mRNA, which contains four major parts, including the 5′ UTR, the protein-coding exon, the noncoding intron, and the 3′ UTR. In broad terms, both the 5′ UTR and the 3′ UTR can be seen as a kind of intron extension; however, their processing during mRNA translation is different from that of the intron located between two proteincoding exons, termed the in-frame intron. The in-frame intron was originally thought to be a huge genetic wasteland in gene transcripts, but this stereotypical misconception was abandoned because of the finding of intronic miRNAs. To this day, the biogenesis of intronic miRNAs remains to be determined (Fig. 1).

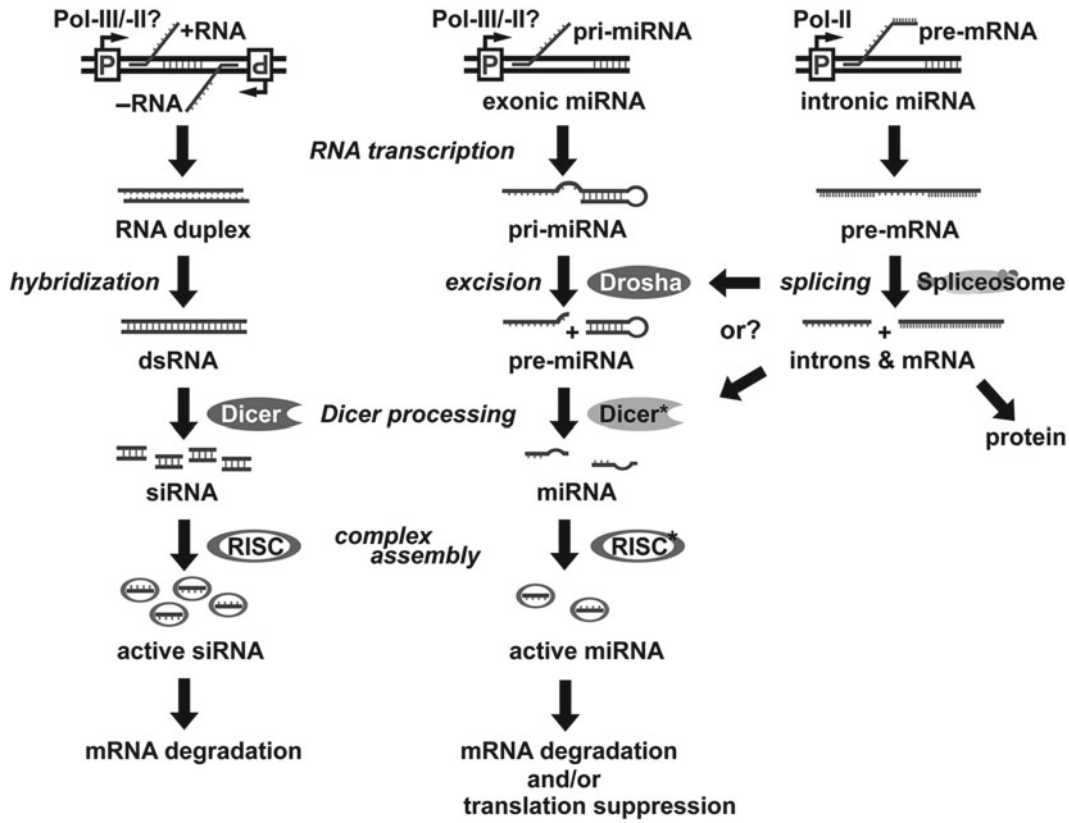

Fig. 1. Comparison of biogenesis and RNA interference mechanisms among small interfering RNA (siRNA), intergenic (exonic) microRNA (miRNA), and intronic miRNA. siRNA is likely formed by two perfectly complementary RNAs transcribed from two different promoters (although this remains to be determined) and further processing into 19- to 22-bp duplexes by the ribonuclease (RNase) III familial endonuclease, Dicer. The biogenesis of intergenic miRNAs, e.g., *lin-4* and *let-7*, involves a long transcript precursor (pre)-miRNA, which is probably generated by type II RNA polymerase (Pol-II) promoters or type III RNA polymerase promoters, whereas intronic miRNAs are transcribed by the Pol-II promoters of its encoded genes and coexpressed in the intron regions of the gene transcripts (pre-mRNA). After RNA splicing and further processing, the spliced intron may function as a primary transcript (pri)-miRNA for intronic miRNA generation. In the nucleus, the pri-miRNA is excised by Drosha RNase to form a hairpin-like pre-miRNA template and is then exported to the cytoplasm for further processing by Dicer* to form mature miRNAs. The Dicers for siRNA and miRNA pathways are different. All three small regulatory RNAs are finally incorporated into an RNA-induced silencing complex, which contains either strand of siRNA or the single strand of miRNA. The effect of miRNA is considered more specific and less adverse than that of siRNA because only one strand is involved. However, siRNAs primarily trigger messenger RNA (mRNA) degradation, whereas miRNAs can induce either mRNA degradation or suppression of protein synthesis, depending on the sequence complementarity to the target gene transcripts.

3. Identification

Currently, there are four major ways to identify miRNAs. They are:

1. Direct cloning.
2. Computer search of the genome.

3. miRNA microarray search in different species.
4. Artificial preparation of miRNA for targeting known gene sequences.

The conventional direct cloning of short RNA molecules, as in the cloning of *let-7* and *lin-4*, is still the method of choice to identify new miRNAs. Conceivably, one can isolate the sRNAs and sequence them individually. Thus far, results have been dominated by a few highly expressed miRNAs. However, once the miRNA is identified, its role in other organisms, including human beings, can be explored. For example, *let-7* was originally identified in *C. elegans*. Subsequently, reduced expression of the *let-7* miRNA (54) and Dicer (55) in human lung cancers suggested that the alteration of *let-7* expression is associated with clinical and biological effects.

There are numerous new computational methods that provide ways to estimate the total number of miRNA genes in different animals (56–59). Fundamentally, each program identifies highly conserved genomic noncoding regions that possess stem-loop structures with specific "seed" sequences and complementarity of the first 8- to 10-nt. Then, the secondary structure is examined in terms of both the forward and reverse complements of the sequence. In addition, the following criteria help to identify miRNAs: the longest helical arm, the free energy of the arm, short internal loops, and asymmetric and bulged loops. The identified miRNAs are usually more heterogeneous than those that are discovered experimentally, suggesting that traditional cloning has a high false-negative rate or miss rate. However, computational techniques may suffer from a high false-alarm rate. Therefore, validation of the identified miRNAs by Northern blot analysis and functional study is critical. These methods are still evolving and there is a possibility of one-to-many and many-to-one relationships between the miRNAs and their targets. Potentially thousands of mammalian targets may be identified with this approach.

To facilitate such investigations, an oligonucleotide microchip for genome-wide miRNA profiling in diverse tissues of various species was developed (60–62). Some of these chips use locked nucleic acid-modified oligonucleotides to allow both miRNA in situ hybridization and miRNA expression profiling (62). Again, this approach can identify regulation via a large class of miRNAs. A good example is the studies of miRNAs regulating brain morphogenesis in zebrafish (63).

4. Applications

Application of miRNA is common in plants. The first experimentally observed RNAi was demonstrated by the discoloration of flowers after introduction of dsRNAs. miRNAs do not solely mediate

gene regulation in flowering plants; the regulation of a similar type of plant gene can be used to silence a fruit-ripening gene. The latter approach has been applied to create hardier tomatoes by engineered repression of the tomato-ripening gene through homology-based silencing.

The following areas are potential applications of miRNAs in vertebrates:

(a) Analysis of gene function.

(b) Evaluation of function and effectiveness of miRNA.

(c) Design and development of novel gene therapy.

(d) Design and development of antiviral vaccines.

(e) Development of loss-of-function transgenic animals.

4.1. Analysis of Gene Function

The human genome contains more than 22 billion bases. Analyzing and understanding these sequences is a challenge to all natural science. Numerous high-throughput screening programs have been developed in the postgenome era. Good examples of the usefulness of such programs are the discoveries that *lin-4* and *let-7* miRNAs, originally identified in *C. elegans*, control the timing of fate specification of neuronal and hypodermal cells during larval development. Subsequently, these miRNAs were found to be conserved in mammals, with potential functions in development and disease (64, 65). Although most programs are focused on global computational screens, others aim at screening or isolating all miRNAs with a specific function in the genome. For instance, retroviral insertional mutagenesis in mouse hematopoietic tumors provides a potent cancer gene-discovery tool in the postgenome sequence era. The multiple high-throughput insertional mutagenesis screening projects were, thus, designed for identifying new cancer genes. Using a short hairpin RNAi expression library against the entire human transcriptome, attempts were made to screen for sRNAs in the genome (66, 67).

With the completion of genome-sequencing projects, a major challenge will be to understand gene function and regulation. Achieving this goal will require determining how miRNAs modulate gene expression. The various gene-silencing mechanisms based on complete or partial complementarity and their intertwined actions are beginning to reveal the sensitive control mechanisms that modify gene expression at the post-transcriptional and RNA turnover levels.

Intronic miRNA also represents a new frontier in genetics research. The evidence of intronic miRNA-induced silencing of gene expression in cell lines, zebrafish, chicken embryos, and mouse skin demonstrates that this ancient intron-mediated gene regulation system is highly conserved in eukaryotes. Intronic regulation of gene expression is mediated through the activation of miRNA-mediated RNAi effects. From an evolutionary vantage point, the genome exhibits a remarkable increase in the complexity

and variety of introns in higher plants and animals; therefore, the influence of intronic gene regulation to facilitate genome stability and gene coordination progressively increases phylogenetically. Conceivably, dysregulation of intronic miRNAs is highly likely to reveal the intertwined actions between intronic miRNA and gene expression, leading to a better understanding of the genetic etiology of human diseases. The success of intronic miRNA generation by artificial means in vivo may provide a powerful tool to study the mechanism by which miRNAs induce diseases and will, hopefully, provide novel gene therapies.

4.2. Evaluation of miRNA Function and Effectiveness

Prediction of miRNA candidates using analytical software has identified thousands of genomic hairpin sequences. For instance, the human DiGeorge syndrome critical region gene 8 (*DGCR8*), and its *Drosophila melanogaster* homolog, were identified in this fashion. The biochemical and whole cell-based data demonstrating the requirement of *DGCR8* for the maturation of miRNA primary transcripts have been acquired. Further, RNAi knockdown experiments with fly and human *DGCR8* showed both accumulation and reduction of pri-miRNAs, as well as mature miRNAs. In this manner, the function of, effectiveness of, and interaction between miRNAs and enzyme processing complexes can be demonstrated (68).

To date, the function of the vast majority of miRNAs so identified remains to be determined. Because direct transfection of hairpin-like pre-miRNAs in mammalian cells is not always sufficient to trigger effective RISC assembly, a key step for RNAi-related gene silencing, our intronic miRNA-expressing system was developed to overcome this problem and, indeed, successfully increased the efficiency and effectiveness of miRNA-associated RNAi induction in vitro and in vivo. Nevertheless, there are still problems in the efficient use of miRNA. Indeed, evaluating the function and effectiveness of the miRNAs identified thus far may contribute greatly to our understanding of gene regulation and the control of the differentiation and development of cells.

Based on the strand complementarity between the designed miRNA and its target gene sequence, we have also developed an miRNA isolation protocol to purify and identify the mature miRNAs generated by the intronic miRNA-expressing system. Several intronic miRNAs have been confirmed active in vitro and in vivo. As shown by this proof-of-principle method, we now have the necessary knowledge to design more efficient and effective pre-miRNA inserts for the intronic miRNA-expressing system.

4.3. Design and Development of Novel Gene Therapy

We are undergoing an epoch-marking transition into the postgenome era, which opens up data sources of unprecedented scale. This information can be used for designing and developing potential drugs as novel gene therapies. Furthermore, the elucidation of genomic control of gene activities mediated via miRNA may play a

crucial role in the characterization and treatment of disease at the molecular level. At the same time, our still very limited knowledge of the biological functions of genes and proteins at different levels of cellular organization is preventing full exploitation of the available data. We believe that the recent discovery of miRNAs will fill the gap and lead to unlimited functional prediction based on the DNA–miRNA and RNA–miRNA paradigm. In theory, oncogene and transgene expression could be inhibited by synthetic miRNAs, a simple, effective gene therapy. Thus, miRNAs or their machineries are now known to be involved in several human diseases, including cancer and neurological disorders. Specific removal of the target genes by miRNAs or their associate mediators can be developed as a simple gene therapy (69).

4.4. Design and Development of Antiviral Vaccine

By the same token, antiviral vaccines can be developed. The problems in preventing viral infections, such as HIV, are as follows: first, the global prevalence of epidemic proportions is mainly caused by the high mutation rate of the HIV genome that gradually generates strains that are more resistant to highly active antiretroviral therapies. Second, the HIV provirus is capable of integrating into a host cellular genome to escape from the inhibitory effects of the treatment, thus inactivating the viral replication cycle rather than destroying the latent viral genome, resulting in an increased number of HIV carriers. Such an increase of drug-resistant HIV strains and their carriers has posed great challenges and financial burdens for the AIDS prevention programs. To alleviate such problems, miRNAs can trigger either translation repression or RNA degradation depending on their degree of complementarity with the target genes. Our approach, using vector-based miRNA to overcome the complications of HIV mutation, is a breakthrough in the field. We think that the knowledge obtained from this research will facilitate the development of antiviral drugs and vaccines against HIV infections (70, 71). Similar approaches for the treatment of viral diseases will target avian flu, severe acute respiratory syndrome, hepatitis B, herpes, and poliomyelitis.

4.5. Development of Loss-of-Function Transgenic Animals

The ability to use miRNA and its machinery for silencing target-gene expression has created much excitement as a novel and simple means to develop loss-of-function transgenic animals. To define the function of a critical molecule in miRNA processing, zebrafish models have been developed that carry loss-of-function mutations. This type of animal model has provided an unprecedented resource for miRNA research because this approach can be used to create miRNAs for use in loss-of-function studies. It is clear that miRNAs also hold great promise as therapeutic tools because of their sequence-specific targeting, particularly against infectious diseases with frequent mutations. Another potential use of transgenic animal models using miRNAs is the testing of gene functions and drug mechanisms in vivo.

Using man-made miRNA, one can establish loss-of-function in zebrafish, chicken, and mice. The loss-of-function transgenic zebrafish could not have been achieved with siRNAs because of promoter incompatibility, but have been developed with intronic miRNAs. The zebrafish, possessing numerous features similar to human biological systems, is most suitable for etiological and pathological studies of human diseases, particularly mechanisms by which the loss of a specific signal molecule causes a disease or disorder. All pharmaceutically developed drugs can be screened with this approach in loss-of-function transgenic zebrafish. In addition, this approach may shed light on the effects of miRNAs on embryonic development, environmental impacts, and micromodulation of gene functions, particularly brain and heart functions. Indeed, insight regarding structure–function features of a candidate gene involved in pathobiology and the mechanisms in which the candidate gene operates can be illuminated with the help of miRNA transgenic animal models carrying a loss-of-function mutation within the candidate gene.

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Chapter 2

Analysis of MicroRNA Length Variety Generated by Recombinant Human Dicer

Julia Starega-Roslan and Wlodzimierz J. Krzyzosiak

Abstract

miRNAs are a large subgroup of noncoding regulatory RNAs, which vary in length within the 20–25 nt range and show substantial length diversity and heterogeneity. To analyze the latter phenomenon, we recently developed high-resolution northern blotting and employed this method to investigate cleavages generated by recombinant human Dicer in the synthetic miRNA precursors. We paid special care to visualize clearly the cleavages generated by the individual RNase III domains of Dicer. We have compared the results of northern blotting with the results of standard analysis with the use of end-labeled RNA and visualization of Dicer cleavage products by autoradiography. The point-by-point steps of substrate preparation, recombinant Dicer cleavage assay, and northern blotting are described in this manuscript.

Key words: miRNA, miRNA precursors, Dicer cleavage, End-labeling, Northern blotting

1. Introduction

miRNA are a group of noncoding RNA, that function as posttranscriptional regulators of gene expression in multicellular organisms. These small ~21–24 nt RNAs are involved in maintaining the normal functioning of eukaryotic cells by regulating the majority of physiological processes including the control of cell differentiation and development.

The biogenesis of human miRNAs includes two RNA cleavage steps in which the activities of RNases Drosha and Dicer are involved (Fig. 1). The biogenesis begins with the nuclear cleavage of the primary transcript (pri-miRNA) by Drosha acting together with DGCR8 within a Microprocessor complex to result in pre-miRNA (1). At this step, Drosha excises a ~60 nt hairpin structure from the primary transcript (2). Many different accessory proteins

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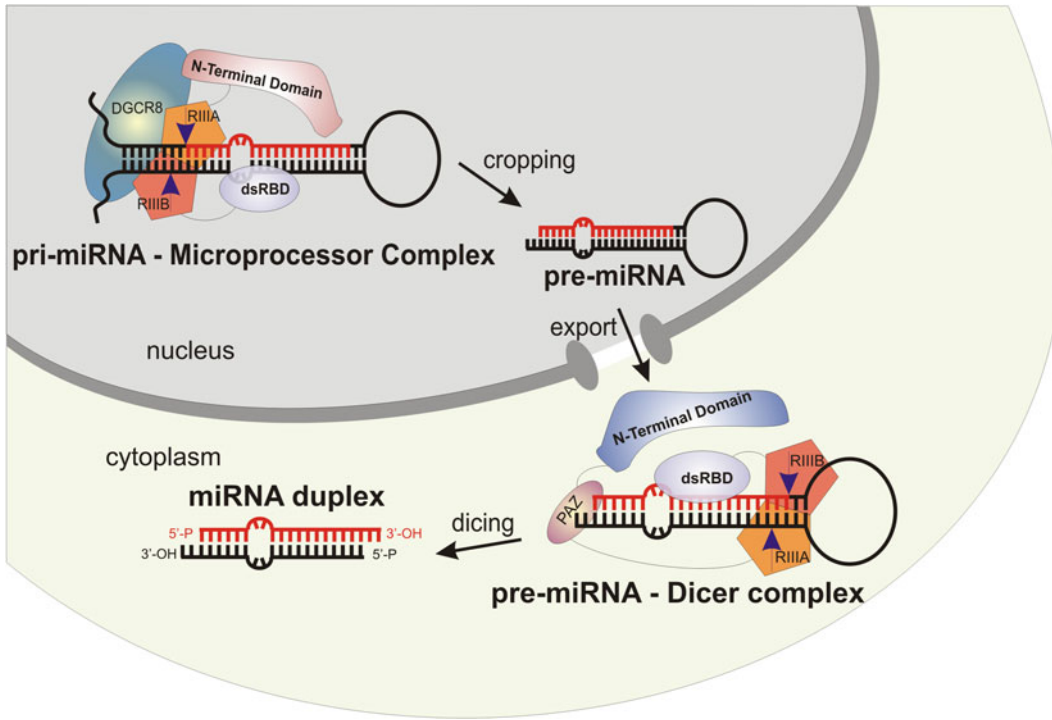

Fig. 1. Two processing steps of miRNA biogenesis.

cooperate with the Drosha/DGCR8 complex in this process in a variety of ways (3). After pre-miRNA export by Exportin-5 from nucleus to cytoplasm (4), the precursor is captured by the RNase Dicer protein complex and Dicer excises the hairpin terminal loop to generate a ~21 nt-long duplex (5).

Dicer is the RNase III enzyme which in human cells cooperates with the TAR-RNA-binding protein (TRBP) and an argonaute family protein (AGO1-4) within the RISC-loading complex (RLC). Dicer contains the PAZ domain with which it anchors to the 3′ terminus of the pre-miRNA. Two RNase III domains that form an intramolecular heterodimeric processing center cleave pre-miRNA about two helical turns away from its ends (6). A typical product of pre-miRNA cleavage is an imperfect duplex composed of miRNA and miRNA* strands that contain 5′ monophosphates and free OH groups at the 2 nt-long, 3′-protruding ends. The architecture of human Dicer suggests that the distance between the RNase III domain processing center and PAZ domain roughly corresponds to 22 nt in chain (7). Therefore, Dicer acts as a molecular ruler in determining cleavage sites within pre-miRNA and dsRNA.

Since the beginning of large-scale miRNA cloning and sequencing efforts, it has been known that mature miRNAs generated from different miRNA genes differ considerably in length, and that most human miRNAs fall within the 20–23 nt range (Fig. 2) (8).

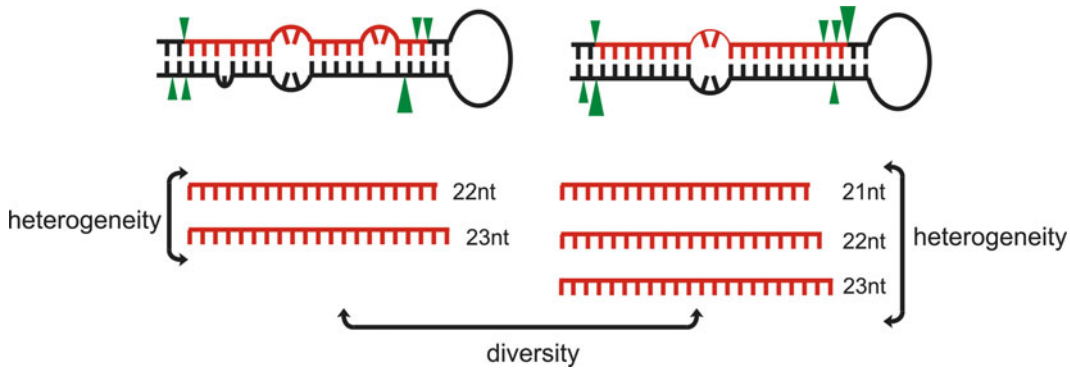

Fig. 2. miRNA length diversity and heterogeneity. miRNA length diversity—miRNAs of diverse lengths are excised from different precursors; miRNA heterogeneity—miRNAs of heterogeneous lengths are excised from single precursor.

This kind of variation (miRNA length diversity) was shown to be attributed to the different structures of the diverse miRNA precursors that are processed by Dicer (9, 10). It is also known from numerous miRNA studies that individual miRNA genes frequently give rise to several miRNA species that differ in their length (miRNA length heterogeneity) (11–13). The hypotheses proposed to explain this kind of miRNA variation included the relaxed specificity of Drosha and Dicer cleavages (10, 12–15), downstream effects such as limited miRNA degradation by exonucleases, the addition of extra nucleotides (11, 15, 16), or miRNA sequencing artifacts (17, 18).

Several different experimental systems have been used thus far to gain an insight into sources of miRNA length heterogeneity. Cell transfections with constructs encoding pri-miRNAs were used to study the specificity of both Drosha and Dicer cleavages (10). The Dicer step alone was also investigated using a variety of systems. The one that utilizes endogenous Dicer functioning together with its protein partners in a cellular environment is the injection of synthetic precursors to the *Xenopus* oocytes (19). Also immunoprecipitated Dicer complexes were used to generate cleavages within synthetic precursors (20), and the Dicer activity present in cellular extracts was tested using such substrates (21–23). Moreover, the human Dicer-TRBP-AGO2 complex reconstituted from recombinant proteins was also used in cleavage assay with synthetic pre-miRNA (24).

We analyzed the potential of recombinant RNase Dicer alone to generate miRNAs of diverse and heterogeneous length from their precursors (10). We used two approaches differing in the way the cleavage products were detected. The first approach depends on the end-labeling of RNA substrate with radioisotope and an analysis of the products released by Dicer by autoradiography after their electrophoretic separation. In the second approach, instead of using end-labeled pre-miRNA, we used an unlabeled precursor

and after Dicer cleavage followed by the separation of products according to their size, visualized cleavage products by northern blotting. The northern blotting with probes specific for the 5′ arm could be considered equivalent to the 5′ end-labeling and used for an analysis of cleavage products generated in synthetic pre-miRNAs by RNase IIIB domain of Dicer. When performing an analysis using 5′ end-labeled RNA, it is often difficult to precisely assign the position of cleavages in the 3′ arm of the precursor. In such a case, 3′ end-labeling could be applied, i.e., pC ligation to pre-miRNA lacking the 3′ terminal C-residue followed by dephosphorylation. However, northern blotting is a better and less expensive alternative that does not require special substrate preparation, and only uses specific-labeled probes detecting 5′ or 3′ arm products. We have shown the results of a comparative analysis for pre-miR-31 and pre-miR-132 using both methods (Fig. 3). When we compared the results of northern blotting with those obtained using ^{32}P end-labeled pre-miRNA, it turned out that the results correspond well with each other. The cleavages in the 5′ arm of end-labeled RNA matched the cleavages detected using a specific probe for the 5′ arm in northern blotting. Therefore, northern blotting turned out to be a good alternative to the ^{32}P end-labeling to detect the cleavage products of synthetic precursors.

To characterize Dicer cleavage products, we introduced a new parameter WALDI (Weighted Average Length of Diced RNA) which helps to define the mean length of excised fragments. The way the value of this parameter is determined is shown here using specific examples. Also in the analysis of deep sequencing data where different events shape overall miRNA variety, the miRNA-WAL parameter may be used to characterize the pool of endogenous miRNAs.

2. Materials

2.1. Oligonucleotides (See Note 1)

1. Northern blotting marker: fragments of RNA in a range of 17–25 nt.
2. Oligo RNA: pre-miRNA (see Note 2).
3. Probes for northern blotting: DNA oligonucleotide complementary to the fragment of RNA, you want to detect, miRNA or miRNA* (see Note 3).

2.2. Reagents Used for Labeling of Oligonucleotides and End-Phosphorylation

1. 5.000 Ci/mmol [γ-^{32}P]ATP (Hartmann Analytics) (see Note 4).
2. Optikinase (USB Corp.).
3. Elution buffer: 0.3 M sodium acetate, pH 5.2, 0.5 M EDTA, 0.1% sodium dodecylsulfate.

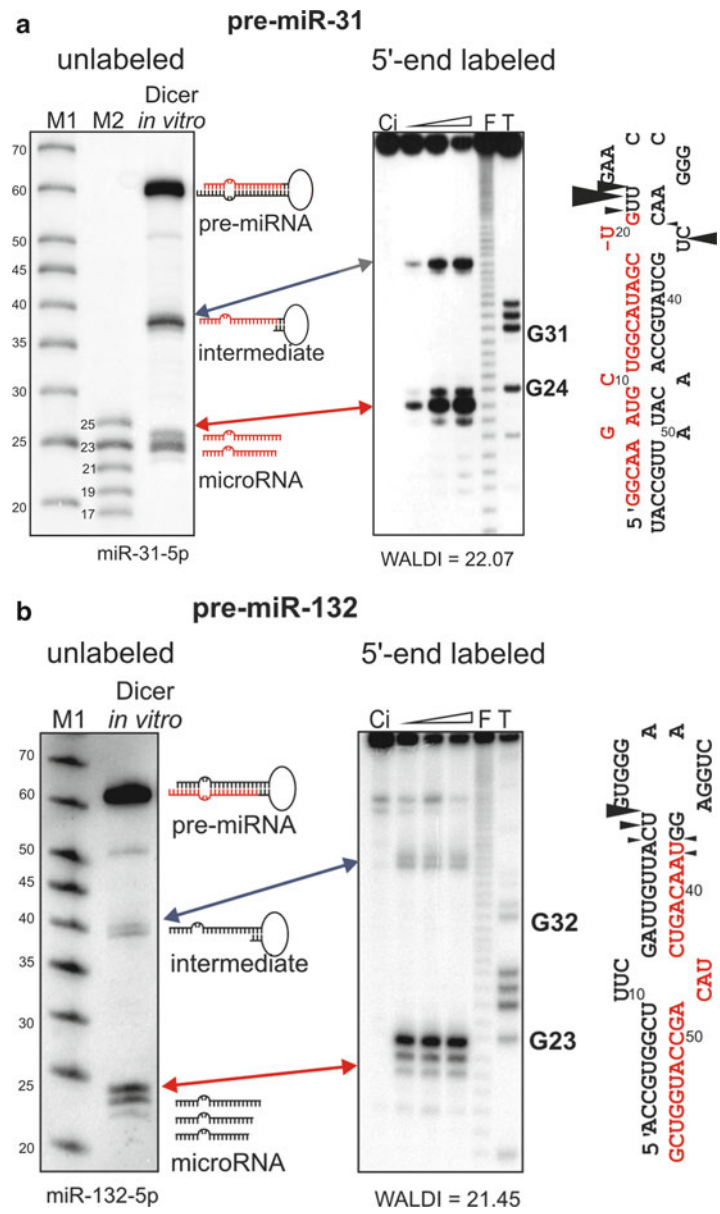

Fig. 3. The high-resolution northern blotting of short RNAs for an investigation of the Dicer cleavage mechanism. The products of Dicer cleavage assay (Ambion) of the unlabeled pre-miR-31 (**a**) and pre-miR-132 (**b**) were subjected to northern blotting with specific 5′ probes detecting miRNA derived from a 5′ arm ("Dicer *in vitro*" lane). Aside, the schematic structure of the products the signals derive from. For a comparison, on the *right hand side*, the structure of pre-miRNA and the result of the 5′ end-labeled precursor *in vitro* cleavage assay by Dicer in time-dependent reaction is shown. The positions of selected G-residues are shown. The Dicer cleavage sites (marked with *arrowheads*, the size of which is proportional to the radioactive signal intensity) are shown in the secondary structure models, where the reported miRNA sequences are marked in *red*. M1—denotes the Low Molecular Weight Marker (USB Corp.); M2–denotes the 17–25 nt RNA marker; Lanes Ci—incubation controls without Dicer; Lanes F—formamide ladders; Lanes T—guanine-specific ladders.

4. X-ray film.
5. EtOH.
6. Illustra MicroSpin G-25 columns (GE HealthCare).
7. Liquid scintillation counter (Hidex).
8. ATP: 10 mM.

2.3. Electrophoresis Reagents

1. Ten and fifteen percent polyacrylamide gel solution: acrylamide and bisacrylamide ratio 19:1, 7.5 M Urea, 1× TBE.
2. Twelve percent polyacrylamide gel solution: acrylamide and bisacrylamide ratio 19:1, 7.5 M Urea, 0.5× TBE.
3. Ammonium persulfate: 10% solution in water (APS).
4. *N*, *N*, *N*, *N*′-tetramethyl-ethylenediamine (TEMED).
5. Electrophoresis buffer: 0.5× TBE, 1× TBE.
6. Vertical electrophoresis gel system 40 × 30 cm (S2 sequencing gel electrophoresis apparatus, Gibco).
7. Vertical electrophoresis gel system 16 × 20 cm (Protrean II xi Cell, BioRad).

2.4. Dicer In Vitro Cleavage Assay Reagents

1. Dicer enzyme: commercially available from Ambion (1 U/μL), Stratagene (0.5 U/μL), a preparation of Dicer enzyme from W. Filipowicz.
2. Urea loading buffer 2×: 7.5 M Urea, 20 mM EDTA, 0.1% bromophenol blue and xylene cyanol dyes.
3. Nuclease S1: 0.2 U/μL (Sigma).
4. S1 buffer: 10 mM Tris–HCl, pH 7.2, 40 mM NaCl, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$.
5. Ribonuclease T1: 0.2 U/μL (Fermentas).
6. T1 buffer: 10 mM sodium citrate, pH 4.5, 0.5 mM EDTA, and 3.5 M Urea.
7. Ribonuclease P1: 1 U/μL (Sigma).
8. P1 Bufer: 6.5 M Urea, 20 mM sodium phosphate, pH 9.0, and 1 mM $ZnCl_2$.
9. Formamide buffer: 99% formamide, 0.5 mM $MgCl_2$.
10. PhosphorImager (FujiFilm).

2.5. Northern Blotting Reagents

1. Formamide loading buffer 2×: 99% formamide, 1 mM EDTA, 0.1% bromophenol blue, and xylene cyanol dyes.
2. Ethidium bromide.
3. Transfer buffer: 0.5× TBE.
4. Semi-dry electroblotter of min. size of 20 × 20 cm (Sigma-Aldrich).

5. Nylon membrane: Genescreen Plus Hybridization Transfer Membrane (Perkin Elmer).
6. Whatman 3MM filter paper.
7. Cross-linker (UVP).
8. Hybridization oven (Labnet).
9. Hybridization bottle ~25 cm long.
10. Hybridization buffer: PerfectHyb Plus buffer (Sigma).
11. Washing buffer: 2× SSC, 0.1% SDS.

3. Methods

3.1. Oligonucleotide Labeling

3.1.1. Preparation of the Pre-miRNA

1. Mix 10 μL of purified pre-miRNA transcript (~1 nmol) with 3 μL of [γ-^{32}P]ATP, 1.5 μL of 10× Optikinase buffer and 1 μL of Optikinase.
2. Incubate the mixture for 10 min in 37 °C.
3. Stop the reaction by adding equal volume of urea loading buffer.
4. Incubate the sample at 95 °C for 1 min, and immediately load the sample on the pre-electrophoresed 10% polyacrylamide gel (S2 apparatus), buffered with 1× TBE. Perform the electrophoresis at 40 mA for 10 min and then at 90 mA for 3 h.
5. After electrophoresis, attach the labeling markers to the gel and place the gel in the autoradiography cassette with the X-ray film for approximately 3 min. After autoradiography, correctly orient the film with respect to the markers and cut out the single gel band corresponding to the desired RNA.
6. Elute the RNA with approximately 500 μL of the elution buffer at 4 °C overnight. Precipitate the RNA with EtOH, and resuspend the pellet in approximately 20 μL of RNase-free water.
7. Store the RNA at –80 °C until use.

3.1.2. Preparation of the RNA Markers for Northern Blotting

1. Mix equal amount of different length of RNA fragments (2 pmol) with 4 μL of [γ-^{32}P]ATP, 2.5 μL of 10× Optikinase buffer and 1 μL of Optikinase.
2. Incubate the mixture for 10 min in 37 °C.
3. Add equal volume of RNase-free water (total volume of 50 μL) and purify by G-25 columns to remove unincorporated [γ-^{32}P] ATP.
4. Measure the intensity of sample in a scintillator counter (see Note 5).

3.1.3. Preparation of Northern Blotting Probe

1. Mix 10–15 pmol of template DNA oligonucleotide (complementary to the fragment of RNA, you want to detect) with 5 μL of [γ-^{32}P]ATP, 2.5 μL of 10× Optikinase buffer, and 1 μL of Optikinase.
2. Incubate for 1 h at 37 °C.
3. Perform further steps as described for RNA marker labeling (Subheading 3.1.2).

3.2. Preparation of the Homologous Ladders

Incubate the 2 μL of end-labeled RNA (~50.000 c.p.m.) with 9 μL of formamide buffer at 100 °C for 10 min. Stop the reaction by adding 9 μL of urea loading buffer.

3.2.1. Alkaline Hydrolysis Ladder

3.2.2. T1 Ladder

Incubate 2 μL of end-labeled RNA (~50.000 c.p.m.) with 3 μL of T1 buffer for 1 min at 100 °C. Place the tube with reaction mixture on ice immediately and add 1 μL of T1 ribonuclease and incubate for 17 min in 55 °C. Stop the reaction by adding 14 μL of urea loading buffer.

3.2.3. P1 Ladder

Incubate 2 μL of end-labeled RNA (~50.000 c.p.m.) with 3 μL of P1 buffer and 4 μL of RNase-free water for 30 s at 100 °C. Place the tube with reaction mixture on ice immediately, add 1 μL of P1 ribonuclease and incubate for 23 min in 55 °C. Stop the reaction by adding 10 μL of urea loading buffer.

3.2.4. S1 Ladder

Incubate 2 μL of end-labeled RNA (~50.000 c.p.m.) with 1 μL of S1 buffer and 5 μL of RNase-free water for 30 s at 80 °C. Place the tube with reaction mixture on ice immediately, add 2 μL of S1 nuclease and incubate for 1 min in 75 °C. Stop the reaction by adding 10 μL of urea loading buffer.

3.3. Dicer In Vitro Cleavage Assay

1. Subject the ^{32}P end-labeled transcript to a denaturation and renaturation procedure before Dicer cleavage. For a single reaction (total volume 4 μL), mix 50.000 c.p.m. (~5 fmol) of labeled RNA with 0.8 μL 5× Dicer buffer.
2. To the mixture add 0.5 μL of RNase-free water (incubation control) or 0.5 U of Dicer (Ambion) (sample).
3. Incubate the control and sample for 3, 7, or 12 min at 37 °C (see Note 6).
4. Stop the reaction by adding equal volume of urea loading buffer.
5. Incubate the sample at 95 °C for 1 min, and immediately load the sample (~4 μL of each sample) on the pre-electrophoresed analytical 15% polyacrylamide gel (S2 apparatus), buffered with 1× TBE. Perform the electrophoresis at 20 mA for 10 min and then at 40 mA for approximately 2 h.

6. Run products of the alkaline hydrolysis and limited T1, S1, or P1 nuclease digestion of the same RNA molecule on the gel (see Note 7).
7. Transfer the gel onto Whatman 3MM paper, cover with plastic wrap, and subject to autoradiography at −80 °C with intensifying screen. One may also dry the gel and place in the PhosphorImager cassette and further analyze the products quantitatively with a PhosphorImager.

3.4. Northern Blotting

3.4.1. Dicer In Vitro Cleavage Assay

1. Prepare unlabeled phosphorylated RNA in the same way as labeled RNA described in Subheading 3.1.1, but instead of using [γ-^{32}P]ATP, use 2 mM cold ATP.
2. Subject the 5 pmol of unlabeled 5′-phosphorylated pre-miRNA to a denaturation and renaturation procedure with 2 μL 5× Dicer buffer.
3. Add RNase-free water to a total volume of 7 μL and add 1U Dicer (Ambion).
4. Incubate the sample for 1 h at 37 °C.
5. Add an equal volume of urea loading buffer.
6. Take 0.2 μL of each sample and mix with excess of Formamide loading buffer.

3.4.2. Gel Electrophoresis

1. Incubate the sample at 95 °C for 1 min, chill on ice, and immediately load the sample on the pre-electrophoresed analytical 12% polyacrylamide gel (BioRad apparatus), buffered with 0.5× TBE.
2. Load on the same gel labeled RNA markers (see Note 8).
3. Perform the electrophoresis at 20 mA for 10 min and then at 40 mA until bromophenol blue dye reaches the end of the gel.
4. One may stain the gel in 0.5× TBE and EtBR (0.5 μg/mL) to check if electrophoresis went well.

3.4.3. Transfer Assembly

1. Cut the membrane and six pieces of Whatman paper to have the same size as the gel with your samples.
2. Wet the nylon membrane and Whatman paper in 0.5× TBE before assemble the transfer sandwich (see Note 9).
3. Dampen and put three filter papers on a blotter followed by the soaked membrane (anode), the gel, and three wet filter papers on the top.
4. Remove air bubbles between the membrane and the gel by gently rolling a pipette several times back and forth over the surface.

5. Set the power supply of the blotter to mAmps (3 mA/cm^2) and limit the voltage to 20 V.
6. After 1 h, disassemble the blotting device.

3.4.4. Immobilization of RNA

1. To fix the RNA by UV cross-linking, expose the side of the damp membrane with the RNA to UV light (120 mJ/cm^2).
2. Put the membrane between two Whatman sheets and bake the membrane at 80 °C for 30 min.

3.4.5. Hybridization of labeled probe

1. Place the membrane RNA-side-up in a hybridization bottle and prehybridize by rotating for 1 h at 37 °C in ~8 mL of PerfectHyb Plus buffer (see Note 10).
2. Denature your probe by 5 min incubation at 95 °C and add to the hybridization buffer.
3. Allow for hybridization with rotation overnight at 37 °C.
4. Pour off the hybridization solution and wash the membrane three times with washing buffer for 20 min at the hybridization temperature (see Note 11).
5. Seal the membrane in a plastic wrap and expose to PhosphorImaging screen overnight.

3.5. WALDI Quantification

1. To determine the value of WALDI parameter, it is required to know what is the intensity of each cleavage generating different length of products.
2. Analyze quantitatively the cleavage products of pre-miRNA using PhosphorImager.
3. Assign the intensities of the cleavages to the length of the excised products taking into consideration that the sum of all intensities is 1.
4. When there are three excised products from one pre-miRNA arm, use the equation:

$$\text{WALDI value} = (\text{length of first fragment} \times \text{intensity of it}) + (\text{length of second fragment} \times \text{intensity of it}) + (\text{length of third fragment} \times \text{intensity of it})$$ (see Note 12).

4. Notes

1. When working with RNA, take special care to use RNase-free equipment and solutions. Prepare all solutions using RNase-free water (deionized water to attain a sensitivity of 18.2 MΩ).
2. RNA transcript can be obtained by *in vitro* transcription. However, the most convenient way is to order a specific RNA

sequence from an appropriate vendor. That way you do not worry about the ends heterogeneity of the RNA after T7 or SP6 transcription. It is essential that pre-miRNA to be Dicer substrate should possess 5′-P and 3′-OH groups after end-labeling.

3. The probe should have a length not shorter than 18 nt, preferably 22 nt, as it was previously shown that probes shorter than 17 nt are ineffective in the northern blotting hybridization step (25). The putative sequence of the detected fragment can be taken from the miRBase database (8).
4. When using radioactive materials take special precautions to store radioactive materials (labeled samples, gels, membranes, probes) in an appropriate, safe way.
5. Having, e.g., five fragments in your marker, label them simultaneously. To obtain the appropriate intensity on your gel, take ~10.000 c.p.m. of the labeled marker (2.000 c.p.m. per each band). Note that you may need to optimize the amount of the marker loaded on a gel depending of the intensities of your pre-miRNA and miRNA fractions.
6. Dicer enzymes from Stratagene and from Ambion contain 20% and 80% of full-length protein, respectively. Dicer preparation from W. Filipowicz contains nearly 100% full-length protein (data not shown). We have compared the specificity of three enzymes from Ambion, Stratagene, and the preparation of Dicer from W. Filipowicz (Fig. 4). All three enzymes generated cleavages in the same position with negligible difference in intensities. However, the incubation time may need to be adjusted experimentally to obtain the desired signal intensity depending on the Dicer preparation used.
7. The products of Dicer cleavage possess 5′-P and 3′-OH groups at its ends. However, the 5′ end-labeled markers (T1 ladder and alkaline hydrolysis ladder) routinely used to assign the length of excised products possess a phosphate group at both ends. Therefore, it is essential to use P1 or S1 ladders or to calibrate the homologous T1 and alkaline hydrolysis ladders with homologous P1 and S1 ladders that have the same end-groups as the Dicer cleavage products. Within the 20–40 nt range, the products of the Dicer cleavages migrate as products of T1 or alkaline hydrolysis with a ~1 nt increase in length (Fig. 4).
8. Use appropriate length markers. It is advisable to use RNA markers and not DNA markers, as the latter will migrate differently from an RNA sample. The best would be a marker that has a homologous sequence to the RNA you wish to detect. In such a case, one hybridization probe will detect both the RNA marker and RNA from a sample. However, make sure that the RNA marker has the same end-groups as miRNA, i.e., 5′-P and 3′-OH.

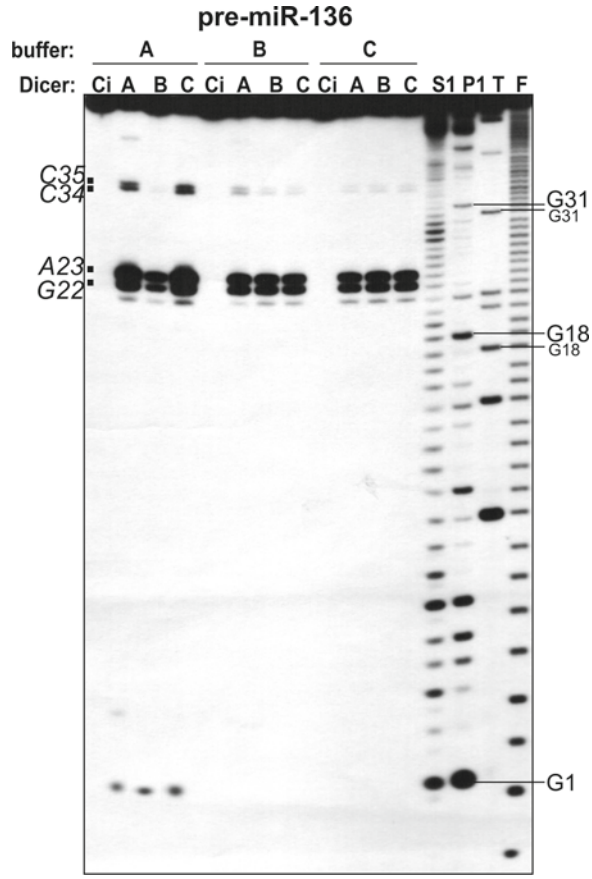

Fig. 4. Comparison of different Dicer preparations with regard to their cleavage specificity. The reactions of the 5′ end-labeled pre-miR-136 transcript using three Dicer preparations (Dicer A was from Stratagene, B was from Ambion, and C was kindly donated by W. Filipowicz) in three different Dicer buffers, as recommended by vendors/donors: buffer A: 150 mM NaCl, 20 mM Tris–HCl pH 8.0, and 2.5 mM $MgCl_2$; buffer B: 300 mM NaCl, 50 mM Tris–HCl pH 9.0, 5 mM $MgCl_2$, and 20 mM HEPES; buffer C: 50 mM NaCl, 30 mM Tris–HCl pH 6.8, 2 mM $MgCl_2$, 0.1% Triton X-100, and 25% glycerol. Lanes Ci—incubation controls without Dicer; Lane S1—S1 ladder; P1—P1 ladder; T—T1 (guanine-specific) ladder; F—formamide ladder. The positions of selected G-residues as well as the positions of Dicer cleavages are shown.

9. Always wear gloves while working with the blotting membranes. Handle the membranes carefully by their edges or by using clean, blunt-ended forceps.
10. At least 10^6 c.p.m./1 mL of the hybridization buffer is required. The probe must be labeled at high-specific activity ($\geq 10^8$ c.p.m./μg template). Too much template used for labeling will lower the specific activity of the probe.
11. Diligently follow all waste disposal regulations when disposing radioactive waste materials.

12. In the same way, calculate the WALDI parameter for the lengths of different fragments:

WALDI = (length of 1-st fragment × intensity of it) + (length of 2-nd fragment × intensity of it) + (length of 3-rd fragment × intensity of it) + (length of 4-th fragment × intensity of it).

If your pre-miRNA is cleaved into two miRNA fragments having 21 and 22 nt of the same intensity, then WALDI will be 21.5.

In the case of pre-miR-31 5′ arm products, the WALDI can be calculated in the following way:

$$\text{WALDI} = (21 \times 0.08) + (22 \times 0.77) + (23 \times 0.15) = 22.07$$

The same way for pre-miR-132:

$$\text{WALDI} = (20 \times 0.06) + (21 \times 0.21) + (22 \times 0.72) = 21.45$$

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Chapter 3

A User-Friendly Computational Workflow for the Analysis of MicroRNA Deep Sequencing Data

Anna Majer, Kyle A. Caligiuri, and Stephanie A. Booth

Abstract

Second-generation high-throughput sequencing is a robust and inexpensive methodology that is becoming an increasingly common technique for the study of microRNA (miRNA) expression levels in the central nervous system. This method allows for the identification of both known and novel miRNAs, reporting on the qualitative and quantitative levels these RNA species represent in any given sample. Numerous bioinformatic programs are currently available to analyze deep sequencing data but many require at least a partial understanding of the command line interface. In this chapter, we describe a user-friendly computational workflow guiding the user through the process from the initial FASTQ deep sequencing file to the identification of known and potentially novel miRNAs in a given experiment, as well as the assessment of the differential expression of these miRNAs between experimental samples. Furthermore, programs that can predict potential targets for these miRNAs are also highlighted.

Key words: MicroRNA, High-throughput sequencing, Central nervous system, Galaxy, miRanalyzer, Ingenuity Pathway Analysis, Program, Workflow, Target prediction, DIANA LAB microT

1. Introduction

The advent of "next generation sequencing" technologies has provided the tools with which to study miRNA expression changes on a global scale and at an unprecedented rate. This platform generates overarching data that provides a detailed glimpse into the global transcriptional events that occur under specific conditions. Nevertheless, this extensive data output, which typically spans millions of sequencing reads, can be challenging to analyze. This bottleneck is the focus of intense activity to design sophisticated software that is specifically tailored to analyze miRNAs. A rapidly increasing knowledge base relating to miRNA biogenesis and function is also driving the refinement of bioinformatic tools. The latest

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advancements allow us to not only identify known miRNA sequences within a sample type and provide both a quantitative and qualitative assessment, but also to predict potentially novel miRNAs. Furthermore, the identification of isomiRs, sequence variants of mature miRNAs generated through RNA processing, or along with the identification of other related small RNA species are also obtainable by deep sequencing methods.

The complete process for miRNA deep sequencing data analysis can be divided into three phases: Primary, secondary, and tertiary analysis (1). Primary analysis covers the initial processing of the image files that are generated during the sequencing run. In this step, each nucleotide base is represented by a different color, resulting in four unique image files. All four images are then aligned to each other and the light intensities are normalized, analyzed, and filtered for base-calling and quality scoring to yield FASTQ and/or FASTA files (2). This analysis is commonly performed by the software that accompanies the sequencer and is beyond the scope of this chapter. One of the most important considerations for miRNA deep sequencing data is the proper exclusion of all sequences that are not miRNAs, such as RNA degradation products, open reading frames, and other noncoding RNA sequences. These steps are performed in both secondary and tertiary phases of analysis (1).

Typically, the secondary analysis encompasses trimming and quality filtering of raw reads and alignment with the reference genome. This part of the analysis can be done using the Galaxy program; an open source, Web-based interface encompassing many different bioinformatic tools (3, 4). In contrast to some protocols, we do not perform the genome alignment during the initial stage of analysis using Galaxy, but rather this is part of the tertiary phase where it forms an important component of the novel miRNA prediction module. Considering that the initial step is to look for known miRNAs, the entire dataset is compared to the annotated sequences in miRBase (5) so as to include as much information as possible. Thus, starting with a FASTQ file, we first remove the 3′ adaptor motif, an artifact of the sequencing process, using Galaxy (Fig. 1). This is executed by the Clip tool which also limits poorly sequenced reads from subsequent filtering steps, and thus, increases the processing speed of the analysis. These imprecise sequencing reads may represent a lack of the 3′ adaptor sequence or consist of less than 15 base pairs in length, too short to be mature miRNAs. Additional filtering performed by the Filter FASTQ tool is used to select for the highest quality reads in the data. More specifically, we employ a Phred33 (6) score of 20; this threshold ensures 99% accuracy in base-calling (7, 8), removing transcripts that contain more than three bases which fall below this threshold. In addition, the filtered output file is converted to a FASTA format, which is readily usable for analysis by many programs, and is easily accomplished using the FASTQ to FASTA tool.

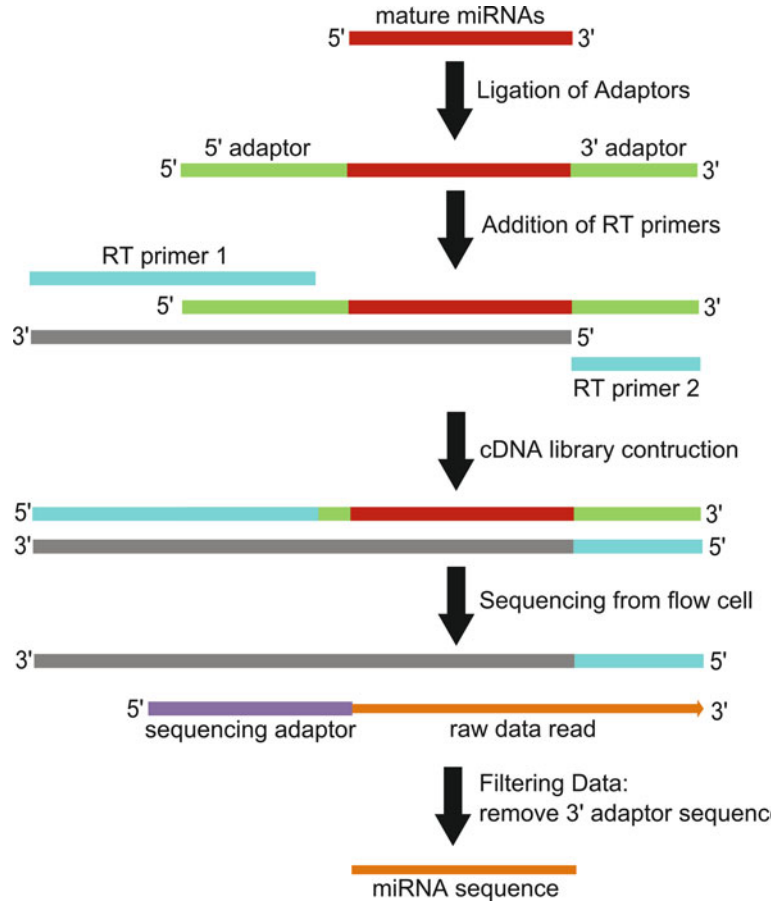

Fig. 1. A brief outline highlighting the sample library preparation. Small RNAs (*red*) are isolated from total RNA followed by the ligation of 3′ and 5′ adaptors (*green*). RT-PCR primers (*blue*) are used to amplify the sample and create a stable cDNA library. The DNA is then denatured and loaded onto the flow cell. The RT primer 2 corresponds to a surface bound primer to attach the antisense strand. The sequencing adaptor (*purple*) binds the antisense strand for read synthesis. The raw data read (*orange*) starts after the sequencing adaptor and incorporates the small RNA and 3′ adaptor sequence. This adaptor sequence must be removed during secondary analysis before aligning to a reference such as miRBase.

The tertiary analysis starts with miRanalyzer (9, 10), a program that aligns all input sequencing reads to libraries of known mature miRNAs such as miRBase. Differential expression analysis of multiple replicates can also be performed via miRanalyzer. The remaining, unmapped sequences are filtered for potential open reading frames and other noncoding RNAs using databases such as RefSeq (11) and Rfam (12). The sequences that do not fall within any of these databases are aligned to the reference genome and further analyzed to predict potentially novel miRNAs identified in at least three different prediction models. The output file containing both known and novel miRNAs can be subjected to additional

bioinformatic analyses to identify the predicted genes of these miRNAs together with any ontological significance.

Prediction of miRNA targets can be done in two ways, depending on the nature of the candidate miRNA. If the miRNA has been annotated previously, target prediction can be done using Ingenuity Pathway Analysis (IPA), which incorporates both predictions from TargetScan (13–15) and experimentally observed miRNA:mRNA associations from TarBase (16, 17). IPA also provides a sophisticated ontological analysis of gene interactions that can be used to further annotate candidate miRNA targets for roles in biological processes involved in central nervous system (CNS) function and dysfunction. If the miRNA of interest is a novel miRNA, the DIANA LAB microT v3.0 program (18, 19) is a suitable starting point to identify potential gene targets.

The protocol described in this chapter encompasses a comprehensive workflow starting with the initial high-throughput sequencing data file and resulting in the identification of known and novel miRNAs. Included in the analysis are the differential expression of these miRNAs between different sample types as well as the prediction of potential target transcripts and their respective functional pathways (Fig. 2).

2. Materials

This protocol was designed to be as user-friendly as possible (advanced computer literacy is not required), and most steps can be performed online. Almost all segments of this protocol are performed explicitly using Windows, although the use of Ubuntu, a version of Unix (which can be found at http://www.ubuntu.com/download), is also required. Freeware programs for processing miRNA deep sequencing data that are highlighted in this chapter include Galaxy (available at http://main.g2.bx.psu.edu/) and miRanalyzer (available at http://bioinfo2.ugr.es/miRanalyzer/miRanalyzer.php). The use of IPA (Ingenuity Systems®, http://www.ingenuity.com) for target pathway exploration of known miRNAs will also be briefly described. Target prediction for novel miRNAs can be done using the DIANA LAB microT v3.0 program (available at http://diana.cslab.ece.ntua.gr/microT/).

3. Methods

3.1. Galaxy: Trimming and Filtering Sequence Reads

1. Large files (>2 GB) must be uploaded via an FTP server by opening any directory or folder within Windows and entering the following into the address bar: ftp://main.g2.bx.psu.

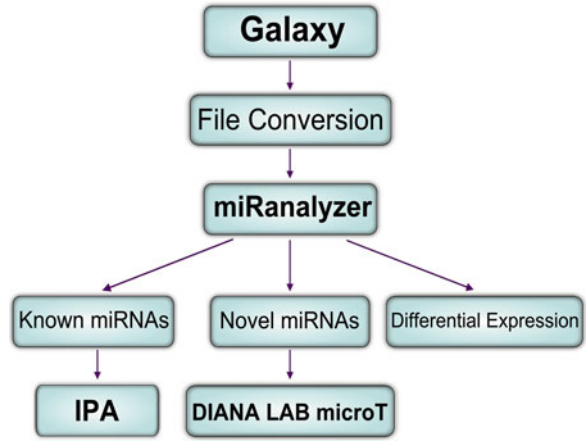

Fig. 2. An overall workflow designed to process miRNA deep sequencing data for both known and novel miRNA sequences, which are then used by additional programs for potential mRNA target identification. Furthermore, differential expression for these high-throughput platforms can also be performed using this workflow. The bolded text represents programs discussed in this chapter.

edu/. A login window will appear prompting you to enter your Galaxy credentials (i.e., user name and password). Press "Log On" and copy and paste your data files into this window.

2. On the Galaxy Web site, go to "User", then "Login". Enter your user name and password then click "Login". In the Tools menu on the left, go to "Get Data" then "Upload File". Under "File Format" select fastqsanger (see Note 1). Choose your files from the "Files uploaded via FTP" section. Under "Genome", the organism from which your sample originated from may be selected by choosing the appropriate descriptor from the pull-down menu (see Note 2). Click "Execute", and you will see your files show up in the History on the right hand side.
3. To trim the 3′ adaptor sequences and to filter your data, import the miRNA workflow that is located at http://main.g2.bx.psu.edu/u/kyle-caligiuri/w/mirna-secondary-analysis by clicking "Import workflow" found on the top right hand side of the main pane. Next, click "Workflow" located at the top of the page and choose the workflow entitled "imported: miRNA Secondary Analysis". Select "Run" from the pop-up menu (see Note 3).
4. Select your data using the drop-down menu below "Library to clip" in "Step 1: Clip" then click "Run workflow".
5. Once all your files have been trimmed and filtered, you can download them by clicking on the name of the file in the green box to expand the box, then clicking the floppy disk icon to Download.

6. To use these files in the miRanalyzer program, the output file(s) need to be converted to a space-separated format which is performed using Ubuntu. Open a terminal in Ubuntu (located under Applications > Accessories) and change to the directory where your files are located. For example, if your files are located in a folder labeled Control in your Documents, type the following and then press Enter:

   ```
   cd Documents/Control
   ```

7. To convert the output data file from Galaxy to the space-separated format, type the following command and press Enter (see Note 4):

   ```
   grep –d ">" input.fasta | sort | uniq –c > sorted.txt
   ```

8. The output file from step 7 needs to be flipped for performing miRanalyzer. This can be achieved by opening the Text Editor (also located under Applications > Accessories) and enter the following two lines:

   ```
   #!/usr/bin/sed –f
   s/^\s\+(\w\+\)\s*\(\w*\)$/\2\1/
   ```

9. Save the file as "flip_script" and ensure it is in the same folder as your datasets (although you can copy and paste the file to other folders if needed).
10. In the terminal, enter the following command for each newly sorted file:

    ```
    cat sorted.txt | sed –f flip_script > output.txt
    ```

3.2. miRanalyzer: Identifies Known miRNAs, Predicts Novel miRNAs, and Compares Datasets to Give you Differential Expression Changes

1. Upload your "output.txt" file by clicking "Browse", and choose the species your sample originated from using the pull-down menu.
2. The following settings should be selected: Zero for the number of mismatches (known miRNAs); one for the number of mismatches (library); select two for the number of mismatches (genome); the threshold of the posterior probability should be set to 0.95; and the minimum number of models which predict the miRNA should be left at the default 3 (see Note 5).
3. Click on the "Launch Program" button for the analysis to commence at which point you will be redirected to an individual page with the following URL:

   http://bioinfo2.ugr.es/miRanalyzer/miRanalyzer.php?launched=true&id=__

   where the "__" represents a unique miRanalyzer job ID number for the submitted analysis (see Note 6).

4. The resulting output analysis, represented by each unique job ID number, will contain sequence information for known and novel miRNAs (see Note 7), which can then be submitted for differential expression analysis by pressing the "Differential Expression" button on the left hand side (see Note 8).
5. Enter the unique miRanalyzer job ID corresponding to the control sample into the text box labeled Group 1 and the treatment job ID into Group 2 and click "Run" (see Note 9).

3.3. Target Prediction for Known and Novel miRNAs Identified from Deep Sequencing Data

1. Make an Excel spreadsheet of the candidate miRNA(s) by listing each unique miRNA in individual rows under the same column with each miRNA being annotated as per miRBase (i.e., mmu-miR-1). Fold change values for each miRNA may also be included in subsequent columns if desired (see Note 10).

3.3.1. IPA: Exploring miRNA Targets and Functional Pathways

2. Upload your miRNA data file by selecting File > Upload Dataset and select the appropriate file and press "Open". Provide information regarding the column headers such that the first column is the miRNA designation or "ID" and if applicable, an additional column contains fold change data that should be designated as "observation 1" and "fold change" in the pull-down menus. Save the file under the appropriate project folder or make a new folder if needed.
3. Once the file is uploaded, the user sees an overall summary of the candidate miRNAs and all additional information. Select "analyze/filter dataset" located in the bottom right corner, then select "miRNA Target Filter" from the pop-up menu to open the analysis window.
4. The "miRNA Target Filter" provides options for filtering candidate miRNA targets and further exploration of the mRNA's functional associations. One can filter mRNA targets for each miRNA by editing the "Relationship > Confidence" column via pressing the "Filter" icon next to that column's title and selecting for moderate, high, or experimentally observed relationships, or a combination of the three. Furthermore, additional columns can be added or removed by using the buttons found on the right hand side of the columns and the data can be sorted accordingly.
5. One of the most important and useful options offered by the miRNA target filter is the inclusion of mRNA data from your own experiments. To do this, select "add/replace mRNA dataset" found at the top, underneath the toolbar > select the mRNA analysis in "My Projects" and press "Next". One may also upload a desired dataset not found in IPA.
6. The "expression pairing" option can be used to filter for the inverse relationship between the miRNA and potential target (i.e., if miRNA is up-regulated the target mRNA should be

down-regulated). This can be achieved by pressing the "expression pairing" button (located next to "add/replace mRNA dataset") and selecting the appropriate data for the miRNA and mRNA expression data columns to use via the pull-down menus and press "OK". Use the "filter" option in the newly-incorporated Expression Pairing column located within the Relationship column such that the fold change of the miRNA is inversely proportional to the mRNA fold change (see Note 11). One can save the analysis by pressing File > Save and/or exporting the data by pressing the "Export Data" icon found at the top of the pane, underneath the Details tab.

7. Once the target gene list has been filtered in the desired manner, pathway associations and functions can be further explored. Select all the genes chosen to be included in the pathway by checking the left hand column boxes and selecting "add to my pathway" above this column, then select "New My Pathway" > "Both" > "Ok". Highlight all the genes by making a box around the newly-created figure, then select "Build". From the "Tool" menu, select "Connect" then press "Apply". Make sure to keep the direct and indirect interactions checked, unless deemed otherwise.
8. For doing a core analysis on the newly selected mRNA candidate list, return to your dataset by pressing "Minimize" at the top right hand corner of the "My Pathways" window. Press "add to my list" > "New My List" > "Both" > "Ok". At this point name and save this file using the "Save My List" button, then press the right arrow icon found on the top right corner of the Lists window, select "Run Core Analysis". Select the appropriate General Settings and other preferences (see Note 12) and then run the analysis by pressing "Run Analysis". You will be prompted to save this analysis, and press "Ok" to continue.

3.3.2. DIANA LAB microT v3.0: Target Prediction for Novel miRNAs

1. On the home page, select the "OR predict for your miRNA sequence" option highlighted in red which is located under the "search for targets of a specific miRNA" panel.
2. Type in your email address to receive the results of the analysis, select the species the novel miRNA originated from and input the sequence in the "file content" window (see Note 13) and select "run job".

4. Notes

1. The data must be in FASTQ Sanger format for this protocol (i.e., Phred33 quality scoring is essential). If your data is already in FASTQ Sanger format and you choose Auto-detect, Galaxy will only establish that it is a FASTQ format but it cannot tell

what scoring method was used. Furthermore, the tools in Galaxy only recognize FASTQ Sanger format, so if your data is not formatted properly, you will have to convert your data to this format after uploading. If this is the case, convert the file format to FASTQ Sanger via "NGS: QC and manipulation" in the Tools pane on the left, then select "FASTQ Groomer" and upload your file.

2. The Genome section is mostly for organizational purposes and is more useful when performing tertiary analysis on Galaxy, which is not the case for this protocol.
3. If desired, the specific settings employed in the workflow can be changed to increase or decrease stringency. In addition, if your data is not from an Illumina sequencer, you will have to change the sequence of the 3′ adaptor that is being trimmed. To do this, select "Edit" from the pop-up menu which will bring you to a workflow of the secondary analysis steps performed by Galaxy. You can click an individual module and its options will display on the right in the Details pane. When you are finished, click Options > Save at the top right of the main pane.
4. The "input.fasta" is the file that was downloaded from Galaxy and "sorted.txt" is the file that will be generated using this command. Sorted.txt is a file containing the number of reads for each transcript and can be renamed if necessary.
5. We did not see any significant differences in our results when setting parameters to the most stringent options (i.e., zero mismatches allowed in "known miRNA", "library" or "genome" and a 0.99 threshold of the posterior probability with three models which predict a novel miRNA). Also, a stand-alone version of miRanalyzer is downloadable where the user can change additional parameters if desired.
6. Avoid moving away from this page until you are able to bookmark the page and/or mark down the jobID so that you can return to your data later.
7. The output analysis produced similar results to an independent analysis performed by LC Sciences (http://www.lcsciences.com/) where the miRNAs that were deemed abundantly present were also the top contenders identified in the analysis done by miRanalyzer.
8. The prediction of novel miRNAs is a very slow process and may take up to one week. Considering that the differential expression analyses take into account novel miRNA reads, it is best not to perform this analysis until the initial job is fully complete.
9. miRanalyzer is able to perform differential expression on multiple replicates of the sequencing runs for the same sample.

This can be performed by separating each job ID using ":". For example, for Group 1 enter jobID1:jobID2:jobID3.

10. It will be advantageous to include fold changes in the upload file because options in IPA exist to pair miRNA expression levels with potential mRNA targets, which further helps to refine the candidate mRNA target list.
11. An inverse relationship between the miRNA and candidate target mRNA is not necessarily true in all cases, hence, caution should be taken when filtering potential miRNA targets in this manner.
12. The General Settings reflect the stringency of the analysis that is being performed on the select list of genes. For CNS work, choose under the "Tissues and Cell Lines" preferences the "Tissues and Primary Cells" > "Nervous System" and the "Cell Line" > "CNS Cell Lines" options. Also, by selecting the species the data originated from in the "Species" preferences, it will make the analysis more relevant to your particular experiment.
13. Up to five novel miRNAs can be analyzed by this program at the same time. The input for all miRNAs must be in the FASTA format which consists of the miRNA name or identifier and the nucleotide sequence as shown below following two lines separated by the "Enter" key:

    >mmu-miR-124

    UAAGGCACGCGGUGAAUGCC

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Chapter 4

The RNA Gene Information: Retroelement-MicroRNA Entangling as the RNA Quantum Code

Yoichi Robertus Fujii

Abstract

MicroRNA (miRNA) and retroelements may be a master of regulator in our life, which are evolutionally involved in the origin of species. To support the Darwinism from the aspect of molecular evolution process, it has tremendously been interested in the molecular information of naive RNA. The RNA wave model 2000 consists of four concepts that have altered from original idea of the miRNA genes for crosstalk among embryonic stem cells, their niche cells, and retroelements as a carrier vesicle of the RNA genes. (1) the miRNA gene as a mobile genetic element induces transcriptional and posttranscriptional silencing via networking-processes (no hierarchical architecture); (2) the RNA information supplied by the miRNA genes expands to intracellular, intercellular, intraorgan, interorgan, intraspecies, and interspecies under the cycle of life into the global environment; (3) the mobile miRNAs can self-proliferate; and (4) cells contain two types information as resident and genomic miRNAs. Based on RNA wave, we have developed an interest in investigation of the transformation from RNA information to quantum bits as physicochemical characters of RNA with the measurement of RNA electron spin. When it would have been given that the fundamental bases for the acquired characters in genetics can be controlled by RNA gene information, it may be available to apply for challenging against RNA gene diseases, such as stress-induced diseases.

Key words: HIV-1, microRNA, Retrotransposon, RNA wave

1. Introduction

One hypothesis of the neutral theory of molecular evolution as the genetic drift was proposed by a geneticist Kimura (1). This hypothesis has been supported by the fact that the DNA genome in human contains the section of "junk," named by Ono (2, 3), to have little purpose. However, two Japanese researchers agreed with natural selection in Darwinism because the evolution of protein was coded

2011.11th.March.14:46. This paper is Requiem for our lost people in Tsunami Japan.

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by the DNA genes that is located in less than 2% of DNA coding region in the human genome. The phylogenetic tree in the protein evolution is able to be elaborated by molecular clocks. Although by the central dogma of Watson-Crick DNA base-pairing, messenger RNA (mRNA) information is read to make a chain of amino acids, the third base of the triplet is insignificant in defining an amino acid. Therefore, the third base would drift aimlessly. However, random gene drift has been ruled out by some significant factor in changing the environment around the gene. In turn, the molecular clock has often indicated different time among the same proteins (4). Further, the protein-coding genes in the genome have been adequately conserved among animals but animal species were tremendously variable (5). Next to the gene drift hypothesis, the selfish gene idea by Dawkins (6) is involved in the continuity on the germ plasm and the protein-coding genes are not modified by the phenotypes that the parents acquired in their own lifetime, but still the single gene is the unit of selection. For all that, there is no address how to inherit an acquired phenotype that would be advantage against natural selection. At first to inherit an acquired phenotype, some factors are transmitted from somatic lines to the germ lines. Secondarily, except for the germ line, mutated gene information has to be transmitted to the next generation by another pathway, such as via milk and/or the placenta (Fig. 1a). What is the factor as genetic information? Now, we know that the junk DNA is not the junk. It contains over thousands of the noncoding microRNA (miRNA) genes in the human genome (miRBase Release 16). Further, milk contains miRNAs (7) and miRNAs pass through the placenta (8). If the profiles of miRNA genes are affected by the pressure of natural selection of the environment, the RNA gene is not randomly drifted and the variation of the RNA genes entail variation in fitness against the environment of life. Before miRNA discovery, the idea of inheritable variation in fitness has not been easy to comprehend. Thus, including the germ line, acquired genetic information, which could be circulated around the environment, may be inherited by the noncoding RNA genes (Fig. 1b).

miRNA is a short length ribonucleotide that does not code polypeptide chain; therefore, bases of miRNA are just information. miRNAs can target hundreds of mRNAs and negatively, sometimes positively control the protein-coding gene expression (9). The miRNA genes are responsible for diverse biological pathways such as cell development, proliferation, differentiation, apoptosis, oncogenesis, metabolism, angiogenesis, inflammation, aging, infection

Fig. 1. (continued) quantum state and could change the basis of miRNA bits. Alteration of miRNA bits can induce miRNA gene expression, then tuning by expressed miRNAs including transcription, translation, splicing, epigenome, mutation, and recombination should be changed within the high-speed terms. Finally, some acquired phenotypes could be obtained and inherited by above description under RNA quantum condition.

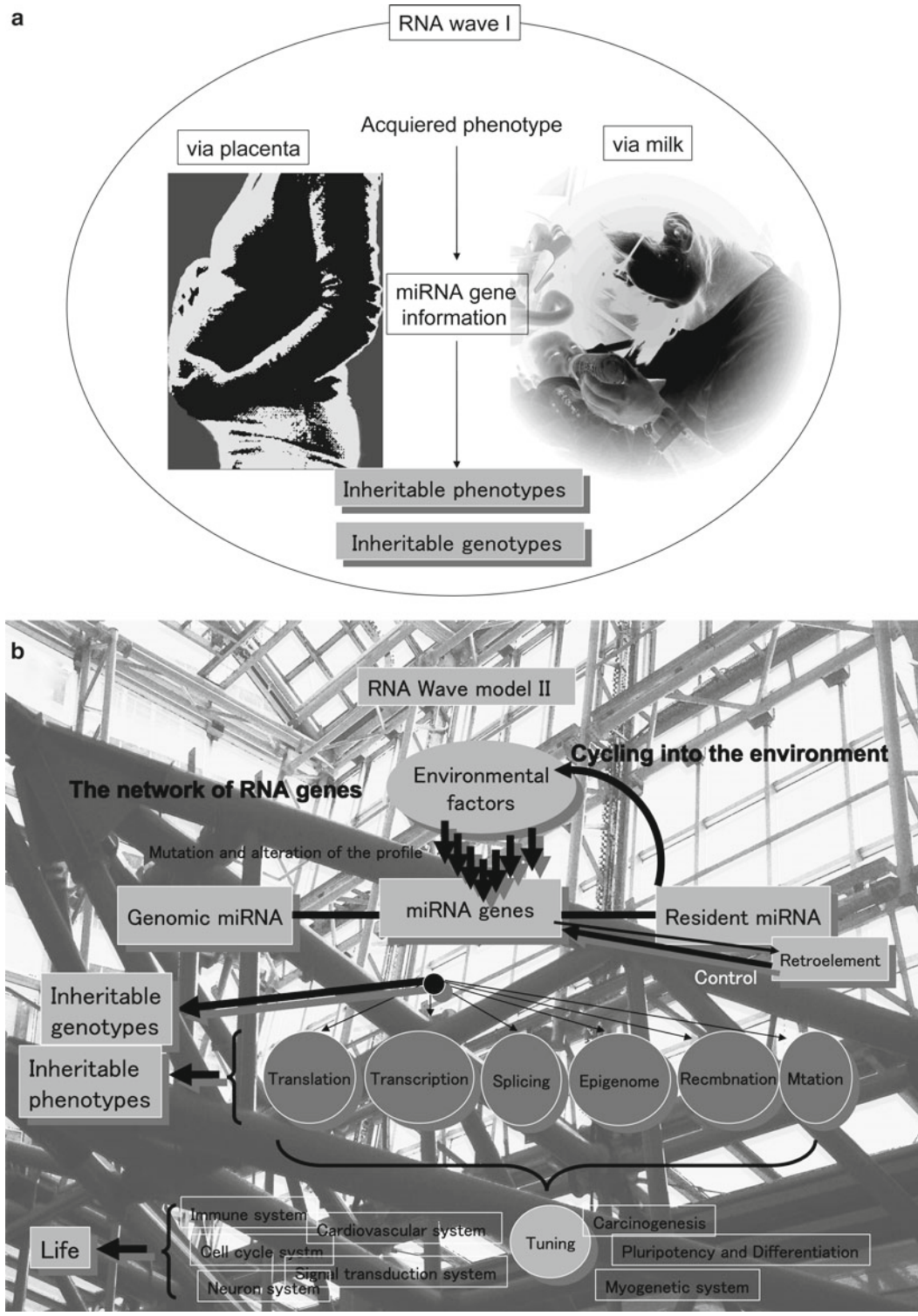

Fig. 1. RNA wave model. (**a**) Acquired characters in part might be somatically inherited by RNA information from a mother to a child. Information on the non-selfish RNA genes including the resident miRNAs and retroelements may move inter somatic and germ cells and reverse-transcribed into the DNA genome. Alteration of miRNA profiling can become an inheritable phenotype. Thus, the robustness of gene regulatory networks would partly rely on vertiginous RNA into RNA pool under the RNA wave. (**b**) Further, environmental factors, such as RNA information and various stresses may affect the host miRNA

and its pathogenicity, the response including DNA damage to environmental factors, such as carcinogens (10–26). Given the researched results including profiling of all the resident and genomic miRNA genes in embryonic stem cell (ESC), the seed sequences of 2- to 7 or 8-nucleotides (nts) in the 5′-end of miRNAs would have been reported to recognize not only the 3′-untranslated regions (3′UTR) to its miRNAs but also the coding regions and 5′UTR of *Oct4*, *Sox2*, and *Nanog* mRNAs. This would be carried out through an imperfect match to repress target mRNA and probable stability of the mRNA target (27); the resident miRNA genes may be transferred from ESCs to the neighboring cells by microvesicles like retrotransposons (28). Since this kind of short seeds is common in the cases described above, there is also a long seed through whole sequences of miRNAs, suggesting that long noncoding RNA and nanoRNA genes could also control gene translation (29). Further, recent cancer research indicates that alteration of miRNA profiles could induce tumor (30). For example, miR-125b-1 was inserted into immunoglobulin heavy chain DNA gene locus by unknown mechanisms in B cell acute lymphoblastic leukemia (31). Although pre-miRNA involves both the guide and the passenger strands and each of them is selected for targeting of mRNAs, a single mutation has been shown that the strand selection is shifted and resulted in altered expression of gene (32). If the RNA gene is a master of regulator in our life, the single base information of the RNA gene may play a critical role in evolution and diversity in fitness according to the acquired phenotype (see Fig. 1).

By the high throughput analysis of the miRNA gene, ESC-specific miRNAs have been cloned and isolated (33). Observations of these experiments suggest that the classical promoter-transcription-protein scheme alone is contradicted because the miRNA gene has also been reported to affect against transcriptional process (34–36). In these complex networking relations, bioinformatics may play a key role albeit in the RNA genes. A subset of the Watson-Crick DNA base-pairing model in the seed sequences is currently used to explain the incomplete or sometimes complete target pairing of miRNA. A rather broader set of additional rules may be useful to explain how some RNA genes to develop bioinformatics approaches. Although the short interfering RNA (siRNA) gene in the plant cells targets mRNA with complete base-pairing using all bases of the endo-siRNA gene (37), it is uncertain whether the miRNA gene in the human cell is completely paired with the target mRNA based on the seed or all base sequences. In zebrafish, miR-214 with no canonical seed pairing can effectively target an mRNA for silencing (38). Recent data also suggest that fine-tuning by the miRNA genes is probably a high-speed and superposing event.

Thus, we add a neo-mechanism for the regulation by the miRNA gene, which can be affected by environmental factors.

DNA-based computing has been reported to solve mathematical problem (39) and a molecular computation with RNA has also been shown to extend the DNA-based computing (40). However, these programs could not be involved in elucidation of the biological function of the nucleotides. To elucidate the biological function of the miRNA gene, we have introduced RNA wave-based model that could be applied for mathematical calculation to miRNA information flow under the quantum-computing steps (41, 42). As far as experimental measurement of the quantum energy, it has already been reported that the photophysical properties of semiconductor quantum dots (QDs) make this approach ideal for spectral labeling and luminescent probing of the methylated nucleotide detection (43, 44). If miRNA is circular like a "torus," miRISC including helicase activities links to an miRNA ring. According to electrostatic potential of each atom of the RNA bases with methyl residues instead of ribose 5-phosphate by the fragment molecular orbit methods (FMO) based on quantum theory, total electrostatic potential of the RNA bases could be calculated (45). The superposing of the magnetic direction of electrons in the high dimensions may be used for the RNA gene coding. Here, we explore and explain an extended RNA wave model based on quantum theory for RNA coding operation without the operon involvement.

2. Materials

2.1. Preparing Softwares

Computing analysis of the oligonucleotide base was performed by the WinMOPAC software version 3.0 (SCIGRESS MO COMPACT PROFESSIONAL: Fujitsu Corp., Tokyo, Japan). Calculated FMO values in radar-circle as solid tori were depictured by KINGSOFT SpreadSheets software (http://www.kingsoft.jp: KINGSOFT Corp., Tokyo, Japan).

2.2. Source of RNA Sequences

The miRNA sequences were prepared from miRBase Release 16 2010 (http://www.mirbase.org).

3. Methods

3.1. microRNA and the Quantum Theory

miRNA may be a master of regulator in our life, which is involved in the origin of species. By analogy between the quantum computation and miRNA function (41, 42), we introduce the Hilbert

space associated with the quantum system at first in the (0, 1, 2, …, $n-1$) states of atoms in an RNA nucleotide.

$$|\psi> = \sum_{i=0}^{n-1} |\psi_i> \omega_i \tag{1}$$

From (1), 2 qubits basis states as an example are shown as follows:

$$|\psi^{(1)}> = |\psi_0{}^{(1)}> \omega_0{}^{(1)} + |\psi_1{}^{(1)}> \omega_1{}^{(1)} \tag{2}$$

$$|\psi^{(2)}> = |\psi_0{}^{(2)}> \omega_0{}^{(2)} + |\psi_1{}^{(2)}> \omega_1{}^{(2)} \tag{3}$$

Tensor product is simplistically described from (2) and (3)

$$|\psi^{(1)}> \otimes |\psi^{(2)}> = |\psi^{(1,2)}>$$

Increasing of dimension as the local qubit basis state of atoms in an RNA nucleotide, (2) is

$$|\psi> = |\psi^{(1)}> \otimes |\psi^{(2)}> \otimes |\psi^{(3)}> \otimes |\psi^{(4)}> \otimes ------ |\psi^{(n-1)}>$$

Each RNA nucleotide qubit basis is represented as,

$$|\psi_a> = |\psi_a{}^{(1)}> \otimes |\psi_a{}^{(2)}> \otimes |\psi_a{}^{(3)}> \otimes |\psi_a{}^{(4)}> \otimes ------ |\psi_a{}^{(n-1)}> \tag{4}$$

$$|\psi_u> = |\psi_u{}^{(1)}> \otimes |\psi_u{}^{(2)}> \otimes |\psi_u{}^{(3)}> \otimes |\psi_u{}^{(4)}> \otimes ------ |\psi_u{}^{(n-1)}> \tag{5}$$

$$|\psi_g> = |\psi_g{}^{(1)}> \otimes |\psi_g{}^{(2)}> \otimes |\psi_g{}^{(3)}> \otimes |\psi_g{}^{(4)}> \otimes ------ |\psi_g{}^{(n-1)}> \tag{6}$$

$$|\psi_c> = |\psi_c{}^{(1)}> \otimes |\psi_c{}^{(2)}> \otimes |\psi_c{}^{(3)}> \otimes |\psi_c{}^{(4)}> \otimes ------ |\psi_c{}^{(n-1)}> \tag{7}$$

Since quantum computation deals with Fourier transformation as a unitary transformation, that state corresponds to binary representations of integers on the computation. The quantum bit is as follows.

$$|\psi> = \alpha|0> + \beta|1>, |\alpha|^2 + |\beta|^2 = 1 \tag{8}$$

$$|\psi> = e^{i\gamma}(\cos\theta/2|0> + e^{i\varphi}\sin\theta/2|1>) \tag{9}$$

We apply the 1s state of the orbit of atom, $e^{i\gamma} = 1$. Therefore, transformation of (9) is

$$|\psi> = \cos\theta/2|0> + e^{i\varphi}\sin\theta/2|1> \tag{10}$$

As shown in Fig. 2a, (10) is represented on bloch sphere.

From these mathematical models of computing, that computation is dependent on the law of physics. Feynman (46) has suggested that integers of the electron wave are the orbits of the electron of atoms, in which quantum mechanisms appear to computation states.

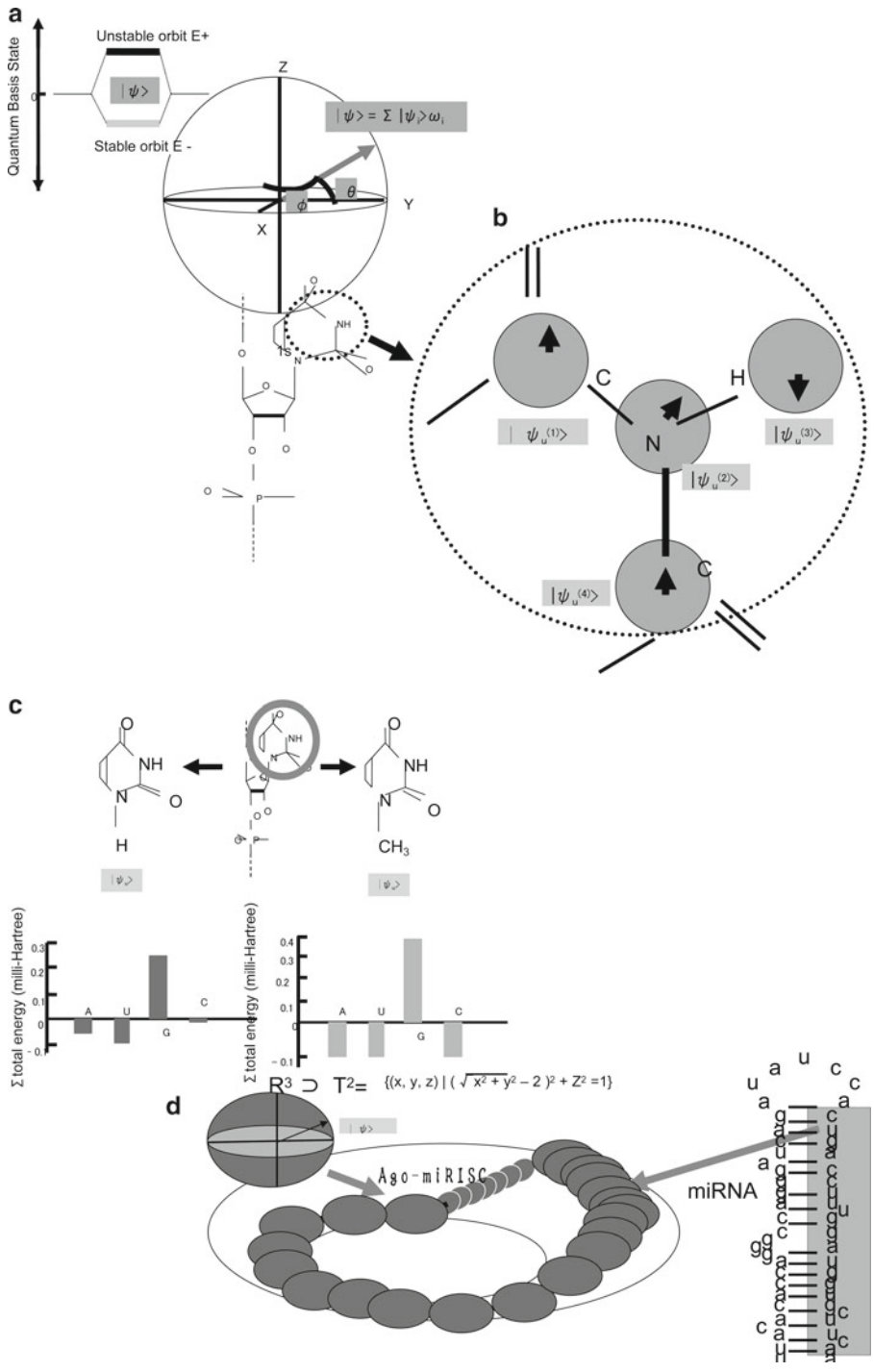

Fig. 2. miRNAs and quantum theory. (**a**) Tow quantum basis states and bloch sphere. (**b**) The quantum basis state of uracil. (**c**) Values of FMO in each atom of 1-methyl-uracil and uracil. (**d**) The FMO values of each nucleotide were corresponding to miRNA RNA sequences such as *MIRN367*.

From general quantum integers,

$$-\frac{\hbar^2}{8\pi^2 m}\frac{d^2\psi}{dx^2}+U_\psi=E_\psi \tag{11}$$

$$-\frac{\hbar^2}{8\pi^2 m}\frac{d^2}{dx^2}+U=H \tag{12}$$

(12) is put into (11),

$$H\Psi=E\Psi \tag{13}$$

As Ψ* is adjoint to ψ, the unitary transformation is used for (13).

$$\Psi^* H\Psi=\Psi^* E\Psi$$

$$\hbar=1.055\times10^{-34}\,\mathrm{J\cdot s},\quad m=9.109\times10^{-31}\,\mathrm{kg}$$

Therefore,

$$E=\frac{\int\Psi^* H\Psi dT}{\int\Psi^*\Psi dT} \tag{14}$$

(8) is applied into (14).

$$E=\frac{\int(\alpha_1|0>+\beta_1|1>)H(\alpha_2|0>+\beta_2|1>)dT}{\int(\alpha_1|0>+\beta_1|1>)(\alpha_2|0>+\beta_2|1>)dT}$$

If we apply only 1s orbit of electron, the amplitudes of $\alpha_1=\alpha_2=\alpha,\beta_1=\beta_2=\beta$.

$$\begin{aligned}&\int(\alpha|0>+\beta|1>)H(\alpha|0>+\beta|1>)dT\\&=\int(\alpha|0>+\beta|1>)^2 H\quad dT\\&=\alpha^2\int|0>H|0>dT+2\alpha\beta\int|0>H|1>dT+\beta^2\int|1>H|1>dT\end{aligned} \tag{15}$$

and,

$$\begin{aligned}&\int(\alpha|0>+\beta|1>)(\alpha|0>+\beta|1>)dT\\&=\int(\alpha|0>+\beta|1>)^2 dT\\&=\alpha^2\int|0>^2\ dT+2\alpha\beta\int|0>|1>dT+\beta^2\int|1>^2\ dT\end{aligned} \tag{16}$$

therefore,

$$E=\frac{\alpha^2\int|0>H|0>dT+2\alpha\beta\int|0>H|1>dT+\beta^2\int|1>H|1>dT}{\alpha^2\int|0>^2\ dT+2\alpha\beta\int|0>|1>dT+\beta^2\int|1>^2\ dT}$$

$$\int |0><H|0><dT = H_{00}, \int |0><H|1><dT = H_{01}, \int |1><H|1><dT = H_{11}$$

$$\int |0>^2 dT = P_{00}, \int |0><|1><dT = P_{01}, \int |1>^2 dT = P_{11}$$

were put into (15) and (16),

$$E = \frac{\alpha^2 H_{00} + 2\alpha\beta H_{01} + \beta^2 H_{11}}{\alpha^2 P_{00} + 2\alpha\beta P_{01} + \beta^2 P_{11}} \tag{17}$$

α and β are the amplitudes; therefore,

$$\partial E / \partial\alpha = 0, \quad \partial E / \partial\beta = 0$$

(17) is transformed as follows:

$$\alpha(H_{00} - EP_{00}) + \beta(H_{01} - EP_{01}) = 0 \tag{18}$$

$$\alpha(H_{01} - EP_{01}) + \beta(H_{11} - EP_{11}) = 0 \tag{19}$$

$H_{00} = H_{11}$ and $P_{00} = P_{11} = 1$ are applied to (18) and (19),

$$\alpha(H_{00} - E) + \beta(H_{01} - EP_{01}) = 0 \tag{20}$$

$$\alpha(H_{01} - EP_{01}) + \beta(H_{11} - E) = 0 \tag{21}$$

Finally, (20) and (21) each is calculated as

$$E+ = \frac{H_{00} + H_{01}}{1 + P_{01}} \tag{22}$$

$$E- = \frac{H_{00} - H_{01}}{1 - P_{01}} \tag{23}$$

Two energy states E in (22) and (23) of each atom into the nucleotide are represented with quantum bits on bloch sphere (Fig. 2a) and these values are computed with winMOPAC software as FMO. *E*+ shows unstable state of electron in a molecular qubit and *E*– is a stable one.

3.2. Fragment Molecular Orbit Method for the Nucleotide

Therefore, values of FMO in each atom of the nucleotide are superposed up from (1, 3–6) of tensor products, such as uracil (Fig. 2b).

$$|\psi_u^{(1)}> \equiv E_{C-4}, |\psi_a^{(2)}> \equiv E_{N-3}, \quad ----------,$$

the ring numbers of atoms in the uracil molecule were given into the entry (*i*),

$$(N-1, C-2, N-3, C-4, C-5, C-6, \cdots) = (0, 1, 2, \cdots, n-1)$$

therefore,

$$|\psi> \equiv \sum_{i=0}^{n-1} E_i \tag{24}$$

In the case of uracil (u) (24), 1-methyl-uracil and uracil are −0.104 and −0.03 milli-Hartree (mH), respectively (Fig. 2c). 9-methyl-guanine and guanine (g) are 0.43 and 0.258, 9-methyl-adenine and adenine (a) are −0.1 and −0.05, and 1-methyl-cytosine and cytosine (c) are −0.101 and −0.002, respectively. The values of (1, 3–6) are approximately represented as

$$|\psi_a> \equiv -0.1$$
$$|\psi_u> \equiv -0.1$$
$$|\psi_g> \equiv +0.4$$
$$|\psi_c> \equiv -0.1$$

3.3. FMO Values of miRNAs

The FMO values of each nucleotide were corresponding to miRNA RNA sequences (Fig. 2d), such as miR-21 from miRBase data base as follows and with Fig. 3a;

```
#include <mirna-21.h>
int_set (int mir-21 [ ], int no)
{
    int u;
    Int a;
    Int g;
    Int c;
    for (u = -0.1; u <0; u ++++)
  return
    for (a = -0.1; a<0; a++++ )
  return
    for (g = 0.4; g<1; g+++ )
  return
    for (c = -0.1; c<0; c++++)
  return
}
```

The miR-21 FMOs were represented as wings by KINGSOFT SpreadSheets 2010 into the below (Fig. 3b). The calculated potencies were obtained in the radar shape. As shown in Fig. 3b, the basis state of potencies of the miRNA genes is shown as very different G spots.

3.4. The Basis State of the RNA Gene Bits

At the time, to think about the basis state of spanning set of bits in vector subspace of miR FMOs, the nucleotide bits were transformed into the binary state 1 and 0 as follows:

$$|g> = \begin{pmatrix} 1 \\ 0 \end{pmatrix} = |1> \qquad |a> = |u> = |c> = \begin{pmatrix} 0 \\ 1 \end{pmatrix} = |0>$$

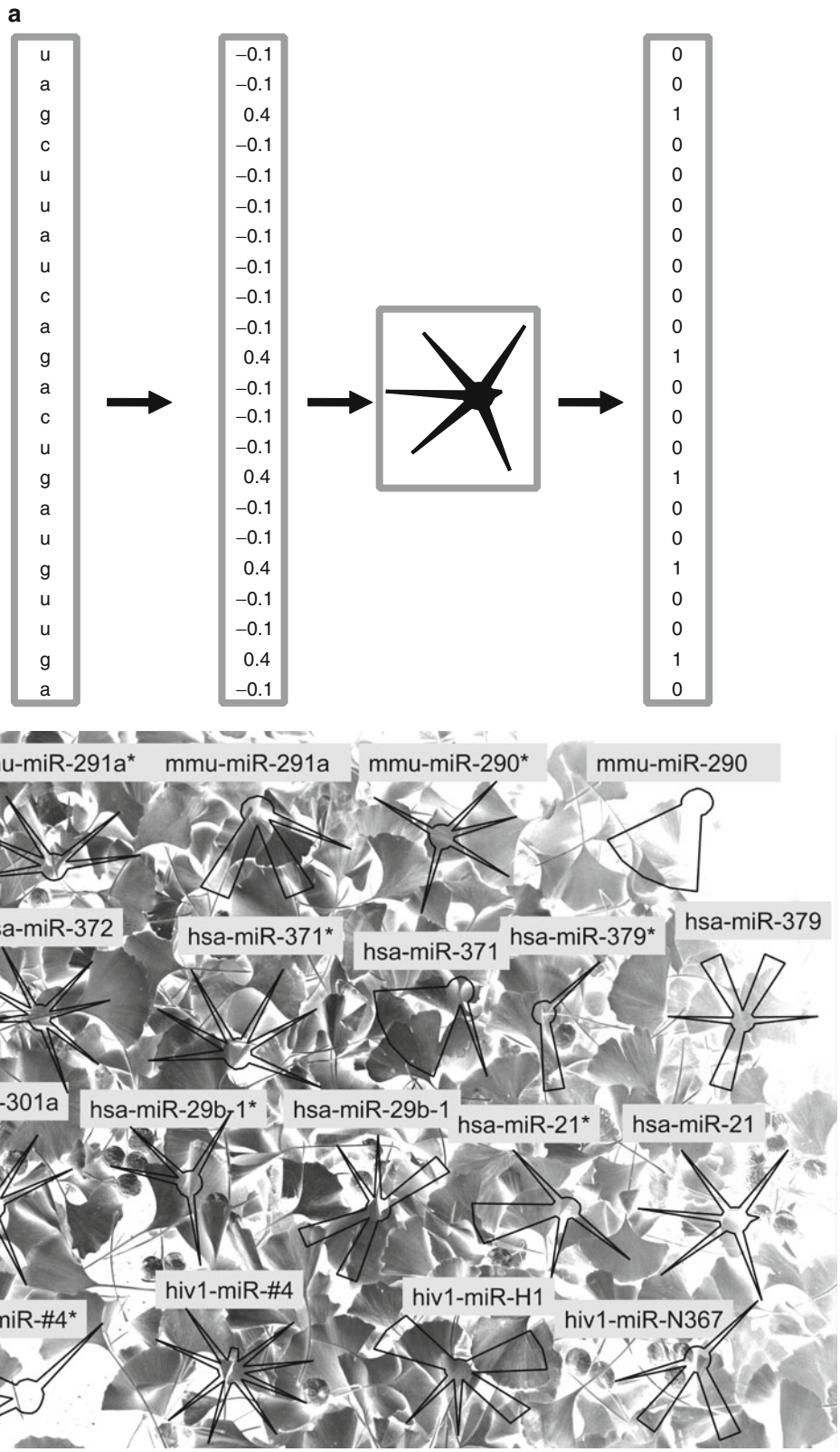

Fig. 3. FMO values of miRNAs to the basis state of miRNA bits. (**a**) Processes of miRNA sequences to miRNA bits by the SpreadSheets program similar to the Excel one (Microsoft Corp. USA). (**b**) The radar shapes of the calculated FMO potencies in miRNAs.

In Hilbert space, one inner product space X of miR-21 and miR-N367 (Fig. 4a) are shown as

$$X_{21} :| 00100000001000100010 >$$

$$X_{N367} :| 00010000001101000000 >$$

In the case of X_{N367}, the HIV-1 3′-LTR should be targeted as shown in Fig. 4a.

$$Y_{LTR} :| 1100111000 \cdots\cdots 0000000100 >$$

As miRNA could tune multigene expression, the relation between X_{n-1} ($n = 1, \ldots, k$) and another inner product space Y of mRNA in the Hilbert space is shown so that the Tensor product could approximately be mapped to the Kroneker product (Fig. 4b),

$$X_{n-1} \otimes Y = \begin{pmatrix} X_0 \\ \vdots \\ X_k \end{pmatrix} \otimes Y \equiv \begin{pmatrix} X_0 \cdot Y \\ \vdots \\ X_k \cdot Y \end{pmatrix} \equiv (X_{n-1}, Y)$$

MIRN367, *MIRH1*, *MIR#4*, and *MIR#4** became imaginarily entangled with HIV-1 3′-LTR (Fig. 4c) because of less representative figure as the higher dimensions of the Hilbert space. Since miR-106, miR-17, miR-20, and miR-93 have been reported as human ESCs-specific miRNAs, we focused on their kets (Table 1). The miR-106a, miR-17, miR-20a, miR-20b, and miR-93 genes showed the same ket, |000010100000001010011001>. "0010100" is involved into the seed region of miRNAs (see Note 1).

4. Notes

1. About transformation from RNA information to quantum information. We showed here that RNA information could be involved in physics according to quantum-computing theory (41, 42). Although the RNA gene potencies based on quantum-computing theory were represented, the superposing of the magnetic field with *MIRN367*, *MIRH1*, *MIR#4*, *MIR#4**, and *HIV-1 3′LTR* may concretely constitute the code of RNA. If the likelihood principle is used (47), we can represent the statements involved in the miRNA gene as follows:

   A: Information is physics.

   B_1: The miRNA genes are information.

   B_2: The miRNA genes are in physics.

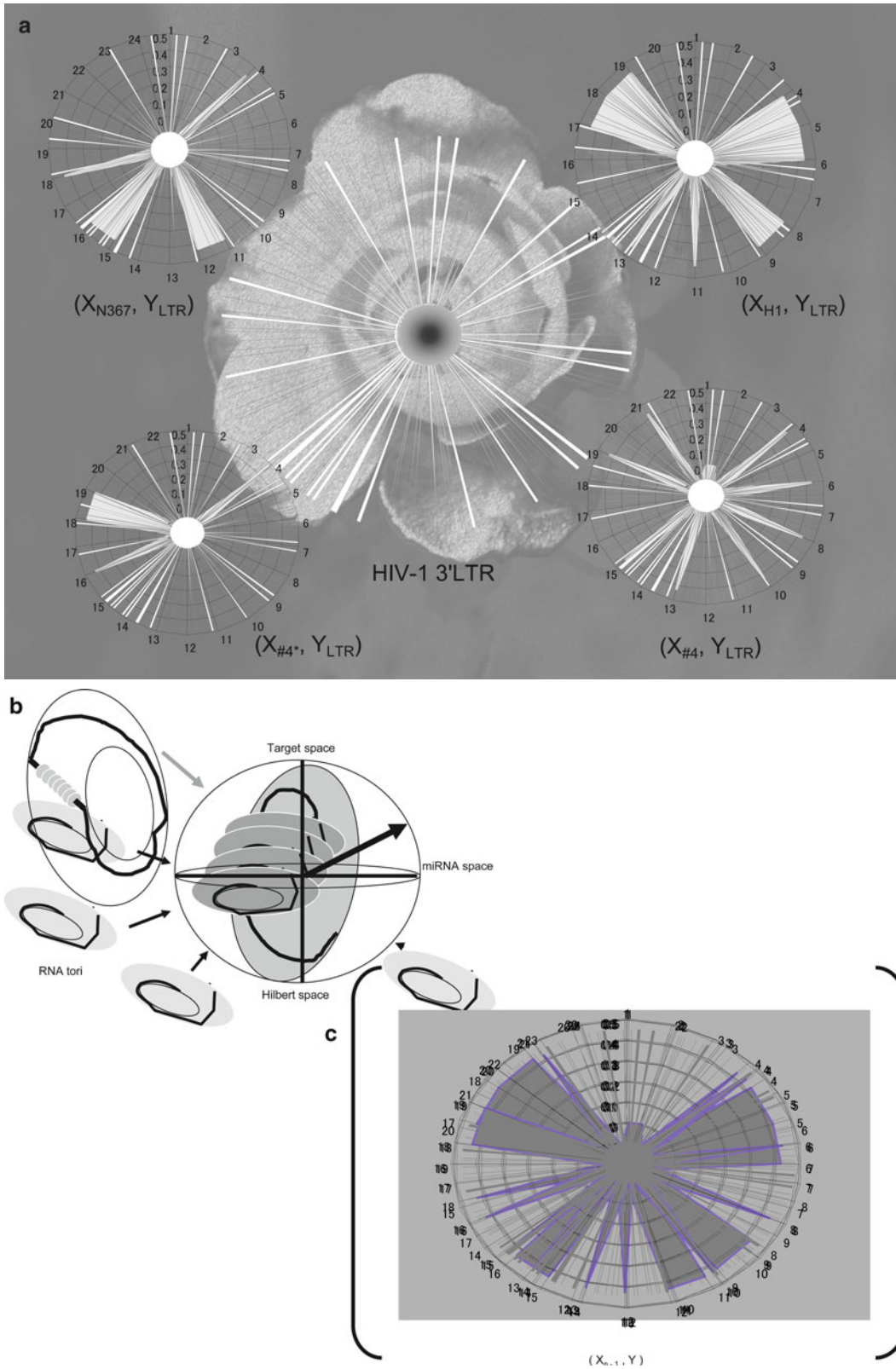

Fig. 4. The basis state of miRNA bits. (**a**) The FMO potencies of HIV-1 derived miRNAs, *MIRN367*, *MIRH1*, *MIR#4*, *MIR#4** (X), and the HIV-1 *3′LTR* (Y) as a target. (**b**) Image of the Hilbert space. (**c**) The relation between miRNA X_{n-1} ($n=1, \ldots, k$) and another inner product space Y of target mRNA in the Hilbert space is represented.

Table 1
Basis state of ket modules of nuclear or cytoplasm expressed miRNAs in human neural stem cell

miRNAs[a]	Sequences	Basis ket modules
The nuclear distribution		
miR-16	UAGCAGCACGUAAAUAUUGGCG	|00100100010000000001101>
miR-30c	UGUAAACAUCCUACACUCUCAGC	|01000000000000000000010>
miR-19a	UGUGCAAAUCUAUGCAAAACUGA	|01010000000001000000010>
miR-30b	UGUAAACAUCCUACACUCAGCU	|01000000000000000000100>
miR-331-3p	GCCCCUGGGCCUAUCCUAGAA	|10000011100000000100>
miR-342-3p	UCUCACACAGAAAUCGCACCCGU	|00000000010000010000010>
miR-345	GCUGACUCCUAGUCCAGGGCUC	|10010000000100001110000>
miR-320	AAAAGCUGGGUUGAGAGGGCGA	|00001001110010101110010>
miR-186	CAAAGAAUUCUCCUUUUGGGCU	|00001000000000000011100>
miR-374	UUAUAAUACAACCUGAUAAGUG	|00000000000000010000101>
miR-140-5p	CAGUGGUUUUACCCUAUGGUAG	|00101100000000000011001>
miR-28-3p	CACUAGAUUGUGAGCUCCUGGA	|00000100001010100000110>
miR-590-5p	GAGCUUAUUCAUAAAAGUGCAG	|10100000000000000101001>
miR-598	UACGUCAUCGUUGUCAUCGUCA	|00010000010010000001000>
miR-197	UUCACCACCUUCUCCACCCAGC	|00000000000000000000010>
miR-339-3p	UGAGCGCCUCGACGACAGAGCCG	|01010100001001000101001>
miR-193b	AACUGGCCCUCAAAGUCCCGCU	|00001100000000010000100>
miR-335	UCAAGAGCAAUAACGAAAAAUGU	|00001010000000100000010>
miR-210	CUGUGCGUGUGACAGCGGCUGA	00101010101000101100l0>

miR-219-1-5p	UGAUUGUCCAAACGCAAUUCU	|01000100000010000000>
The cytoplasmic distribution		
miR-106a	AAAAGUGCUUACAGUGCAGGUAG	|00001010000001010011001>
miR-17	CAAAGUGCUUACAGUGCAGGUAG	|00001010000001010011001>
miR-20a	UAAAGUGCUUAUAGUGCAGGUAG	|00001010000001010011001>
miR-20b	CAAAGUGCUCAUAGUGCAGGUAG	|00001010000001010011001>
miR-93	CAAAGUGCUGUUCGUGCAGGUAG	|00001010000001010011001>
miR-26a	UUCAAGUAAUCCAGGAUAGGCU	|00000100000000110001100>
miR-21	UAGCUUAUCAGACUGAUGUUGA	|00100000001000100010010>
miR-130a	CAGUGCAAUGUUAAAAGGGCAU	|00101000010000000111000>
miR-18a	UAAGGUGCAUCUAGUGCAGAUAG	|00011010000001010010001>
miR-15b	UAGCAGCACAUCAUGGUUUACA	|00100100000000011000000>
miR-301b	CAGUGCAAUGAUAUUGUCAAAGC	|00101000010000010000010>
miR-125b	UCCCUGAGACCCUAACUUGUGA	|00000101000000000001010>
miR-221	AGCUACAUUGUCUGCUGGGUUUC	|01000000010001001110000>
miR-135a	UAUGGCUUUUUAUUCCUAUGUGA	|00011000000000000001010>
miR-103	AGCAGCAUUGUACAGGGCUAUGA	|01001000010000111000010>
miR-100	AACCCGUAGAUCCGAACUUGUG	|00000100100001000000101>
miR-25	CAUUGCACUUGUCUCGGUCUGA	|00001000001000011000010>
miR-99a	AACCCGUAGAUCCGAUCUUGUG	|00000100100001000000101>
miR-744	UGCGGGGCUAGGGCUAACAGCA	|01011110001110000000100>
miR-532-5p	CAUGCCUUGAGUGUAGGACCGU	|00010000101010011000010>

(continued)

Table 1
(continued)

miRNAs[a]	Sequences	Basis ket modules
miR-324-5p	CGCAUCCCCUAGGGCAUUGGUGU	|01000000000111000011010>
miR-28-5p	AAGGAGCUCACAGUCUAUUGAG	|00110100000010000000101>
miR-652	AAUGGCGCCACUAGGGUUGUG	|00011010000001110010 1>
miR-219-2-3p	AGAAUUGUGGCUGGACAUCUGU	|01000010110011000000010>
miR-18b	UAAGGUGCAUCUAGUGCAGUUAG	|00011010000001010010001>
miR-363	AAUUGCACGGUAUCCAUCUGUA	|00001000110000000000100>
miR-152	UCAGUGCAUGACAGAACUUGG	|00001010001000100000011>
miR-27b	UUCACAGUGGCUAAGUUCUGC	|00000010110000100001 0>
miR-34a	UGGCAGUGUCUUAGCUGGUUGU	|01100101000001001100 10>
miR-128	UCACAGUGAACCGGUCUCUUU	|00000101000011000000 0>
miR-361-5p	UUAUCAGAAUCUCCAGGGGUAC	|00000010000000011110 00>

[a]Nuclear and cytoplasmic hsa-miRNAs were determined by Jeffries et al. (52)

If we agree with A, we claim that $P(A|B_1) \equiv P(A|B_2)$. The target sequences of miRNAs as information have been investigated into the short-seed model (nt 7–8); however, the retroelement, Alu RNA can directly cause human pathology, such as geographic atrophy (48) and tumor cells release an abundance of microvesicles containing miRNAs as well as LINE or Alu RNA (49), suggesting that retroelement RNA would not have the seed region but its whole RNA would be information for diseases. Therefore, further to understand the RNA code even if long RNA genes would be involved in cause of diseases, this argument should rest on an analogy between quantum-computing and the miRNA gene information. It has actually been reported that migratory birds can use a physiological magnetic compass to orient their position on the map of the earth based upon the biochemical pathway in the birds (50). This report suggests that the physical information could fall within a bird's cognizance as the biophysical information such as RNA quantum codes. Thus, we have investigated the RNA information as a sensor against environment according to physics.

Recently miRNAs have been found in the human cell nuclear (51, 52). Therefore, the genomic RNA genes contain two types of genes, both DNA and RNA genes. These research groups have explored that a 6 nts sequence "aguguu" and 4 nts sequence "aSuS (S = c or g)" at the 3′ end of miRNAs are occasionally responsible for preferential localization in the nucleus and the nuclear, respectively. Further, siRNAs that mimic the genomic miRNA genes have been reported to target noncoding RNAs, suggesting that the nuclear miRNAs could be involved in a similar regulatory role of the plant endogenous miRNA and siRNA genes, such as direct transcriptional regulation as previously shown in the RNA wave model (45). In that case, miRNA base-pairing seed rule may differ from those that could have done in the cytoplasm of human cells. The basis state of qubits of the nuclear and cytoplasm miRNAs are represented as very different kets, except for identical five cytoplasm miRNAs of miR-106a, miR-17, miR-20a, miR-20b, and miR-93. Since excitation and emission states of the ket basis state are able to be changed by the environmental quantum stress as the input bits, all miRNA genes could be translocated into the nuclear to be robust for RNA–RNA interaction. Further, the subcellular localization of miRNAs may be due to quantity and quality of the quantum energy of miRNA molecules as the output bits rather than qualitative one only, such as a nuclear localization motif based on Watson-Crick DNA base-pairing (53). Therefore, we have to investigate experimental support to confirm this hypothesis. It should be noted that epigenetic DNA methylation would be similar to above miRNA quantum characters and the epigenetic programming of the sequences could have contained the stable and heritable genetic information that would be related with an acquired characters in responsible to environmental stress (54).

Interaction between CpG methylation, histone modification, and RNA genes has been reported to be involved in epigenetic silencing (55, 56). What controls the quantitative methylation-mark changing on DNA according to the physical atomic theory (57)?

Information on the non-selfish RNA genes including the resident miRNAs and retroelements may be regarded as fundamental unit of the evolution to move inter somatic and germ cells in genomic space (45). The mobile RNA gene information contains two types of the gene information, these are both protein and RNA itself (58–61). Information of the RNA genes would horizontally transpose not only from cell to cell but also from our environment by feeding of RNA genes-containing foods including milk, retroviruses, etc., and via placenta from a mother to her own child (7, 8, 58) (see Fig. 1a). It is different from nonmobile DNA information. An acquired information by the resident miRNA genes could directly control transcription (62) and posttranscriptional pathways, epigenome, recombination, and mutation in host. The acquired information of the retroposable RNA genes might create phenotype by fine-tuning of gene expression in the genomic space (63–68), in which its genotype can be vertically inherited under pressures of the environment to fit into Darwinism (69). Thus, under the quantum RNA condition and occasion, acquired characters could be controlled by the RNA genes that have an important role for the laws of inheritance in human.

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Chapter 5

Setting Up an Intronic miRNA Database

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Abstract

In the recent past, intragenic microRNAs (miRNAs) have gained significant attention. Due to the unique linkage to their host gene's transcription, these miRNAs offer more information than intergenic miRNAs as they associate with some of their hosts' properties. However, genome wide analysis of intronic miRNA data can be very challenging, especially if it relies on Web-based tools only. We therefore describe in this chapter how to set a database and how to link the different publicly available information resources on miRNAs and host genes. We also provide an example of a simple, but useful analysis technique. The basic structures and ideas suggested in this chapter can easily be extended to integrating other data and be applied to different analysis techniques.

Key words: Intronic miRNAs, miRNA bioinformatics, Biological databases, miRBase bioinformatics, Information retrieval, Kyoto encyclopedia of genes and genomes analysis, Gene ontology analysis

1. Introduction

MiRNAs are not a homogeneous class of small, noncoding regulatory RNA molecules. Depending on their genomic location, they can be subcategorized as intra- and intergenic (1, 2). Approximately half of the miRNAs are found in intergenic regions, whereas the overwhelming majority of the other half are localized in intronic regions of protein-coding genes, while a minority are even found within exons (3). Intergenic miRNAs have their own promoters and can either be transcribed as a single gene, or as polycistronic transcripts (4–6). Intragenic miRNAs map to a genomic region either annotated as an intron or exon of another gene. Unlike intergenic miRNAs, a significant number of these miRNAs appears to be co-regulated with their host gene via co-transcription (7). This phenomenon raised the question whether intronic miRNAs and their

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host genes are functionally associated. Indeed, increasing evidence points to a link between host gene and intragenic miRNA, for example in terms of a feedback loop (3, 7, 8). Due to the close connection of intronic miRNAs and their host genes, interpretation of intronic miRNA expression is only feasible in the context of their host genes. This, however, can be a daunting task when relying solely on online tools such as miRGen or miRBase (9–14), especially if individual questions would require modifications. In this chapter, we therefore describe how to set up an miRNA database with a focus on the analysis of intragenic miRNAs. We also present an example of a simple intronic miRNA host gene analysis for the human organism. These basic structures can easily be extended to the integration of additional species to perform more complex analyses. Advantages of the implementation of such a system include the possibility (a) to perform queries across all protein-coding and miRNA genes, (b) to easily update the database whenever needed, and (c) to adjust the system according to your needs.

2. Materials

2.1. Hardware

There are no specific requirements. We suggest to implement the database system on a computer with at least 4 GB of RAM, 2.0 GHz dual-core processor, 10 GB of free harddisk space, and a fast internet connection.

2.2. Software

1. Operating System: Preferably a Unix-based operating system such as Linux, Solaris, FreeBSD, or Mac OS.
2. Database: Any SQL-based database system, such as MySQL version 5.0 (or higher) or PostgreSQL version 8.0 (or higher). Our implementation uses MySQL.
3. Programming Language: Python 2.7 (or higher) or Perl 5.14 (or higher). Packages needed for python may include "MySQLdb" to access the database and "Pygr" (15) to parse FASTA files and retrieve sequences.
4. Statistical Processing: We encourage the use of a recent release of the statistical programming software R release 2.7 or higher (current version 2.14) (16). We use the following R libraries: "GOstats," "org.Hs.eg.db," "KEGG.db," and "GO.db" (17, 18).

3. Methods

3.1. Setting Up the Database

1. First, a grid needs to be established. A great resource for gene information is the ftp-server of the National Center for Biotechnology Information (NCBI). The file containing

general gene information for *Homo sapiens* can be found at ftp://ftp.ncbi.nlm.nigh.gov/gene/DATA/GENE_INFO/Mammalia/Homo_sapiens.gene_info.gz. This file allows the import of information such as the "Entrez-ID," "Ensembl-ID," "Prot-ID," "Official Symbol," the gene description and gene type into one table. This table builds the backbone of the database, as all other entries concerning protein-coding genes are linked to it.

2. In a second step, structural information about the genes needs to be integrated. NCBI's Reference Sequence collection (RefSeq; http://www.ncbi.nlm.nih.gov/RefSeq) (19) is a nonredundant collection of annotated genes that can easily be accessed through the UCSC Genome Browser (http://genome.ucsc.edu) (20, 21) (the full link for downloading RefSeq data for the hg19 genome assembly is http://genome.ucsc.edu/cgi-bin/hgTables?hgsid=188637351&clade=mammal&org=Human&db=hg19&hgta_group=rna&hgta_track=mrna&hgta_table=refGene&hgta_regionType=genome&position=chr21%3A33031597-33041570&hgta_outputType=primaryTable&hgta_outFileName=refseq_genes_human_hg19.txt). The file contains detailed gene information such as the position of the gene on the genome (chromosome name, strand of sequence alignment, transcription start and end coordinates, CDS start and end coordinates) and the start and end coordinates of known exons for that gene. We suggest creating two tables from this file, one containing the structural gene information and the other storing the exon positions for these genes.
3. In order to be able to derive sequences from genome coordinates, the latest human genome assembly (currently GRCh37/hg19) can be downloaded in 2bit-format from UCSC's ftp-server at ftp://hgdownload.cse.ucsc.edu/goldenPath/currentGenomes/Homo_sapiens/bigZips/hg19.2bit and needs then to be converted to a FASTA file with the tool "twoBitToFa," which can be downloaded from http://hgdownload.cse.ucsc.edu/admin/exe/. There are different libraries that will allow a user to parse and read FASTA files. We found the python library "Pygr" to be convenient (15).
4. MirBase (10–13) is the official miRNA registry. It currently contains a total of 16772 registered miRNAs across 153 different organisms (1,424 for human; release 17). Central to the analysis of intragenic miRNAs is the correct mapping to potential host genes. First, download genomic coordinates of known miRNAs at ftp://mirbase.org/pub/mirbase/CURRENT/genomes/hsa.gff. The file contains the chromosome number, strand, start and end coordinates for each known human miRNA stemloop. We strongly encourage extending this information by directly mapping the miRNA

coordinates to RefSeq gene information. This can be done by following these steps:

(a) Check, if the miRNA stemloop coordinates overlap with an annotated RefSeq region (transcription start and transcription end). Based on this mapping, classify the miRNA as intra- or intergenic.

(b) Check if the miRNA coordinates overlap with any exon coordinates and subclassify the miRNA as intronic or exonic.

(c) Add information on whether the miRNA is on the same strand as the host gene.

(d) Count introns and exons starting from 1 at the 5′-end and calculate the distance to the exon upstream. Add both, intron-/exon number that contains the miRNA as well as the distance upstream. This is especially important since with greater distance co-transcription may be less likely, given that the miRNA may have its own promoter.

5. A table containing the miRNA stemloop sequences and miRNA family information needs to be created. Three files are necessary for this step:

   (a) A file containing the stemloop sequences of all miRNAs (ftp://mirbase.org/pub/mirbase/CURRENT/database_files/mirna.txt.gz).

   (b) A file containing the miRNA family classification (ftp://mirbase.org/pub/mirbase/CURRENT/database_files/mirna_prefam.txt.gz).

   (c) A file for mapping miRNA family classification to miRNA stemloop sequences (ftp://mirbase.org/pub/mirbase/CURRENT/database_files/mirna_2_prefam.txt.gz). The ID in the first column of "mirna_2_prefam.txt" refers to the ID in the first column of "mirna.txt," the second column to the first column of "mirna_prefam.txt."

6. Finally, the mature miRNA sequences can be integrated. Therefore, the files "mirna_mature.txt" and "mirna_pre_mature.txt" need to be downloaded from ftp://mirbase.org/pub/mirbase/CURRENT/database_files/. The first file contains the names and positions of the mature miRNA sequences. Importantly, these are relative positions to the miRNA stemloop coordinates. For example, hsa-mir-21 is located on chromosome 17, ±strand, 57,918,627–57,918,698. The mature sequence is denoted to be base 8–29. Hence, the mature sequence is given by the following coordinates: chromosome 17, ±strand, 57,918,634–57,918,655 (UAGCUUAUCAGACUGAUGUUGA). The second file allows matching of the mature sequences and the stemloop sequences. This is necessary,

as the name of the mature sequences for example for "hsa-miR-1-1" and "hsa-miR-1-2" is "hsa-miR-1" in either case. The derived mature sequences should be identical.

7. A central aspect of the analysis of miRNAs is knowing their targets. A variety of target prediction tools exist, most of which provide precomputed predictions for download. We use two tables for implementing target prediction information. One table stores summarized information on a predicted miRNA–target interaction, including a link to the corresponding entry in the miRNA-table, a link to the corresponding entry in the gene-information table (the predicted target), the number of predicted binding sites, the maximum or minimum score, depending on the scoring system. The second table contains the predicted binding sites, including the binding coordinates of the mature miRNA sequence and of the 3′-UTR of the targeted gene, the score or free binding energy of this particular interaction, and a reference to the summarized information in the first table. In most cases the summarized information will be sufficient for identification of miRNA targets.

3.2. Example of a KEGG and GO Analysis

Given functional linkage between intragenic miRNAs and host genes, it is reasonable to assume that knowledge of the host genes reveals information about the biological function of their intragenic miRNAs. A common way of analyzing functional annotations of genes is the mapping to a biomolecular ontology. Two major ontologies include the Kyoto Encyclopedia of Genes and Genomes (KEGG) (22, 23) and the Gene Ontology (GO) (24). The latter consists of the three ontologies, "Molecular function (MF)," "Biological process (BP)," and "Cellular component (CC)." The analysis can be applied to miRNA targets and host genes likewise. We use the statistical programming tool R in combination with the GOstats library from the BioConductor project (18). The following sample code illustrates how to perform this type of analysis.

1. Load the required libraries:

   ```
   > library(GOstats).
   > library(org.Hs.eg.db).
   > library(KEGG.db).
   > library(GO.db).
   ```

2. Load the set of host genes of interest, for example from a matrix as described in the Subheading 4. Then extract the Entrez Gene identifiers.

   ```
   > host.matrix <– read.csv("/Path/to/HostGeneMatrix.csv").
   > genes_of_interest <– as.character(host.matrix$EntrezId).
   ```

3. Define the universe of genes to be tested. In cases where you analyze the full genome, this will be the set of known genes. All mappable ENTREZ gene identifiers can be extracted as follows:

   >gene_universe<-as.character(unlist(as.list(org.Hs.egSYMBOL2EG))).

4. Define the parameters to either perform a KEGG or a GO analysis (for the latter you need to specify the ontology="MF," "BP," or "CC"):

   > paramsKEGG <- new("KEGGHyperGParams," geneIds = genes_of_interest, universeGeneIds = gene_universe, annotation = "org.Hs.eg.db," pvalueCutoff = 0.05, testDirection = "over").

   > paramsGO <- new ("GOHyperGParams," ontology="MF," geneIds = genes_of_interest, universeGeneIds = gene_universe, annotation = "org.Hs.eg.dbm" pvalueCutoff = 0.05, testDirection = "over").

5. Perform the analysis

   > kegg.analysis <- hyperGTest(paramsKEGG).

   > go.analysis <- hyperGTest(paramsGO).

6. Analyze the results.
   The variables "kegg.analysis"/"go.analysis" contain the results of the analyses. The mapping between the KEGG or GO pathway identifiers and the *p*-values can be accessed through kegg.analysis@pvalues or go.analysis@pvalues. Similarly, odds ratios as well as the mapping of the individual genes to the pathways can be found at kegg.analysis@oddsRatios and kegg.analysis@catToGeneId, respectively. You can plot the resulting graph of a GO analysis with the following command (nodes are colored according to significance):

   > plotGOTermGraph(go.analysis).

4. Notes

4.1. Database

The file "Homo_sapiens.gene_info.txt" also contains synonyms for each gene (separated by "|"), which we recommend to store in a separate table and link to the official gene for easy access.

It is important to know that genomic start coordinates downloaded from the UCSC Web site (including the FASTA- and RefSeq files) start counting at "0," whereas general convention is to start counting at "1" (e.g., mirBase miRNA coordinates).

When analyzing expression profiles of intragenic miRNAs, we found the following matrix generated from this database structure helpful: name of the miRNA, name of the host gene, Entrez-identifier of the host gene, classification of the miRNA (intronic, exonic), distance to the upstream exon- and intron number.

Target predictions from tools such as TargetScan or miRanda (25, 26) or conservation information can easily be integrated in the described framework. We store target predictions from different target prediction tools in one table that contains the fields "miRNA-ID," "Gene-ID," "Number of Binding-Sites," "Score," and "Prediction Method," as these have been shown to be of relevance when evaluating a predicted miRNA–target interaction (27, 28).

4.2. KEGG and GO Analysis

The library "org.Hs.eg.db" is updated biannually. In case you want to create your own annotation file, you can use the R-library "AnnBuilder." Details can be found at (29).

The gene universe is important, as it significantly influences the result of the analysis. As described above, we use the set of all genes when performing whole-genome studies. However, individual studies may require a different universe.

We recommend using q-values instead of p-values generated by GOstats to control for a False Discovery Rate. Alternatively, Bonferroni correction can be used as a more conservative measure.

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Chapter 6

Identify Intronic MicroRNA with Bioinformatics

Chih-Hao Kuo, Mark D. Goldberg, Shi-Lung Lin, Shao-Yao Ying, and Jiang F. Zhong

Abstract

MicroRNAs (miRNAs) are single-strand nonprotein coding RAN with 18 to 25-nucleotides long. With complementary sequence to target messenger RNA (mRNA), miRNA regulates mRNA degradation and protein translation. miRNAs have been identified in various organisms ranging from virus to human. Increasing evidence indicates that mammalian gene regulation has multiple layers and the availability of mRNA is not the sole regulation mechanism. The evolutionally conserved miRNA may be a primary regulation mechanism of gene expression. Its role in directing embryo development and stem cell differentiation should not be underestimated.

Due to the small size of miRNA, identifying it with experimental approach (e.g., direct cloning) is difficult. The cell type and developmental-specific expression of miRNA make the experimental approach even more difficult. Consequently, bioinformatics approaches have been developed to identify novel miRNA. In human miRNA study, many studies search for the mostly complete human genomic sequence. Here, we report a rapid bioinformatics approach to mine miRNA from gene specific introns. Intronic miRNA may directly regulate the expression of its target genes during development. The reported bioinformatics approach not only identifies the potential miRNA, but also provides the intron location of these miRNA like sequence. This information is critical for studying the gene–gene interaction via miRNA, and facilitates the study of miRNA in gene expression regulation.

Key words: Human microRNA, In silico microRNA, Pattern recognition

1. Introduction

1.1. MicroRNA

MicroRNAs (miRNAs) are small-sized regulatory RNAs which are usually composed of 18–25 nucleotides (1). This single-stranded noncoding RNAs are able to inhibit protein expression post-transcription through either perfect or partially complementary binding to target sequences, a process known as RNA-interference (RNAi). More than 95% of human genome is the noncoding DNA

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region (2). Among these regions, many have been reported to encode miRNAs (3). It is then conceivable that miRNAs may play pivotal roles in the communication and regulation of various cellular events. It is now clear that miRNAs are involved in various cellular processes such as cell cycle, cell proliferation, apoptosis, development, and differentiation (4–6). More importantly, abnormal miRNA expression has been associated with different types of cancer (7, 8) and neurodegenerative disease (9). Despite increasing numbers of miRNAs being discovered, the exact functions of many miRNAs so far are still not elusive.

1.2. Biogenesis of MiRNA

Both miRNA and small interfering RNA (siRNA) are generated by Dicer (DCR), a multidomain enzyme of the RNase III family. DCR cuts long, double-stranded RNA (dsRNA) into siRNAs and chops short precursor miRNAs with imperfect stem-loop structure (1, 10–12). It is believed that siRNA is produced from double-stranded, perfectly complementary RNAs that are transcribed by two separate promoters whereas exonic or intronic miRNA is generated from one promoter and contains multiple base mismatches. The primary-microRNA (pri-miRNA) is first transcribed by RNA polymerase II. This long hairpin-like miRNA predecessor is then processed by RNase III endonuclease Drosha to form precursor-microRNA (pre-microRNA) which is about 60–70 nucleotides in length. After excised by Drosha RNase, the pre-microRNA is further exported out of nucleus by RAN-GTP and Exportin-5. In the cytoplasm, another endonuclease DCR can recognize the unique secondary structure of pre-microRNA and cut it into mature 18–25 nucleotides miRNA. In order to elicit its gene-silencing effect, mature miRNA has to be incorporated into ribonucleoproteins which consist of multiple components and forms RNA-induced silencing complex (RISC). The RISC then target the 3′ untranslated region of mRNA transcript and either blocks the protein translation machinery or leads the target to degradation based on the degree of complementarity. It is believed that the majority of miRNAs suppresses the gene function through partial complementarity with their targets sequences. Therefore, it is conceivable that there are multiple targets for a single miRNA and many miRNAs may regulate one gene.

1.3. Role of miRNAs in Cancer

MiRNAs have been found deregulated in several cancer models. miRNA can be oncogenic or tumor-suppressive gene depending on the nature of their target sequences (13). Overexpression of an miRNA targeting a tumor suppressor gene may decrease its anti-tumor activity. On the other hand, down-regulation of an miRNA that can silence an oncogenic gene may elevate the expression of this oncogenic protein. For instance, miR15a-16 cluster and miR143/145 were found to be tumor-suppressive and deregulated in chronic lymphocytic leukemias (CLL) (14) whereas miRNAs

such as miR-17/92 cluster (15), miR-21 (16), and miR-155/BIC (17) may possess oncogenic potentials. A recent study showed that miR-34a suppresses tumor development and mastasis by targeting c-Myc transcriptional complexes in human renal cell carcinoma (18). These findings demonstrate the potential of miRNAs as diagnostic and prognostic marker in early cancer diagnosis and therapeutic targets for cancer treatment.

1.4. MicroRNA-302 Cluster and Stem Cell

The use of human embryonic stem cell has been shown to have great potential in tissue repair and transplantation therapy. Nevertheless, research on human embryonic stem cell still faces difficulties. It is challenging to obtain pure sample without the contamination of feeder cell. Ethical issues also arise when dealing with human embryos. Recent discovery on generating induced pluripotent stem (iPS) cell reveal a new field for creating human embryonic stem cell-like cells from somatic cells without the use of human embryo. The first iPS cell was generated using retroviral transfection of four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) (19). Nevertheless, there are still problems regarding this procedure. The introduction of ongenic genes such as c-Myc or Klf4 may increase the tumorigenicity of produced iPS cells and will limit potential future applications. Furthermore, simultaneous introduction of four genes is difficult to control and the copy numbers of each factors may not be the same and consistent among different iPS cells. Although, Nanog was found to be sufficient for the replacement of Klf4, the problem of tumorigenicity still exists.

MicroRNA-302 (miR-302) family is composed of miR-302b, miR-302c, miR-302a, and mir302d which are found to be much conserved among mammals such as mouse and human (20). Overexpression of miR-302 family was found in the mouse oocyte and human embryonic stem cell. Many of their targets are involved in the signal for differentiation in embryonic development (21). It is for this finding that a novel intronic miR-302s expression system was developed to study the potential of miR-302 family to reprogram somatic cell into iPS (22). Many miR-302s-induced pluripotent stem (miRPS) cell lines were successfully developed from PC-3, LNCaP, and MCF-7 (23). Several characteristics were examined to evaluate the similarities between human embryonic stem cells and miRPS cells. Embryonic stem cell markers including Oct3/4, SSEA-3, and SSEA-4 were found to be overexpressed in these cell lines and microarray analysis showed similar expression pattern between miRPS cells and human embryonic stem cells H1 and H9 (23).

The introduction of single miR-302 family cluster gene makes it easy to control the level of expression and eliminate the problem of tumorigenicity observed in iPS cells generated using traditional four transcription factors (24). Given the above reasons, miRNA-induced pluripotent stem cell may offer opportunity and greater advantages in the field of regenerative medicine.

2. Materials

2.1. Hardware

For searching the whole human genomic DNA sequence including intronic DNS sequence, we used the Caltech UNIX computer station to run a pattern search perl script.

2.2. Software

1. *Operating System:* A Unix-based operating system such as Linux, Solaris, FreeBSD, or Mac OS.
2. *Database:* The human genomic sequence was downloaded from UCSC Genome Bioinformatics Site (http://hgdownload.cse.ucsc.edu) and ftp into the Caltech Unix-based system for performing the pattern search.

3. Methods

3.1. Setting Up the Database

A file with human genomic DNA sequence in fasta format was FTP to Caltech Unix-based ITS UNIX Cluster.

3.2. Pattern Match Algorithm

We defined a "pattern" as a sequence of multiple "pattern units." For example, pattern units are P1 P2 P3 P4 … in a pattern P. The scan of a sequence "Genome" begins by setting the current position of the fasta format sequence to 1. Then, try to match P1 starting at the current position. Each attempt to match a pattern unit will be a success or a failure. If it succeeds, then it will try to match the next unit. If it fails, then the program will find an alternative match for the immediately preceding pattern unit P2. If this succeeds, then it will forward again to the next unit. If it fails, it will go back to the preceding unit. This algorithm is the so-called backtracking. If there are no previous units, then the current position is incremented by one, and everything starts again. This process proceeds until either the current position goes past the end of the sequence or all of the pattern units succeed. On success, the program will report the "hit," and the current position is set just past the hit, and it will try to find another hit.

3.3. Define a Potential miRNA Pattern

Identifying miRNA is critically important in understanding their roles in human diseases. There are features in the noncoding regions of human DNA can be screened with bioinformatics to identify potential miRNA precursors. These potential precursors can then be verified and used to isolate corresponding miRNA from cells. Once isolated, the biological functions of these miRNA can be studied. These miRNA precursors are 60–80 nucleotides in length (25) and are transcribed as long primary miRNA (26).

Our program looks for targets located 50–100 nucleotides downstream of the start codon in the genome. Searching for specific sequence motifs that have a high RNAi probability such as AA(N19)TT or NA(N21), or NAR(N17)YNN, where N is any nucleotide, R are the purines, and Y are the pyrimidines. Look for target sequences that have GC content between 35 and 60%, since larger GC content indicated other secondary structure. Eliminate any sequences that have long stretches of nucleotide repeats. Finally, avoid sequences that share homology with other related genes.

4. Results and Discussion

With bioinformatics, we identified DNA sequences that are potential miRNA precursors and post the result in our Web site (http://www-hsc.usc.edu/~jzhong/). These precursor sequences are listed in a separated file and will need to be verified by RT-PCR. The confirmed candidates will be isolated for studying their biological functions in human cells.

5. Notes

1. The genomic data must be in FASTA format. If the genomic data is in other format, it must be converted to FASTA format.
2. In order for the rapid screening, we loaded the entire genomic sequence to the RAM. Therefore, the computer station must have sufficient memory RAM to accommodate the entire genomic sequence.
3. The screen pattern can be modified to increase or decrease stringency. Lower stringency will result in more hits but many hits may not be the miRNA candidates.
4. Repeat intronic DNA sequence may be selected for multiple times because their locations are different. It will be helpful to screen all the hits and eliminate the repeat hits. That can be done with perl scripts or in Excel.
5. The identified miRNA candidate must be verified with wet-laboratories experiments. Similar to other *in silico* analyses, our data-base only provides candidate sequence and their genomic location (27, 28). These candidates must be verified for biological function.

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Chapter 7

Experimental miRNA Target Validation

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Abstract

In the recent past, microRNAs (miRNAs) have gained significant attention as potent regulators of gene expression. These small noncoding RNA molecules are currently of major interest when investigating regulatory circuits of the cell. After identification of potential miRNA–target gene interactions (e.g., using computational algorithms), biomolecular validation is necessary. In the current chapter, we present a protocol for validation of an miRNA target interaction implying cloning of a dual-luciferase miRNA target expression vector, transfection of cells with this vector and a precursor miRNA (pre-miRNA), and the subsequent luciferase assay.

Key words: miRNA target interaction, miRNA target validation, Luciferase assay, miRNA transfection, Mutagenesis

1. Introduction

MicroRNAs (miRNAs) are potent regulators of gene expression by either repression of translation or mRNA degradation (1). Target recognition appears to be mainly mediated through base-pairing of an 8 nucleotide short sequence in the 5′-region of the miRNA (2). While this process is not yet fully understood, evidence from multiple studies suggests that a single miRNA may regulate up to several hundreds of target genes (3, 4). Due to the lack of high-throughput target identification methods, computational algorithms are the driving force in predicting miRNA target genes (2, 5, 6). However, these algorithms suffer from high false positive rates and may be subject to individual biases (7, 8). Therefore, experimental validation is necessary (9). This is commonly done by cloning the 3′-UTR sequence of a putative target gene into a

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reporter gene vector, followed by transfection of this vector and a precursor miRNA (pre-miRNA), and measurement of resulting changes in reporter activity. Several different approaches are commonly utilized to confirm miRNA target interaction. Within this chapter, we describe the experimental validation of miRNA predicted targets using a modern and highly accurate three-step approach: (1) Construction of a dual-luciferase miRNA target expression vector, (2) Transfection of host cells with the pre-miRNA and the vector by electroporation using the Neon™ Transfection System (10), and (3) Measurement of changes in gene expression using a dual-luciferase (firefly and renilla) reporter assay (11). Advantages of this process include utilization of established cloning protocols (12), the application of highly effective up-to-date technology, such as the Neon™ Transfection System (13), and accurate gene expression quantification for generation of high quality data (14).

2. Materials

2.1. DNA Constructs

2.1.1. Amplification

1. Specific primers for PCR amplification (see Note 1).
2. PfuUltra II fusion HS DNA polymerase (Agilent Technologies).
3. 10× PfuUltra II Reaction Buffer (Agilent Technologies).
4. dNTP mix: ready to use premixed solution (25 mM) of dATP, dCTP, dGTP, and dTTP (Roche).
5. Distilled water (dH_2O).

2.1.2. Cloning

1. pSC-B-amp/kan Blunt PCR Cloning Vector (Stratagene).
2. StrataClone Ultra Blunt PCR Cloning Kit (Stratagene).
3. pmirGlo Dual-Luciferase miRNA Target Expression Vector (Promega).
4. T4 DNA ligase and T4 ligase buffer (Promega).
5. *Wizard*® SV *Gel* and PCR Clean-Up System (Promega).
6. Restriction enzymes (depending on the insert): PmeI, DraI, EcoI, SacI, NheI, XhoI, XbaI, SalI, AccI, SbfI (NEB—see Note 2).
7. *Escherichia coli* cloning system: K12 heat shock competent cells (XL 10 Gold ultracompetent cells—Stratagene), LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g NaCl), and ampicillin.
8. Distilled water (dH_2O).

2.2. Mutagenesis

1. Specific primers for mutagenesis.
2. QuickChange® Lightning Enzyme (Stratagene).

3. 10× QuickChange® Lightning Buffer (Stratagene).
4. dNTP mix (Stratagene).
5. QuickSolution® reagent (Stratagene).
6. DpnI restriction enzyme (Stratagene).
7. Distilled water (dH_2O).

2.3. Transfection and Cell-Culture

1. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1× penicillin-streptomycin-glutamine (Gibco).
2. Solution of 0.25% trypsin and 1 mM EDTA (Gibco).
3. Phosphate-buffered saline (PBS).
4. Pre-miR™ miRNA precursor (see Note 3) and Pre-miR™ negative control (Ambion).
5. Neon™ Transfection System with a 10 μL Neon™ Tip (Invitrogen).

2.4. Luciferase Assay

1. Transfected and incubated HEK 293 cells.
2. Dual-Glo® Luciferase Buffer (Promega).
3. Dual-Glo® Luciferase Substrate (Promega).
4. Dual-Glo® Stop&Glo® Buffer (Promega).
5. Dual-Glo® Stop&Glo® Substrate (Promega).

3. Methods

3.1. Amplification and Cloning

1. Obtain the 3′-UTR of your target gene (including the seed sequence of the miRNA of interest) by PCR amplification using the PfuUltra II fusion HS DNA polymerase.
2. Insert distilled water (40.5 μL), 10× PfuUltra II reaction buffer (5 μL), dNTP mix (0.5 μL), template cDNA (5–30 ng/μL to 1 μL), first primer (1 μL to 10 nM), second primer (1 μL to 10 nM), and PfuUltra II fusion HS DNA polymerase (1 μL) into a sterile PCR tube (total reaction volume: 50 μL).
3. Perform amplification by PCR with the following cycling conditions: 95°C for 5 min denaturing; 36 cycles of 95°C for 30 s, Primer T_m—5°C for 30 s, 72°C for 30 s/kb of plasmid length, and a final extension at 72°C for 5 min.
4. Separate PCR products on 2% agarose gel (stained with ethidium bromide) to verify production of the expected fragment.
5. If gel analysis confirms a specific amplification, prepare a 1:10 dilution of the PCR reaction in distilled water. Ligate PCR product into the pSC-B-amp/kan using the UltraBlunt PCR

Cloning Kit (Stratagene); bring this gene product into XL 10 Gold ultracompetent cells, plate overnight, pick colonies, transfer the colonies into a tube with 3 mL LB medium (with ampicillin), and place the tube on a shaker (225 rpm) at 37°C overnight.

6. Perform miniprep (of pSC-B-amp/kan and pmirGlo) using QIAprep Spin *Miniprep* Kit (Quiagen) followed by digestion with suitable restriction enzymes. Digestion using the enzymes *XhoI* and *PmeI* (as an example) requires distilled water (27.5 μL), NEB buffer 4 (5 μL), BSA (0.5 μL), template DNA (2 μg to 15 μL), XhoI (1 μL), and PmeI (1 μL) in a sterile tube (total reaction volume: 50 μL), followed by incubation for 60 min at 37°C and separation on 2% agarose gel.
7. Extract and purify the DNA fragments from the agarose gel with the Wizard® SV Gel and PCR Clean-Up System.
8. Blend the ligation reaction mixture using the following components: 1 μL T4 ligase, 1 μL T4 ligase buffer, and 8 μL of the vector-insert DNA mixture. The recommended molar ratio of vector:insert DNA is 1:1, 1:3, or 3:1. Incubate the reaction at 15°C for 4–18 h.
9. For transformation of competent cells, pipette 10 μL ligation reaction and 50 μL of XL 10 Gold ultracompetent cells (thawed on ice) into a prechilled 14 mL tube. After mixing gently, incubate the transformation mixture for 20 min on ice. Heat-shock the mixture at 42°C for 45 s and incubate the tube on ice for 2 min. Add 250 μL of LB medium (pre-warmed to 42°C) into the tube and place the tube on a shaker (225 rpm) at 37°C for 1 h. Plate the complete transformation mixture on a LB-ampicillin plate at 37°C in a humified atmosphere of 5% CO_2 in the air overnight.
10. Prepare miniprep from selected colonies (see Note 4).

3.2. Mutagenesis

1. In order to test the interaction of the miRNA and its predicted seeding sequence, perform point mutation of the seed sequence within the 3′-UTR clones.
2. Design two complimentary primers containing the favored mutation and ambient unmodified nucleotide sequences (for primer requirements go to http://www.stratagene.com/qcprimerdesign).
3. Prepare the reaction mixture using: 5 μL of 10× reaction buffer, 1 μL plasmid DNA (pmirGlo containing the 3′-UTR (10–100 ng), 1 μL of the first primer (125 ng), 1 μL of the second primer (125 ng), 1 μL dNTP mix, 1 μL QuickSolution reagent, ddH_2O to a final volume of 50 μL, and 1 μL QuickChange Lightning Enzyme.

4. Perform PCR with the following cycling conditions: 95°C for 2 min denaturing; 36 cycles of 95°C for 20 s, 60°C for 10 s, 68°C for 30 s/kb of plasmid length, and a final extension at 68°C for 5 min.
5. After PCR add 1 μL of DpnI restriction enzyme into each reaction tube. Gently mix the reaction mixture, briefly spin down the mixture, and incubate for 5 min at 37°C to digest the parental dsDNA.
6. Perform a transformation of XL 10 Gold ultracompetent cells as described in Amplification and Cloning.
7. Prepare miniprep from selected colonies (see Note 5).

3.3. Transfection of Constructs

1. Cultivate the required number of HEK 293 cells (cells should be 70–90% confluent on the day of the experiment)—for a 12-well plate, $1–2 \times 10^5$ cells per well are needed (see Note 6).
2. Prepare 12-well plates by filling the wells with 1 mL of culture medium without antibiotics. Pre-incubate these plates at 37°C in a humified atmosphere of 5% CO_2 until usage.
3. Aspirate the media, trypsinize the cells using Trypsin/EDTA, harvest the cells in medium, centrifuge the cells at $100–400 \times g$ for 5 min, discard the supernatant, wash the cells with PBS, centrifuge the cells again at $100–400 \times g$ for 5 min, discard the supernatant, resuspend the pellet with Resuspension Buffer R at a density of 1×10^7 cells/mL (see Note 7).
4. Fill the Neon™ Tube with 3 mL Electrolytic Buffer (Buffer E for the 10 μL Neon™ Tip) and put it into the Neon™ Pipette Station (see Note 8).
5. Adjust the pulse conditions dependent on the cell type (a list with the required conditions can be found at http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Culture/Transfection/Transfection___Selection-Misc/Neon-Transfection-System/Neon-Protocols-Cell-Line-Data.html).
6. Pipette 0.5–3 μg of plasmid DNA, 50 nM Pre-miR, and the prepared cells into a sterile tube (total volume for each reaction 10 μL) and gently mix.
7. Insert the Neon™ Tip into the Neon™ Pipette and aspirate the cell-plasmid DNA-Pre-miR mixture into the Neon™ Tip (without any air bubbles—see Note 9). Place the Neon™ Pipette into the Neon™ Tube placed at the Neon™ Pipette Station. Start the loaded electroporation protocol by pressing "Start" on the touch screen.
8. Remove the Neon™ Pipette from the Neon™ Pipette Station after "Complete" is displayed on the touch screen and discharge the Neon™ Tip immediately into the prepared and pre-warmed

12-well plate. Gently rock the plate in order to distribute the cells.

9. In order to analyze the regulation of gene expression by miRNAs, four different transfection experiments have to be performed for each target region: (1) Pre-miR and the pmirGlo harboring the putative binding site, (2) Pre-miR negative control and the pmirGlo harboring the putative binding site, (3) Pre-miR and the pmirGlo harboring the mutated binding site, and (4) Pre-miR negative control and the pmirGlo harboring the mutated binding site.
10. Incubate the 12-well plate at 37°C in a humified atmosphere of 5% CO_2 for 48 h.

3.4. Luciferase Assay

1. Aspirate the media, trypsinize the cells using Trypsin/EDTA, harvest the cells in medium, centrifuge the cells at 100–400 × *g* for 5 min, discard the supernatant, wash the cells with PBS, centrifuge the cells again at 100–400 × *g* for 5 min, discard the supernatant, resuspend the pellet in 15 µL medium.
2. For luciferase assay, add 15 µL (volume equal to the volume of the culture medium) of Dual-Glo® Luciferase Reagent (see Notes 10–12) into each well (mix gently 2–3 times).
3. Incubate for 10 min and perform measurement of (Firefly) luciferase activity. The measurement should be performed within 2 h.
4. Add 15 µL Dual-Glo® Stop&Glo® Reagent (volume equal to the volume of the culture medium) into each well and mix gently 2–3 times.
5. Incubate for 10 min and perform measurement of (Renilla) luciferase activity as described before.
6. Calculate the ratio of firefly to renilla luciferase activity. Normalize this ratio to the ratio of a series of control wells that are treated consistently on all plates.
7. Reduction of luciferase activity of the pmirGlo harboring the putative miRNA binding site (in relation to the negative control) suggests that the analyzed miRNA molecule is able to interact with the predicted seed sequence and modulates gene expression (see Note 13).

4. Notes

1. When designing primers for DNA amplification, attach a sequence at the end of the primer which is accessible for specific restriction enzymes. These sequences are important in order to

excise the DNA fragment from the topovector (pSC-B-amp/kan) and to clone this fragment into the reporter vector (pmirGlo).

2. Before choosing restriction enzymes, make sure that no sequences within your 3′-UTR-DNA fragment exists which are accessible for the specific restriction enzymes. A helpful tool can be found at http://tools.neb.com/NEBcutter2/.
3. After cloning the DNA fragment into the expression vector, check if the fragment was effectively cloned into the vector by sequence analysis.
4. Construction of an expression vector for pre-miRNAs can be used instead of Pre-miR™ miRNA precursors. However, Pre-miR™ miRNA precursors show some advantages including simple handling and addition of a specific amount of pre-miRNA.
5. Sequence analysis should be also performed after mutagenesis.
6. Expression of miRNAs is different within particular cell lines. Therefore, analyzing the expression of the miRNA (you are interested in) of this cell line by real-time PCR is crucial. In the case of over-expression of the regarding miRNA, reconsider the selection of your cell line.
7. Storing the cell suspension within the Resuspension Buffer for more than 30 min at room temperature reduces cell viability and transfection efficiency.
8. Make sure that you use the correct Electrolytic Buffer for electroporation (use the Buffer E for the 10 μL Neon™ Tip and Buffer E2 for the 100 μL Neon™ Tip).
9. Avoid air bubbles during pipetting within the Neon™ Tip as these bubbles may cause arcing during electroporation which lowers transfection efficiency.
10. Do not thaw the reagents at temperatures above 25°C. The reagents are stable at room temperature for several hours.
11. The Dual-Glo® Luciferase Reagent can be exposed to five freeze-thaw cycles with little or no negative effect on potency.
12. After adding the Dual-Glo® Luciferase Reagent during luciferase assay do not vortex the samples. Vortexing may coat the sides with a microfilm of luminescent solution, which potentially can escape mixing with the Dual-Glo® Stop&Glo® Reagent and leads to incorrect measurement results.
13. Although the ability of miRNAs to repress luciferase activity of a 3′-UTR-vector construct is a useful screening tool, it remains a surrogate for testing the direct effects of miRNAs in vivo on their putative targets' gene expression. Results of reporter gene

assays benefit from additional miRNA transfection experiments to evaluate the impact on mRNA and protein expression changes of the target gene.

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Chapter 8

Enrichment Analysis of miRNA Targets

Jianzhen Xu and Chi-Wai Wong

Abstract

MicroRNAs (miRNAs) are a class of ~22-nucleotide endogenous noncoding RNAs which regulate target gene expression via repressing translation or promoting mRNAs degradation. Any individual mammalian miRNA often has more than a hundred predicted mRNA targets and that close to one thirds of all mRNA transcripts bear one or more conserved miRNA binding sites in their 3′-untranslated region. Enrichment analysis of miRNA targets has become a standard technique to elucidating hierarchical functions of miRNAs in gene regulatory networks. In this protocol, we discuss analytical methods and use of computational tools in a step-by-step manner. Important details are also provided to help researchers choose more appropriate tools for a given type of analysis. Available Web-based resources for enrichment analysis of miRNA targets are summarized.

Key words: MicroRNA, MicroRNA targets, Enrichment analysis, Computational prediction, Functional annotation, Web resources

1. Introduction

MicroRNAs (miRNAs) are a class of ~22-nucleotide endogenous noncoding RNAs which form imperfect base pairing with targeting sequences on the 3′-untranslated region (3′ UTR) of target mRNAs (1). In most instances, miRNAs repress translation or promote degradation of target mRNAs; thus, miRNAs are generally involved in suppressing gene expression (2). Residing in protein-coding, intronic, and intergenic regions throughout the genome, miRNAs are mainly transcribed into long primary miRNAs (pri-miRNAs) by RNA polymerase II (3). In the nucleus, pri-miRNAs are capped, polyadenylated, and processed by a series of enzymes including RNase III enzyme Drosha into about 70-nucleotide hairpins called pre-miRNAs (4). A transporter protein exportin-5

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then exports pre-miRNAs to the cytoplasm where they are further cleaved by another RNase III enzyme Dicer to generate mature miRNA duplexes (5). A broad range of biological processes such as developmental timing, differentiation, and tissue morphogenesis are regulated by miRNAs (6). Not surprisingly, some miRNAs also play crucial roles in diseases such as cancer exemplified by aberrant expressions of oncogenic and tumor suppressor miRNAs (7).

MiRNA-directed gene silencing is mediated via RNA-induced silencing complex (RISC), the generic name for an Argonaute (AGO)-small RNA complex, a multi-subunit RNA binding protein complex including AGO and GW182 (glycine-tryptophan (GW) repeat-containing protein of 182 kDa) family proteins (2). One strand of miRNA duplex, referred to as the guide, preferentially enters into miRNA-induced silencing complexes (miRISCs) and guides the complex to recognize its target genes (8). Previous studies indicate that target recognition by a particular miRNA mainly functions via base pairing between the 5′-proximal 2–8 bases sequence of that particular mature miRNA, referred to as the "seed" region, with the targeting sequences on the 3′ UTR of target mRNA (9, 10). Several mechanisms such as interfering with eIF4F-cap recognition, blocking 40S small ribosomal subunit recruitment, antagonizing 60S subunit joining, and preventing 80S ribosomal complex formation to initiate mRNA translation are collectively responsible for suppressing gene expression post-miRNA-mRNA pairing by miRISCs (2). In addition, miRISCs may inhibit translation at post-initiation steps by inhibiting ribosome elongation, inducing ribosome drop-off, or facilitating proteolysis of nascent polypeptides (2). Besides, miRISCs can interact with deadenylase complex to facilitate deadenylation of the poly(A) tail of target mRNA resulting in miRNA-mediated mRNA decay (2).

Computational approaches have played an important role in miRNA studies and dozens of prediction tools for miRNA gene finding and miRNA target prediction have been developed (11). As the seed region is primarily responsible for determination of miRNA target specificity, it is commonly used in most bioinformatics algorithms to search for complementary sequences in the 3′ UTRs of potential target mRNAs (12). A general picture emerges that any individual mammalian miRNA often has more than a hundred predicted mRNA targets and that close to 1/3 of all mRNA transcripts bear one or more conserved miRNA binding sites in their 3′ UTRs (1). In addition, target mRNAs may potentially be regulated through miRNAs binding to atypical sites with imperfect seeds or non-conserved sites, further expanding the target networks. Amidst these substantial networks regulated by miRNAs, experimental verification of all predicted mRNA targets seems daunting; thus, improvement of bioinformatics analysis and refinement of algorithms for predicating targets are continuingly being developed.

Enrichment analysis of miRNA targets is a statistical technique used to elucidate higher levels of miRNA functions. A biological basis of enrichment analysis is that genes interact to form a module or interaction network. For instance, cell fates are determined by concerted actions of many functionally related genes in modular fashions (13, 14); thus, when an individual gene is disturbed, this signal could propagate within a gene interaction network and finally affect an entire functional module. It is reasonable to expect that genes, which are prone to be regulated or disturbed, are enriched in functional modules; thus, enrichment-based analysis becomes important to identifying functional modular changes and elucidating underlying functional mechanisms. This concept originates from gene microarray analysis. In that setting, after identifying a set of genes that are differentially expressed in two conditions, for example normal tissue vs. cancer tissue, researchers are then interested in exploring underlying perturbed pathways via enrichment analysis of differentially expressed genes (15, 16). In analyzing miRNA targets, the target set of one or a collection of miRNAs can be regarded as differential expressed genes. Based on functional modules or pathways enriched in miRNA targets, one can elucidate potential miRNA functions in gene regulation networks. Previously, we and others have introduced this concept into target analysis for a single miRNA or a cluster of miRNAs (12, 17). In the past 2 years, enrichment analysis of miRNA targets has become a standard practice in elucidating higher-order functional modules of target genes. A variety of tools have been developed to facilitate easy use under specific circumstances (18–20). Although they carry the core spirit and use the same general approach, they differ in certain aspects for specific purposes. In order to help researchers choose more appropriate tools for a given type of analysis, we describe in the following sections the capabilities of these tools and the statistical models employed as well as a practical guide in detail.

2. Materials

1. Hardware (64-bit computer; 4 GB of RAM (16 GB preferred)).
2. Macintosh, Unix/Linux or Windows operating system, with access to the Internet.
3. Depending on the experimental goals and selection of analytic tools (see Subheadings 3.2 and 3.3), you may require to install the Perl programming language (available from http://www.perl.org) or Java Runtime Environment (available from http://www.java.com) locally.

3. Methods

There are four main procedures involved in enrichment analysis of miRNA targets (Fig. 8.1). First, users should have one or a set of miRNAs of interest, for example from mining microarray assays with unique expression pattern. Then, users should predict the target genes and examine their cellular functions. Finally, combining various functional annotations and statistical models, users should explore functional modules related to these miRNAs via enrichment analysis of the targets. In each procedure, users should carefully choose the most efficient tool and appropriately set relevant parameters.

1. Obtaining one or a set of miRNAs.
2. Selecting target prediction tools and parameters.
3. Obtaining functional annotations.
4. Selecting enrichment models and parameters.

3.1. Obtaining One or a Set of miRNAs

Known miRNAs can be accessed from major repositories such as miRbase (21). It currently contains published miRNA sequences and associated annotations for 16,772 entries including 1,424 human miRNAs (Release 17: April 2011). Plant microRNA database (PRMD) is another online archive of plant miRNAs (22). It currently contains sequence information, secondary structures, target genes, and expression profiles for over 8,000 miRNAs collected from 121 plant species.

The mature sequence of an miRNA plays a pivotal role in target prediction (see Subheading 3.2). Even a single difference in nucleic acid sequence significantly affects miRNA-target pairing. To distinguish among miRNA species, a standard nomenclature system has been set up via an international collaboration (23). A prefix "miR" linked to a unique identifier is used for naming a mature miRNA product. Identical or nearly identical orthologs are given the same number designated with a three-letter prefix. For instance, hsa-miR-153 and rno-miR-153 represent human (*Homo sapiens*) and rat (*Rattus norvegicus*) miR-153 gene, respectively. Identical sequences that reside at different genomic positions within a species are given the same number with their genes distinguished by additional dash-number suffixes. For example, hsa-miR-153-1 and hsa-miR-153-2 are homologs located in the second and seventh chromosome, respectively. MiRNAs harboring slightly different sequences but belonging to the same gene family are annotated with an additional lower case letter. For example, miR-181a is closely related to miR-181b. Besides, a -3p or -5p suffix is used to denote mature miRNAs originating from opposite arms of the same hairpin structure. Furthermore, an asterisk following a name indicates

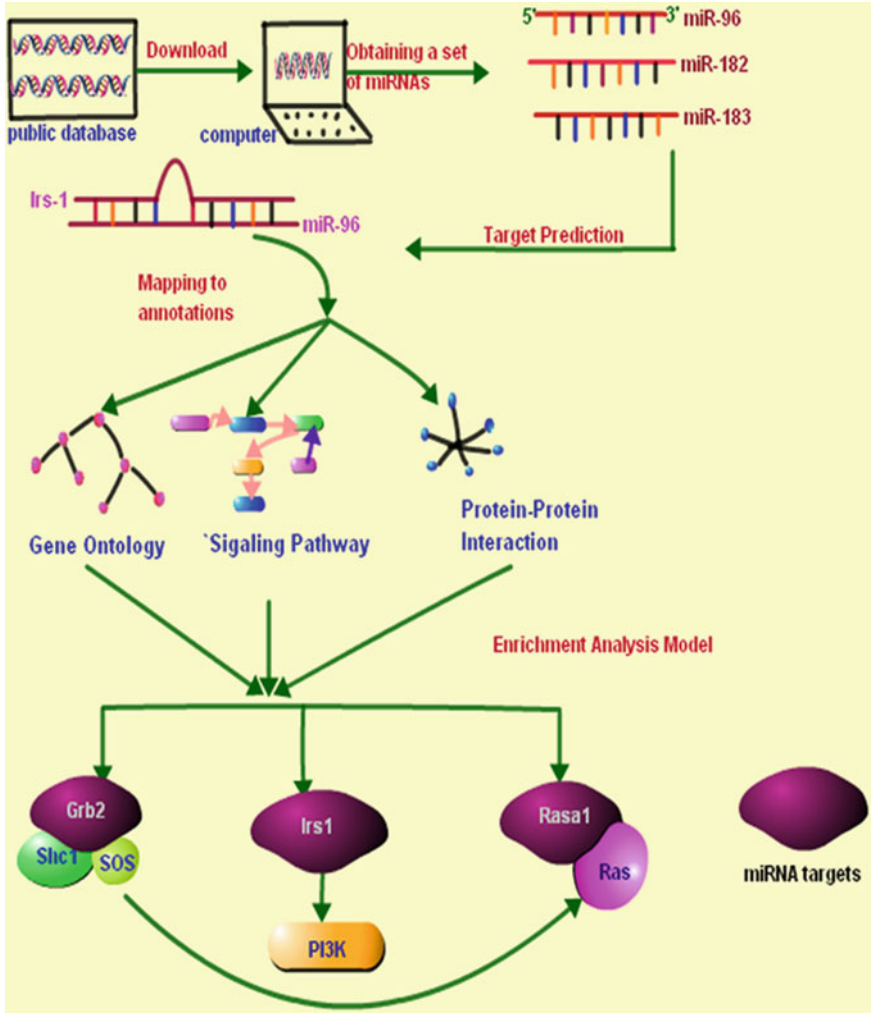

Fig. 1. Enrichment analysis of MiRNA targets. Steps involved in (1) obtaining one or a set of miRNAs from public miRNA databases; (2) selecting miRNAs targets prediction tools and setting suitable parameters to predict the targets; (3) choosing appropriate biological functional annotations such as Gene Ontology or Biocarta signaling pathways; (4) selecting enrichment models and parameters to identify functional annotation enriched with miRNA targets.

that the miRNA is expressed at a low level compared to the one in the opposite arm of a hairpin. For example, miR-19 and miR-19* share the same duplex but more miR-19 is expressed.

Recent studies have reported experimental instances that a set of miRNAs can cooperatively regulate a group of functionally related genes instead of a single gene (24–27). Specifically, Dews et al. found that in Myc over-expressing tumors, two anti-angiogenesis genes Tsp1 and Ctgf are down-regulated by miR-19 and miR-18. Noticeably, these two miRNAs are located in adjacent chromosome positions forming an miRNA cluster. In a theoretical model, an individual miRNA may only exert moderate effects on its target genes while a collection of miRNAs may act in a

combinatorial fashion to shut down a biological process (28, 29). However, a key issue is that these regulatory miRNAs should themselves be regulated coordinately. Gusev et al. directly mined miRNA profiling data and identified five co-expressed miRNAs sets (30).

Alternatively, we reasoned that certain mechanisms, related to transcriptional control, must exist to guarantee coordinated expression of these miRNAs. Mammalian miRNA genes are often clustered along the genome displaying conservation of patterns (31). In human, mouse, rat, and chicken, even at a very conservative maximum inter-miRNA distance (MID) of 1 kb, over 30% of all miRNAs are organized into clusters; whereas, over half of all miRNAs are clustered on chromosomes if the MID extends to 50 kb (32). These clustered miRNAs are believed to be coordinately transcribed as pri-miRNAs ranging from hundreds to thousands of nucleotides in length and processed into multiple mature miRNAs. Expression profiling analysis indeed showed that proximal pairs of miRNAs are generally co-expressed and an abrupt transition in the correlation between pairs of expressed miRNAs occurs at a distance of 50 kb (33). According to their chromosomal positions, several miRNA clusters have also been experimentally confirmed by RT-PCR or Northern blotting (3, 34, 35). These evidences strongly suggest that members of a particular miRNA cluster, which are defined by their chromosomal positions, are highly likely to be processed as co-transcribed units. Thus, we merged combinatorial regulation concepts with clustering properties of miRNAs to extract clustered miRNAs and investigated signaling pathways that are targeted by or enriched with miRNA clusters (17) (see Note 1).

3.2. Selecting Target Prediction Tools and Parameters

Various computational algorithms have been developed to predict animal miRNA target sites. Prediction is usually based on the following criteria: (1) the sequence complementarity between the miRNAs and the 3′ UTRs of their potential targets, with an emphasis on the 5′ seed of the miRNA (nucleotide positions 2–8 of the miRNA); (2) the miRNA–mRNA duplex has a higher negative folding free energy and stable thermodynamics. Some algorithms also consider evolutionary conservation of the binding sites in order to improve prediction accuracy (11). For example, PicTar filters miRNA-mRNA pairs according to the alignments of 3′ UTRs matching to miRNA seeds and their thermodynamic stabilities. A HMM maximum-likelihood fit approach is used to score and rank each predicted target (36). TargetScan is similar to PicTar, but it also provides conserved targeting sites in the open reading frames (ORFs) of vertebrate genomes (12). Unlike the perfect match for TargetScan and Pictar, miRanda allows occasional mismatches and G:U base-pairs; thus, it usually predicts more target sites (37) and is useful in detecting non-conserved seed match.

An earlier comparison among different prediction strategies indicated that overlapped predictions by multiple algorithms achieved the highest specificity but the lowest sensitivity. The intersection of PicTar and TargetScanS predictions was recommended in order to achieve both high sensitivity and high specificity (38). Recently, two independent groups conducted proteomics analysis to measure protein expression changes in response to miRNA perturbation. These studies indicated that seed sites are critical in predicting targets and popular prediction algorithms such as PicTar and TargetScan achieve the best evaluated results (39, 40) (see Note 2).

There are plenty of publicly available miRNA target prediction tools. They provide Web service for target prediction or archive of pre-computated results. Some prediction programs can also be downloaded (12, 36, 37, 41–47). Some useful online resources for accessing miRNAs sequences and predicting targets are summarized in Table 1 (see Note 3).

3.3. Obtaining Functional Annotations

Gene function annotations come from diverse resources such as gene ontology, signaling pathway, or large-scale gene set annotation project. These resources are usually contained in enrichment analysis tools (see Subheading 3.4). Users should be very familiar with the scope and power of each annotation; therefore they can choose more appropriate annotations.

3.3.1. Gene Ontology

Gene Ontology (GO) project describes gene function in three structurally controlled vocabularies (ontologies); namely, their associated biological processes, cellular components, and molecular functions in a species-independent manner (48). For example, Bcl-2 is annotated with "anti-apoptosis" node (GO:0006916) in biological process sub-ontology, "mitochondrial outer membrane" node (GO:0005741) in cellular component sub-ontology, and "BH3 domain binding" (GO:0051434) in molecular function sub-ontology. GO effectively categorizes genes with similar biological processes, cellular components, and molecular functions into groups. For example, 254 genes are annotated with "anti-apoptosis" node (GO:0006916), 104 genes are annotated with "mitochondrial outer membrane" (GO:0005741), and 4 genes are annotated with "BH3 domain binding" (GO:0051434).

3.3.2. KEGG

KEGG PATHWAY is a collection of hundreds of manually drawn pathway maps cataloged into ten subsections such as metabolism, drug development, and human disease (49).

3.3.3. Biocarta

Biocarta demonstrates the molecular relationships in a pathway in dynamic graphical models. It is manually curated by scientists. It covers hundreds of signaling pathways and over 120,000 genes from multiple species (http://www.biocarta.com/genes/index.asp).

Table 1
Online resources for accessing miRNAs sequences and predicting targets

Categories	Resources	Availability	Notes	References
Genomic and expression information databases	miRbase	http://www.mirbase.org/		(21)
	PRMD	http://bioinformatics.cau.edu.cn/PMRD/		(22)
Tools for vertebrates miRNA target prediction	PicTar	http://pictar.mdc-berlin.de/	Pre-computated results	(36) (12)
	TargetScan	http://www.targetscan.org/	Pre-computated results and Web server	(37) (44) (45)
	miRanda	http://www.microrna.org	Pre-computated results and program	(43) (47)
	DIANA-MicroT	http://diana.pcbi.upenn.edu/DIANA-microT	Web server	
	RNA22	http://cbcsrv.watson.ibm.com/rna22.html	Web server	
	PITA	http://genie.weizmann.ac.il/pubs/mir07	Pre-computated results and program	
	RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid	Web server and program	
Tools for plant miRNA target prediction	psRNATarget	http://plantgrn.noble.org/psRNATarget/	Web server	(42)
	Target Finder	http://jcclab.science.oregonstate.edu/node/view/56334	Program	(41) (46)
	UEA sRNA toolkit	http://srna-tools.cmp.uea.ac.uk/plant/cgi-bin/srna-tools.cgi	Web server and program	

3.3.4. Molecular Signatures Database

Molecular Signatures Database (MSigDB) is a collection of annotated gene sets for use with GSEA pipeline (50). It currently contains 6,769 gene sets which are further divided into five major collections: (1) C1 positional gene sets corresponding to each human chromosome and each cytogenetic band; (2) C2 pathways gene sets coming from various resources such as KEGG, BioCarta, STKE databases, etc.; (3) Motif gene sets including genes with conserved *cis*-regulatory motif; (4) GO gene sets from GO annotations; (5) Computational identified gene sets from large-scale microarray data.

3.4. Selecting Enrichment Models and Parameters

In traditional biological research, one gene or a few genes are investigated in details at a time. However, with the completion of genome projects for many species, high-throughput omics scanning approaches such as expression microarray, proteomics, and ChIP-on-CHIPs are routinely used. One of the main purposes of such large scanning is to obtain a global view of gene expression changes and to further elucidate underlying functional mechanisms that drive these changes. From a statistical viewpoint, the problem is to select significant functional categories by comparing the observed number of interested genes in a specific category with the number of genes that might appear in the same category if a random selection is performed from the same pool. Similar concept is applicable in the analysis of miRNAs targets, i.e., identifying the enriched functional categories with a set of miRNAs targets. In recent years, many tools have been developed. Regardless of their distinctive features, these tools generally use a list of miRNA target genes under appropriate statistical models to select significant results from back-end annotation databases. Commonly used statistical models include hyper-geometric, binomial, Fisher's exact test, and permutation analysis. Another crucial statistical issue in enrichment analysis of miRNAs targets is the correction for multiple tests since many functional categories are considered at the same time. P values are usually adjusted by the Benjamini–Hochberg procedure or a more conserved Bonferroni method (51, 52) (see Note 4). For example, miTALOS identifies miRNA-pathway associations based on Fisher's exact test and the significance is corrected by Benjamini–Hochberg procedure (58). ToppGene Suite uses permutation analysis to assign a p value for each miRNA-annotation pair and then provides options to adjust the p value to select the significantly enriched miRNA-annotation pair. Some easy-to-use Web service and programs for enrichment analysis of miRNA targets are summarized in Table 2.

4. Notes

1. Sets of co-expressed miRNAs are usually operationally defined and can be mined from miRNAs expression profiles via simple statistical testing. For example, Gusev et al. defined a set of co-expressed miRNAs as being over-expressed in cancer samples (30). Similarly, there is no clear definition of cluster miRNAs since the transcriptional control of miRNAs cluster remains elusive. Previously, we identified co-regulated mouse miRNAs clusters based on the following evidences: (1) members within a cluster should have adjacent chromosomal positions (in our settings, MID was set to 5 kb); (2) the expression

Table 2
Web servers and programs for enrichment analysis of miRNA targets

Resources	Availability	Notes	References
ToppGene Suite	http://toppgene.cchmc.org/enrichment.jsp	Web server; comprehensive annotations	(56)
David	http://david.abcc.ncifcrf.gov/home.jsp	Web server; program; comprehensive annotations	(51)
miTALOS	http://mips.helmholtz-muenchen.de/mitalos/index.jsp	Web server; KEGG annotations; tissue-specific analysis	(18)
GOmiR	http://www.bioacademy.gr/bioinformatics/projects/GOmir/	Program; GO annotations;	(19)
sigenrich	http://sigterms.sourceforge.net	Excel macros; plenty of annotations;	(57)
CORNA	http://corna.sourceforge.net/tutorial.html	Implemented in R platform; plenty of annotations;	(20)

profiles of miRNAs within this cluster should be similar (In our settings, we filtered out such clusters that the mean correlation measure is less than those of the background correlation in at least two of the three analyzed expression profiles); (3) the inter-miRNA region of the same cluster should not contain a transcription initiation signal (in our settings, we examined for the presence of regulatory elements such as transcription start site, CpG window, or predicted first exon among the inter-miRNA region within the same cluster. We filtered out the clusters that these regulatory elements were found) (17).

2. Each target prediction algorithm usually predicts hundreds of potential targets. In enrichment analysis of targets, an entire target set is used to select significant functional categories. However, in some circumstances, researchers need to select a few candidate targets for follow-up wet lab confirmation. The nuts and bolts for target priority are based on the following points. First, prediction results are ranked according to a computed score and the top predictions are thought to be more likely bona fide targets. Indeed, this ranking is usually correlated with protein down-regulation, indicating that consideration of only the top few predictions should be effective (39, 40). Second, one can select promising targets based on previous experimental results. For example, miRNA-153 is a brain-specific miRNA which is significantly repressed in glioblastoma (GBM). Our experiment results showed that miRNA-153 inhibits cell proliferation and induces apoptosis. Three targets gene, B-cell lymphoma 2 (Bcl-2), myeloid cell, leukemia

sequence 1 (Mcl-1), and insulin receptor substrate-2 (Irs-2), were ranked in the priority list because they participate in cell growth and death control. All of these targets were confirmed by follow-up experiments (53, 54).

3. Recent study has indicated that vertebrate miRNA* species play regulatory roles in gene networks based on both computational and experimental evidences (55). However, some precomputated target results do not contain the results for miRNA* species. For users who are interested in predicting targets for miRNA*, we recommend directly downloading the miRNA* sequences from public databases and search for its targets with local programs which can be downloaded. Afterwards, users can conduct enrichment analysis with the targets genes of miRNA* species.
4. Some tools offer more than one correction method. Depending on the study purpose and the corresponding data structure, researchers can select the most suitable correction model. In general, Benjamini–Hochberg procedure is the priority choice if multiple functional categories are assessed at the same time. Bonferroni method is only suitable when a few categories are simultaneously considered because researchers usually end up with no significant results when too many categories are taken into account under such conservative condition (52).

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Chapter 9

MicroRNA Expression Profiling During Neural Differentiation of Mouse Embryonic Carcinoma P19 Cells

Botao Zhao, Bing Huang, Wei Li, and Youxin Jin

Abstract

The Mouse P19 cell line was derived from an embryonal carcinoma. The pluripotent P19 cells can be induced to differentiate into neuronal and glial cells. Here, we describe an miRNA microarray method to monitor the miRNA expression profiles during the course of P19 cells neuronal differentiation. The results indicated that there were significant differences between the miRNA expression in P19 EC cells and the resultant differentiated neural stem cells.

Key words: Mouse embryonic carcinoma (EC) P19 cell, Neural differentiation of mouse P19 cells, MicroRNA, MicroRNA profile, Small RNA library, Microarray

1. Introduction

Mouse embryonic carcinoma (EC) P19 cell is a teratocarcinoma cell line derived from transplanted epiblast stage cells of mouse embryo. P19 EC cells are pluripotent cells with the ability to differentiate into derivatives of three germ layers in response to different chemical inducers in vitro (see ref. (1)). They have been widely used to dissect the mechanism of embryonic development during gastrulation stages (see ref. (2)). Aggregated P19 EC cells can be induced to differentiate into neurons and glial cells in the presence of retinoic acid (RA) (see ref. (3)). Recently, Xia et al. establish a feasible induction system that allows rapid and efficient derivation of a high percentage (~95%) of neural stem cells from P19 EC cell in N2B27 serum-free medium (see ref. (4)). This system facilitates more accurate and reliable analysis of gene expression during neural differentiation in vitro. Here, we applied a microRNA microarray

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to profile microRNA expression on the in vitro model of neural differentiation of mouse P19 cells (see ref. (5)). Total RNAs from differentiated and undifferentiated P19 cells were constructed to small RNA libraries, respectively. Fluorescence labeling was performed by PCR reaction on the small RNA libraries. Labeled targets were subjected to hybridization on the microarray fabricated in our lab. MicroRNAs microarray data were further confirmed by northern blot. The results demonstrated significant microRNA expression differences between P19 EC cells and the resultant differentiated neural stem cells.

2. Materials

2.1. Total RNA Preparation

1. Mouse embryonic carcinoma (EC) P19 cell line.
2. Trypsin, 0.25% (1×) with EDTA 4Na, liquid (Invitrogen, Carlsbad, CA, USA).
3. N2B27 medium: 1:1 mix of DMEM/F12 supplemented with 25 mg/mL insulin, 100 mg/mL apo-transferrin, 6 ng/mL progesterone, 16 mg/mL putrescine, 30 nM sodium selenite, 50 mg/mL bovine serum albumin fraction V, and neurobasal medium supplemented with B27 without Vitamin A. All chemicals were from Sigma (St. Louis, MO, USA), except the BSA and B27 media, which were from Invitrogen (Carlsbad, CA, USA).
4. TRI regent (Sigma St. Louis, MO, USA).
5. Chloroform (Merck, Darmstadt, Germany).
6. RNase-free water (Ambion, Austin, TX, USA).
7. 75% Ethanol: prepared with RNase-free water.

2.2. Construction of Small RNA Library

1. 20% Polyacrylamide/urea gel stock buffer: 19:1 Acrylamide: Bisacrylamide in 7 M urea solution. Filter through a 0.45 μm filter and store at 4°C in a brown bottle (see Note 1).
2. 2× Denature loading buffer: 10 mM EDTA, pH 8.0, 0.025% Xylene Cyanol FF and 0.025% bromophenol blue in formamide deionized.
3. 1× TBE buffer: 0.089 M Tris Base, 0.089 M Borate, and 0.002 M EDTA, pH 8.3.
4. Gel elution buffer: add 1/10 volume 3 M NaAc, pH 5.5 to RNase-free water.
5. 10 mg/mL Glycogen solution (Fermentas, Vilnius, Lithuania).
6. T4 RNA ligase supplied with 1 mg/mL BSA Solution (Fermentas, Vilnius, Lithuania).

7. 100 mM ATP solution (Fermentas, Vilnius, Lithuania).
8. RNase inhibitor (Takara, Japanese).
9. 50% PEG 6000 in RNase-free water.
10. Terminal Deoxynucleotidyl Transferase (TdT) (Fermentas, Vilnius, Lithuania).
11. 2′,3′-Dideoxyadenosine 5′-Triphosphate, 100 mM Solution (ddATP) (GE Healthcare, Piscataway, NJ, USA).
12. T4 Polynucleotide Kinase (T4 PNK) (Fermentas, Vilnius, Lithuania).
13. Tris–HCl saturated phenol pH 8.0 (Sangon, Shanghai, China).
14. Access Quick RT-PCR System (Promega, Madison, WI, USA).
15. ssDNA ladder (20-nt, 30-nt, 38-nt, 52-nt, 55-nt, and 75-nt) (Invitrogen, Carlsbad, CA, USA) (see Note 2).
16. 5′ Adapter & primer: ACCGAATTCACAGTCAGACC; 3′ adapter: GCAGATCGTCAGAATTCCAG; 3′ primer: CTGGAATTCTGACGATCTGC (Invitrogen, Carlsbad, CA, USA).

2.3. MicroRNA Detection by Microarray

1. 5′-C6-Amine-labeled probes (Invitrogen, Carlsbad, CA, USA).
2. Array printing buffer: 2× SSC, 1 M betaine hydrochloride, 0.01% SDS, 5% formamide deionized.
3. Epoxy-coated glass slides (ArrayIt, Sunnyvale, CA, USA).
4. Cy5-labeled 5′ primer (Takara, Japanese) (see Note 3).
5. Deep Vent DNA polymerase (NEB, Ipswich, MA, USA).
6. dNTP solution (Fermentas, Vilnius, Lithuania).
7. Sephadex G50 (GE Healthcare, Piscataway, NJ, USA).
8. Array blocking buffer: 0.1 M Tris–HCl pH8.5, 50 mM ethanolamine.
9. 2× Array hybridization buffer: 8× SSC, 0.2%SDS.
10. Array wash buffer 1: 2× SSC pH7.0, 0.03%SDS; Array wash buffer 2: 0.2× SSC pH7.0; Array wash buffer 3: 0.1× SSC pH7.0.

3. Methods

3.1. Total RNA Preparation of P19 Cells

1. Dissociate nearly confluent P19 cells into single cells by Trypsin-EDTA and allow the cells to aggregate in the cell

culture dishes at a seeding density of 1×10^5 cells/mL in N2B27 medium for 4 days.

2. Harvest differentiated and undifferentiated P19 cells and remove the culture medium.
3. Add TRI regent directly to the cell culture dish (1 mL per 10 cm^2 culture plate surface area) and homogenize by pipetting several times (see Note 4).
4. Allow sample to stand for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes.
5. Transfer the sample into a 1.5-mL centrifuge tube. Add 200 μL of chloroform per 1 mL TRI regent used and shake vigorously for 15 s. Allow the sample to incubate at room temperature for an additional 2–3 min.
6. Centrifuge the resulting mixture at 12,000 × *g* for 15 min at 4°C.
7. Carefully transfer the upper aqueous phase to a new tube. Add 2.5-fold volume ethanol to the tube and at –20°C for at least 2 h.
8. Pellet the RNA by centrifuging the sample at 16,000 × *g* for 15 min at 4°C.
9. Discard the supernatant. Add 1 mL of 75% ethanol to the pellet and invert the tube twice then incubate at room temperature for 5 min.
10. Spin at 16,000 × *g* for 5 min at 4°C, and discard supernatant. Re-spin for 10 s and completely remove all liquid. Briefly dry the RNA pellet for 2–5 min.
11. Add an appropriate volume of RNase-free water to dissolve the RNA pellet.
12. Quantify the RNA on a NanoDrop 2000 Spectrophotometer.

3.2. Construction of Small RNA Library

1. Pour a 15% polyacrylamide/urea gel (15 cm length). Pre-run the gel for 15–30 min at 200 V, then wash the wells with 1× TBE buffer using a syringe.
2. Combine 20 μg of total RNA and 10 μL of 2× denature loading buffer to a final volume of 20 μL.
3. Prepare ssDNA oligo ladder by combining 1 μg of 20-nt and 30-nt oligo each in 20 μL 1× loading buffer (see Note 5).
4. Denature the sample and oligo ladder at 80°C for 5 min and put on ice immediately.
5. Centrifuge to collect volume to bottom of tube and load the sample and oligo ladder onto the gel.
6. Run the gel at 10W until the bromophenol blue reaches the bottom of the gel with 1× TBE buffer (see Note 6).

7. Dismantle the gel-running apparatus and sandwich the gel with clear plastic wrap.
8. Place the gel on top of the dull white side of a thin layer chromatography (TLC) fluor-coated plate. Carefully position and mark the ladders and lanes on the plastic wrap under a hand-held UV light (254 nm) (see Note 7).
9. Turn the gel over and remove the unmarked plastic wrap. Excise the gel slice between the 20-nt and 30-nt ssDNA ladders with a clean and burned scalpel blade.
10. Cut the gel slice into small pieces (1 mm^3 cubes), transfer to a new microcentrifuge tube.
11. Add appropriate amount of gel elution buffer to ensure immersing all the gel pieces, and elute the RNA by rotating the tube gently at room temperature for at least 4 h (to overnight).
12. Transfer the supernatant to a new tube and repeat the elution for 2 h.
13. Combine the supernatants and remove the gel residual by centrifugation at 10,000 × *g* for 5 min.
14. Transfer the supernatant to a new tube, precipitate the RNA by adding glycogen to a final concentration of 10 μg/mL and three volumes of absolute ethanol at overnight –20°C.
15. Recover the RNA pellet as described in steps 8–11 of Subheading 3.1 (see Note 8).
16. Add ligation reaction system (2 μL of 10× T4 RNA ligase buffer, 1 μL of 1 mg/mL BSA, 1 μL of 10 mM ATP, 1 μg of 5′ adapter, 20 U of RNase inhibitor, 10 U of T4 RNA ligase, 10 μL of 50% PEG6000, and water to 20 μL) to the tube, mix gently and incubate the tube for 4–8 h at 16°C (see Note 9).
17. Add equal volume of 2× gel-loading solution and denature the nucleic acids by incubating the tube for 5 min at 80°C. Put sample on ice immediately.
18. Load samples and ssDNA oligo ladders (1 μg of 38-nt and 52-nt oligo each) onto a 10% polyacrylamide/urea gel and run at 10W until the bromophenol blue reaches the bottom of the gel.
19. Excise the gel between the 38-nt and 52-nt ssDNA ladders and recover the RNA as steps 7–15.
20. Block 3′ adapter by adding a ddATP residue to its 3′ end. Combine the reaction system (5 μL of 5× TdT buffer, 5 nmol of 3′ adapter, 25 nmol of ddATP, 40 U of TdT, and water to 25 μL) and incubate at 37°C for at least 4 h (to overnight).
21. Purify the blocked adapter as steps 4–15 (see Note 10).
22. Phosphorylate the blocked 3′ adapter (5 μL of 5× T4 PNK forward reaction buffer, 1 mM ATP, 10 μL of adapter, 20 U of

T4 PNK and ddH2O to 25 μL) and incubate at 37°C for 4 h.

23. Add 175 μL ddH2O to the tube, extract by phenol/chloroform and chloroform (see Note 10).
24. Remove the aqueous phase to a new tube, precipitate the adapter by adding 1/10 volume 3M NaAc, pH 5.5, and two volumes of ethanol.
25. After incubating at –20°C for 2 h, recover the treated adapter pellet as described in steps 8–11 of Subheading 3.1.
26. Ligate the 3′ adapter to small RNA as step 16. Substitute the 5′ adapter by the blocked and phosphorylated 3′ adapter in the ligation reaction system.
27. Recover the ligated RNA as steps 17–19 by using 55-nt and 75-nt ssDNA ladders.
28. Perform RT-PCR on small RNA ligated with 3′ and 5′ adapters by Access Quick RT-PCR System (25 μL of 2× master mix, 0.5 μM of each primer, purified ligated RNA, 5 U of AMV, and RNase-free water to 50 μL). Incubate the reaction at 48°C for 30 min and at 94°C for 2 min, and then cycle 10–20 times at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s.
29. Take 5 μL from the RT-PCR reaction to run a 2% agarose gel. A good band in the 60-nt size range can be seen.

3.3. MicroRNA Detection by Microarray

1. Extract miRNA sequences from miRbase 9.0. Synthesize miRNA complementary sequences as microarray probes (see Note 11). Add 10 deoxyadenosine residues to the 5′ end of the probe as spacer. Probes are 5′-C6-Amine labeled.
2. Dissolve the miRNA probes in array printing buffer at a concentration of 20 μM.
3. Print the probes on epoxy-coated glass slides at 60% humidity with PixSys 5500 spotting robot. Spaces of dot and subarray are 200 μm and 4.5 mm, respectively.
4. Leave the printed slides in the printing platform at 60% humidity overnight to complete cross-linking reaction. Store the printed slides dryly for later use.
5. Label small RNA by PCR with a Cy5-labeled 5′ primer and 3′ primer. Set up a 100 μL PCR reaction (10 μL of 10× PCR buffer, 0.2 mM of dNTP, 0.4 μM of each primer, 1–2 μL of template from step 28 of Subheading 3.2, 1 U of Deep Vent DNA polymerase, and water to 100 μL). Run a PCR program as step 28 of Subheading 3.2 ignoring the 48°C incubation.
6. Precipitate and recover the PCR product as steps 24 and 25 of Subheading 3.2. Dissolve in 10 μL ddH2O.

7. Swell 1 g Sephadex G50 in 10 mL of ddH2O overnight. Transfer 400 μL of G50 slurry to a spin column. Spin 2 min at 1,000 × *g*. Transfer the column to a new tube, load the Cy5-labeled PCR product on the G50 column. Spin 2 min again at 1,000 × *g*. Collect the cleaned PCR product.
8. Take a microarray slide, and mark the printed area at the reverse side.
9. Block the slide in array blocking buffer at 50°C for 30–60 min.
10. Rinse the slide in ultra-pure water by gentle shake for 10 min.
11. Put the slide in a 50-mL culture tube and centrifuge at 2,000 × *g* for 1 min to remove any drop of water (see Note 12).
12. Denature the Cy5-labeled PCR product at 90°C for 3 min and cool to 4°C on a PCR machine.
13. Mix equal volume of 2× array hybridization buffer to the PCR product and apply to the array area.
14. Carefully cover a slide cover on it to avoid any bubbles.
15. Clean and dry an array hybridization cassette. Add 10 μL of water to each reservoir inside the cassette chamber.
16. Put the slide in an array hybridization cassette. Cover the clear plastic cassette lid by tightening the sealing screws.
17. Submerge the hybridization cassette in a water bath at 48°C in dark overnight (see Note 13).
18. Remove excess water from the outside of the cassette to prevent incubator water from flowing into the cassette chamber and reaching the array slide before the sealing screws are loosened.
19. Loosen the sealing screws and remove the lid.
20. Quickly transfer the slide to a beaker containing the initial wash buffer 1. Gently agitate the wash buffer to make the cover slip float free from the microarray surface.
21. Gently rinse the array slide in wash buffer 1 for 2 min, in wash buffer 2 for 2 min, and in wash buffer 3 for 1 min at room temperature.
22. Dry the slide as step 11. Slide can be stored at dark and dry for short term.
23. Scan the array by ScanArray 4000 at 5 μm resolution (Fig. 1).
24. Extract the dot intensities by QuantArray.
25. Normalize the array data by total intensity. Remove the bad dots by Coefficient of Variation (CV) evaluation. Calculate the adjusted *p* values.

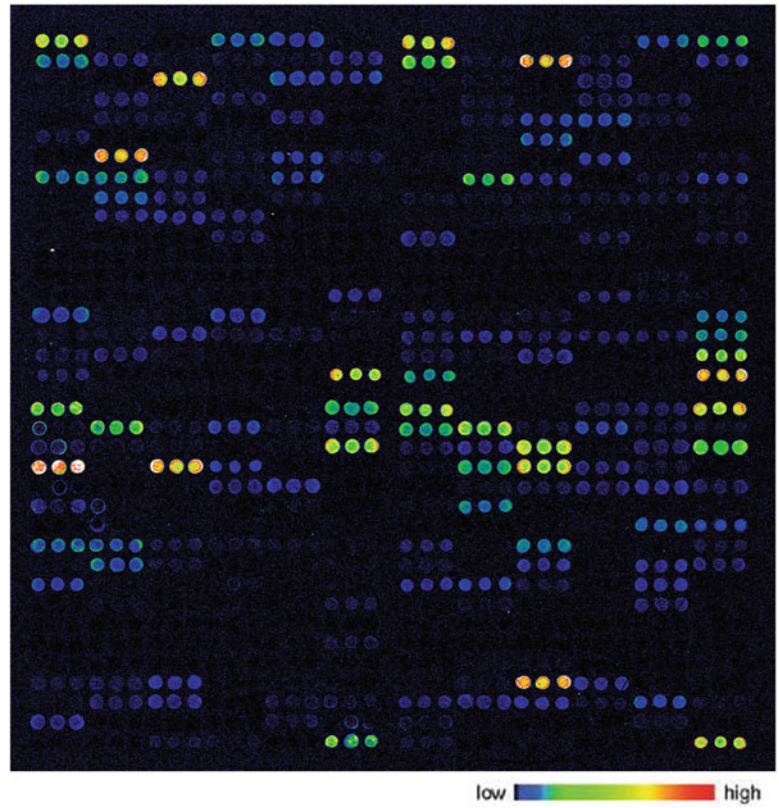

Fig. 1. A sample image scaned by ScanArray 4000.

26. MicroRNAs differentially expressed in the P19 cells and the induced P19 neural stem cells are listed in Table 1. Changes of some of microRNAs are confirmed by northern blot (see Note 14) (Fig. 2).

4. Notes

1. Urea may precipitate from the stock solution when kept in refrigerator. Resolve urea by warming at 37°C before use.
2. ssDNA ladders can be any sequences with balanced base composition. It is better to use PAGE purified oligonucleotides as ssDNA ladders.
3. Reconstitute Cy5-primer in ultra-pure water and dry by vacuum in small aliquots. Store dried primers in dark and cold for long-term storage.
4. If not special noted (e.g., mirVana™ miRNA Isolation Kit), total RNA purified by the popular column procedure will be significantly depleted in small RNAs. Trizol/TriReagent

Table 1
List of miRNAs showing differential expression in the non-induced P19 cells compared with the induced P19 neural stem cells (reproduced from (5) with permission from Oxford University Press)

Up-regulated		Down-regulated	
miRNA name	**P19 N/P19**	**miRNA name**	**P19 N/P19**
mmu-miR-103	2.14	mmu-miR-182	0.2255
mmu-miR-106b	2.062	mmu-miR-183	0.2114
mmu-miR-124	20.31	mmu-miR-200b	02151
mmu-miR-130a	2.623	mmu-miR-22	0.2402
mmu-miR-130b	2.037	mmu-miR-23a	0.306
mmu-miR-134	2.03	mmu-miR-23b	0.1901
mmu-miR135a	3.019	mmu-miR-24	0.2163
mmu-miR-135b	2.544	mmu-miR-27a	0.2576
mmu-miR-153	7.773	mmu-miR-27b	0.2496
mmu-miR-154	2.382	mmu-miR-29a	0.2827
mmu-miR-16	2.377	mmu-miR-29b	0.4937
mmu-miR-181a	3.066	mmu-miR-302a	0.2939
mmu-miR-181b	3.058	mmu-miR-302b	0.2189
mmu-miR-181c	2.98	mmu-miR-302c	0.2662
mmu-miR-191	5.966	mmu-miR-302d	0.2721
mmu-miR-20a	2.107	mmu-miR-31	0.2876
mmu-miR-210	4.212	mmu-miR-499	0.3616
mmu-miR-219	8.036	mmu-miR-7	0.3457
mmu-miR-221	2.312	mmu-miR-96	0.1544
mmu-miR-26a	2.231	mmu-miR-10b	0.4221
mmu-miR-26b	2.243	mmu-miR-290-5p	0.0951
mmu-miR-335-5p	8.425	mmu-miR-291a-2p	0.0626
mmu-miR-33	2.407	mmu-miR-291a-5p, mmu-miR-291b-5p	0.1476
mmu-miR-369-3p	2.891	mmu-miR-292-3p	0.1082
mmu-miR-381	2.827	mmu-miR-292-5p	0.0653
mmu-miR-382	2.433	mmu-miR-293	0.0561
mmu-miR-410	2.462	mmu-miR-294	0.0775

(continued)

Table 1
(continued)

Up-regulated		Down-regulated	
miRNA name	**P19 N/P19**	**miRNA name**	**P19 N/P19**
mmu-miR-151-3p	2.362	mmu-miR-295	0.0705
mmu-miR-153	7.497	mmu-miR-298	0.2364
mmu-miR-300	2.013	mmu-miR-346	0.4983
mmu-miR-341	2.293	mmu-miR-96	0.1882
mmu-miR-376a	3.663		
mmu-miR-410	2.094		
mmu-miR-9	2.136		

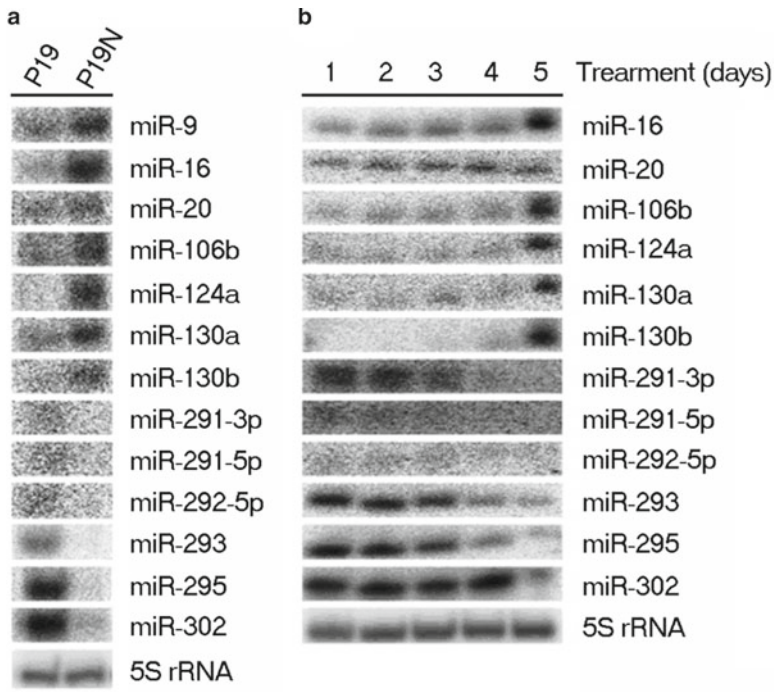

Fig. 2. Validation of the results from miRNA microarray studies by northern blot analysis. (**a**) miRNA expression in non-induced P19 cells (P19) and induced P19 neural stem cells (P19 N). (**b**) miRNA expression during 4 days of P19 induction. The names of the miRNAs are mentioned at the right side of each panel. 5S rRNA served as a loading control (reproduced from (5) with permission from Oxford University Press).

extraction procedures are the recommended methods. However, we recommend to substitute isopropanol precipitation with ethanol precipitation for better recovery small RNAs.

5. If total RNA is enough, use a combination of ssDNA oligo and additional 20 μL of total RNA as ladder. It avoids migration difference due to the different amount loaded in lanes, and therefore facilitates excising gel slices of expected size more accurately.
6. Attach a metal plate at one side of the gel to improve the dissipation of heat, which makes the electrophoretic bands more neatly and the location of the expected gel slide more accurately.
7. EB staining also can be used for ladder visual. However, the UV shadowing method is strongly recommended because it avoids RNase and cross contamination effectively.
8. Do not pour the supernatant directly because the pellet of RNA and glycogen attached very loosely to the tube bottom. Remove supernatant carefully by pipette to avoid any loss of RNA pellet.
9. PEG6000 stimulates the ligation reaction greatly in the indicated concentration. We have tested several T4 RNA ligases from different sources and found that T4 RNA ligase purchased from Fermentas showed the best performance in our situation.
10. Adapter blocked with a ddATP has one more nucleotide than the original adapter while phosphorylated adapter migrates about half a nucleotide faster than the un-phosphorylated adapter. Therefore, blocked and phosphorylated adapter can be separated easily with the untreated one on a PAGE/urea gel longer than 30 cm.
11. The T_ms of all probes are adjusted to 55 ± 3°C as below. If the probe T_m is higher than 58°C, ignore some nucleotides at both end of the probe to lower the T_m. If it is lower than 52°C, then add nucleotides at 3′ end of the 5′ primer to the 5′ end of the probe to raise the T_m.
12. Place the glass slides at the direction parallel to the radius of centrifuge rotor; otherwise, the slides are cracked when centrifuging.
13. The hybridization is 7°C lower than the average T_m of the probes in our experiment, which is a compromise of the signal intensity and hybridization specificity described in Zhao et al. (6).
14. Real-time PCR also can be used to validate the microarray result. However, northern blot is recommended because of its high reliability.

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Chapter 10

Evaluating the MicroRNA Targeting Sites by Luciferase Reporter Gene Assay

Yi Jin, Zujian Chen, Xiqiang Liu, and Xiaofeng Zhou

Abstract

MicroRNAs are post-transcriptional regulators that control mRNA stability and the translation efficiency of their target genes. Mature microRNAs are approximately 22-nucleotide in length. They mediate post-transcriptional gene regulation by binding to the imperfect complementary sequences (a.k.a. microRNA regulatory elements, MRE) in the target mRNAs. It is estimated that more than one-third of the protein-coding genes in the human genome are regulated by microRNAs. The experimental methods to examine the interaction between the microRNA and its targeting site(s) in the mRNA are important for understanding microRNA functions. The luciferase reporter gene assay has recently been adapted to test the effect of microRNAs. In this chapter, we use a previously identified miR-138 targeting site in the 3′-untranslated region (3′-UTR) of the RhoC mRNA as an example to describe a quick method for testing the interaction of microRNA and mRNA.

Key words: MicroRNA, MicroRNA targeting sequence, MicroRNA regulatory element, Luciferase reporter gene assay, miR-138, RhoC

1. Introduction

MicroRNAs (miRNAs) are endogenously expressed, single-stranded noncoding RNAs (approximately 20–24 nucleotides in length) found in almost all eukaryotic cells. miRNAs have been shown to regulate many developmental and physiological processes, and the deregulation of miRNAs has been linked to a number of disease processes (1, 2). miRNAs constitute an important class of fine-tuning gene expression regulators referred to as “dimmer switches” because of their ability to

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repress gene expression without completely silencing it. They are post-transcriptional regulators that bind to imperfect complementary sequences (a.k.a. miRNA regulatory element, MRE) on the target messenger RNA transcripts (mRNAs) and usually result in translational repression and gene silencing (2). Animal miRNAs usually bind to sites in the 3′-untranslated region (3′-UTR), whereas plant miRNAs usually bind to coding regions of mRNAs. A number of bioinformatics tools are available to predict the miRNA targeting sequences (3). However, to understand the roles of microRNA in complex biological processes, it is important to experimentally assess the functional relevance of the predicted miRNA targeting site(s).

MicroRNA-138 (miR-138) has been shown to regulate a number of essential biological processes, including the development of the mammary gland (4), regulation of dendritic spine morphogenesis (5), modulation of cardiac patterning during embryonic development (6), and thermo-tolerance acquisition (7). The deregulation of miR-138 has been frequently observed in a number of cancer types, including thyroid cancer (8), lung cancer (9), leukemia (10), and head and neck/oral cancers (11–15). Down-regulation of miR-138 is associated with enhanced RhoC expression and cell migration and invasion in oral cancer cells (12, 16). A targeting sequence for miR-138 has recently been identified in the 3′-UTR of the RhoC mRNA (Fig. 1) (12). In this chapter, for illustration, we will test the interaction of miR-138 and its targeting sequence in the 3′-UTR of the RhoC mRNA.

Firefly luciferase is commonly used as a reporter to assess the transcriptional activity in intact cells. The most common application of luciferase reporter gene assay is to examine the regulation of transcriptional activities by promoters and transcription factors. Recently, this assay has also been adapted for testing the effect of miRNA-mediated, post-transcriptional regulation on target genes. This is achieved by engineering a luciferase gene construct containing the predicted miRNA targeting sequence from the target gene (often located in the 3′-UTR). For many human genes, luciferase constructs containing the entire 3′-UTR can be obtained from a number of commercial sources (e.g., OriGene Technologies, Inc, GeneCopoeia, Inc, and SwitchGear Genomics). However, the 3′-UTRs may contain multiple targeting sequences and other regulatory elements. Specific assays to test each miRNA targeting sequences are needed. In this chapter, we describe a quick method to test the interaction of miRNA and the specific target sequence. We also present a simple strategy for creating mutant construct as the negative control.

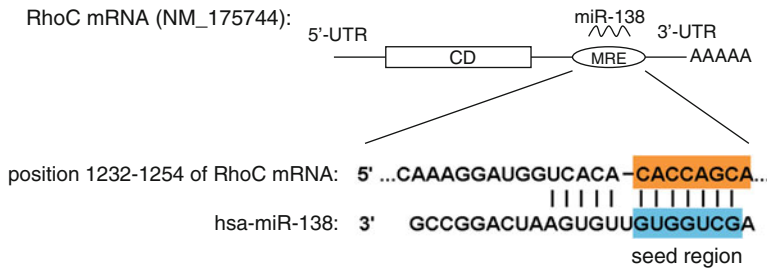

Fig. 1. The miR-138 targeting sequence located in the 3′-untranslated region of RhoC mRNA.

2. Materials

2.1. pGL3 Luciferase Reporter Vector

There are a number of available luciferase reporter vectors, including recently introduced ones that are designed for testing miRNA-mediated gene silencing. In this chapter, we use pGL3-Control Vector (Promega), one of the most commonly used luciferase reporter vectors.

2.1.1. pGL3-Control Firefly Luciferase Control Vector (Promega)

2.1.2. pRL-TK Vector (Renilla Luciferase Control Reporter Vectors) (Promega)

The pRL-TK vector provides constitutive expression of Renilla luciferase. pRL-TK vector co-transfected with firefly luciferase vector provides an internal control for normalization of the transfection efficiency.

2.2. pGL3 Luciferase Reporter Constructs

pGL3 luciferase reporter constructs are created by cloning the specific miRNA binding sequence (wild-type/mutants) into the XbaI site located at 3′-UTR of pGL3-Control vector.

2.2.1. Digestion of pGL3-Control Vector

1. Restriction enzyme XbaI (Fermentas).
2. Alkaline phosphatase, calf intestinal (CIP, Fermentas).

2.2.2. Oligonucleotides Synthesis (Sigma) and Annealing

1. Sense and antisense oligos were synthesized with XbaI site at 5′ and 3′-end.
2. 10× Annealing buffer: 100 mM Tris–HCl, pH 7.5, 1 M NaCl, 10 mM EDTA.

2.2.3. Agarose Gel Electrophoresis for DNA Analysis

1. 10× TBE buffer (Tris-Borate electrophoresis buffer: 108 mg/mL Tris base, 55 mg/mL Boric acid, 9.3 mg/mL EDTA).
2. Ethidium bromide (5 mg/mL) (EtBr).
3. Prepare 1.5% agarose gel: Add 0.75 g agarose to 50 mL 1× TBE. Heat the solution in the microwave to dissolve the agarose. When the solution is cooling down, add 5 μL of EtBr to the dissolved agarose and mix well in a fume hood. Pool the gel to the gel tray and let it cool down completely.

4. Running buffer: 1× TBE.
5. Electrophoresis equipment.
6. Gel loading dye (6×): 50 mM EDTA, 0.2% SDS, 50% glycerol, 0.05% w/v bromophenol blue.
7. 1 kb and 100 bp DNA ladder.

2.2.4. Quick Ligation Kit (New England Biolabs)

(a) Quick T4 DNA ligase; 2× Quick ligation reaction buffer.

2.2.5. Transformation

1. XL1-blue Supercompetent cells (Stratagene).
2. SOC media (Cellgro).
3. LB-agar plates with 100 μg/mL ampicillin: 5.0 g tryptone, 2.5 g yeast extract, 5.0 g NaCl, 7.5 g agar; adjust volume to 500 mL with dH_2O. After autoclaving, add ampicillin to make final concentration 100 μg/mL.

2.2.6. DNA Purification

(a) GeneJet Plasmid Miniprep Kit (Fermentas).

2.3. Transfection Reagent and miRNA Mimic

2.3.1. Hela Cell

HeLa is an immortal cell line used for transfection experiments. Other comparable cell lines can also be used based on experimental design.

2.3.2. Medium and Transfection Reagent

(a) Lipofectamine™ 2000 transfection reagent (Invitrogen).
(b) Opti-MEM I reduced serum medium (Gibco).

2.3.3. miRNA Mimic

1. hsa-miR-138 mimic: 5′-AGCUGGUGUUGUGAAUCAGG CCG (Thermo scientific-Dharmacon).
2. Negative control mimic #1: cel-miR-67, 5′-UCACAACCUCC UAGAAAGAGUAGA (Thermo scientific-Dharmacon).

2.4. DUAL-Luciferase Reporter Assay System

Dual-luciferase Reporter Assay kit provides an optimized system and all the necessary reagents for the sequential assay of firefly and Renilla luciferase activity.

2.4.1. Dual-Luciferase Reporter Assay Kit (Promega)

1. 1× Passive Lysis Buffer (PLB): Dilute 5× PLB with dH_2O.
2. LAR II: Resuspend the lyophilized Luciferase Assay Substrate in Luciferase Assay Buffer II. Store at –20°C (up to 1 month) or –70°C (up to 1 year).
3. Stop & Glo Reagent: Add 50× Stop & Glo Substrate to Stop & Glo buffer. Store at –20°C for 15 days.

2.4.2. Glomax 20/20 Luminometer (Promega)

Other comparable luminometer can also be used.

3. Methods

3.1. Cloning Method

3.1.1. Design the Wild-Type and the Mutant miRNA Targeting Sites (Oligonucleotides Synthesis)

Chemical-based oligonucleotide synthesis provides a rapid and inexpensive access to custom-made oligonucleotides of the desired sequence (up to several hundred nucleotide residues). To simplify our cloning strategy, we will take the advantage of vastly available resources for synthesizing oligonucleotides (e.g., Integrated DNA Technologies, Sigma-Genosys). To illustrate, we will design a set of oligonucleotides to test the previously described hsa-miR-138 targeting sequence in the 3′-UTR of the RhoC mRNA (12). Sense and antisense sequences corresponding to a 62-bp fragment from the 3′-UTR of RhoC mRNA (position 1210–1271, NM_175744) will be used. Partial sequences for the XbaI site are appended to the ends of the oligo for creating sticky ends upon annealing. The 5′ phosphorylation is also required to facilitate the ligation. The sequences of these oligonucleotides are listed below. The XbaI sites are indicated by bold font, and the seed regions of the hsa-miR-138 targeting site are indicated by underlining.

1. Wild-type sense: 5′-p**CTAGA**TCTTGCCCCCTTTGACCT T C C C C A A A G G A T G G T C A C A C A C C A G C ACTTTATACACTTCTGGC**T**-3′.
2. Wild-type antisense: 5′-p**CTAGA**GCCAGAAGTGTATAAAGT G C T G G T G T G T G A C C A T C C T T T G G G G A A G G T C AAAGGGGGCAAGA**T**-3′.

   As a negative control, we also designed oligonucleotides containing a mutated hsa-miR-138 targeting site. The mutants vare created by replacing the seed regions of the hsa-miR-138 targeting site with $T_{(7)}$ (see Note 1).
3. Mutant sense: 5′-p**CTAGA**TCTTGCCCCCTTTGACCTT C C C C A A A G G A T G G T C A C A T T T T T T T A C T T T A T ACACTTCTGGC**T**-3′.
4. Mutant antisense: 5′-p**CTAGA**GCCAGAAGTGTATAAAGT A A A A A A A T G T G A C C A T C C T T T G G G G A A G G TCAAAGGGGGCAAGA**T**-3′.

3.1.2. Oligonucleotides Annealing

1. Resuspend both sense and antisense oligos in 1× annealing buffer to make the final concentration 100 μM.
2. Mix 1 μL of each strand with 1 μL of 10× annealing buffer and 7 μL of H_2O.
3. Incubate the reaction mixture at 95°C for 6 min, and then place at room temperature for 30 min.
4. Store on ice or at 4°C until ready to use.
5. Dilute the annealed oligos twice before performing ligation: for the first dilution, dilute 20× with dH_2O. For the second dilution, dilute 100× with 1× annealing buffer (final concentration is 5 nM).

3.1.3. pGL3-Control Vector Linearization and Purification

1. pGL3-Control vector is digested by XbaI (Fermentas) and dephosphorylated by Alkaline phosphatase, and calf intestinal (CIP, Fermentas) simultaneously. To cut the vector, 1 μg of PGL3 DNA is incubated with 1 μL of XbaI, 1 μL of CIP, 1 μL of 10× digestion buffer, and 7 μL of dH_2O (total volume 10 μL) at 37°C for 1 h. Then incubate the mixture at 75°C for 5 min to inactivate the enzymes.
2. Add 6× loading dye to the digested product and load to 1.5% agarose gel.
3. Electrophorese the sample at 100 V for 20 min. Check the gel frequently while it is running to make sure it is not getting too hot, as this will distort the bands or melt the agarose. If the DNA fragment is ≤200 bp, check the gel every 5 min to make sure the band is not running out of gel.
4. Following electrophoresis, check the gel under the UV light and take a picture as a record.
5. Cut the DNA fragment from agarose gel with a clean, sharp scalpel.
6. Follow the Qiaquick gel extraction kit protocol (Qiagen) to retrieve and purify the DNA fragment (linearized pGL3-Control vector).
7. Measure the concentration (μg/μL) of DNA and convert it to molar concentration.

3.1.4. Ligation

Insert synthesized oligos into pGL3-Control vector at XbaI site.

1. Combine 1 μL XbaI digested, and dephosphorylated pGL3-Control vector (1 nM) with 1 μL of insert (5 nM). The vector and insert ratio is 1:5. Adjust volume to 5 μL with dH_2O.
2. Add 5 μL of 2× quick ligation reaction buffer and 0.5 μL of quick T4 DNA ligase and mix thoroughly.
3. Centrifuge briefly and incubate at room temperature for 30 min.
4. Chill on ice, the sample is ready for transformation or can be stored at −20°C (see Note 2).

3.1.5. Transformation, Pick up Clones, and Isolation of Plasmid DNA

The ligation products are ready for transformation. Many different transformation methods could be used. We use XL1-blue supercompetent cells from Stratagene to perform transformation. We briefly describe the procedure here:

1. Prechill two 14 mL BD falcon polypropylene round-bottom tubes on ice. (One for ligation product transformation and one for control experiment). Preheat SOC medium to 42°C.
2. Thaw XL1-blue competent cells on ice. Then add 30 μL of cells into each of prechilled tubes and mix gently.

3. Add 0.5 μL of β-mercaptoethanol to each tube and swirl gently. Incubate tubes on ice for 10 min while gently swirling every 2 min.
4. Add 1–2 μL of ligation product to one tube and add the same amount of control product to the other tube. Mix gently by pipetting up and down. Incubate the tubes on ice for 30 min.
5. Heat shock for 45 s in 42°C water bath, then chill on ice for 5 min. The precise duration of the heat shock (45 s) is critical for transformation efficiency.
6. Add 300 μL of preheated SOC medium and incubate at 37°C for 1 h while shaking at 250–300 rpm.
7. Spread 50–100 μL of transformation mixture on LB agar plates (100 μg/mL ampicillin).
8. Incubate the plate overnight (≥16 h) at 37°C.
9. Check the plate for clone growth next day.
10. Pick up several clones to grow overnight at 37°C with shaking at 250–300 rpm.
11. Purify plasmid DNA with GeneJet Plasimd Miniprep Kit (Fermentas).
12. Check the plasmid DNA on 1.5% agarose gel. If the size of the plasmid DNA is as expected, sequence the plasmid to check the orientation of the insert and detecting any mutation (see Note 3).

3.2. Co-transfection of Plasmid DNA and miRNA Mimic Along with pRL-TK Vector into Mammalian Cells

1. One day prior to transfection, seed $5–10 \times 10^4$ Hela cells to each well of a 24-well plate. Approximately 20–40% confluence of the cells is anticipated at the time of transfection (16 h after seeding).
2. Incubate cells at 37°C with 5% CO_2 overnight.

3.2.1. Seed Hela Cells

3.2.2. Lipofectamine 2000 Transfection

All volumes are multiplied by 3.5 to account for the triplicate samples and loss during pipetting.

1. Prepare stock pGL3 plasmid (250 μg/mL), pRL-TK (5 μg/mL), hsa-miR-138 mimic (25 μM), and negative control mimic (25 μM) in RNase-free water (see Note 4).
2. Prepare transfection reagent: for each transfection, 2 μL of lipofectamine 2000 is incubated with 400 μL of Opti-MEM medium at room temperature for 5 min (Solution 1).
3. Mix plasmid and the appropriate miRNA mimic.

   For our experiment testing the hsa-miR-138 targeting site in RhoC mRNA, the following experimental groups are used:

   Negative control mimic (1.6 μL) + pGL3-WT (4 μL) + pRL-TK (1 μL).

hsa-miR-138 mimic (1.6 μL) + pGL3-WT (4 μL) + pRL-TK (1 μL).

Negative control mimic (1.6 μL) + pGL3-Mutant (4 μL) + pRL-TK (1 μL).

hsa-miR-138 mimic (1.6 μL) + pGL3-Mutant (4 μL) + pRL-TK (1 μL).

This will make the final working concentrations at 100 nM miRNA mimic, 2.5 ng/μL pGL3 plasmid, and 12.5 pg/μL pRL-TK.

4. After 5 min, add Solution 1 into each tube and mix the contents of all tubes gently by pipetting carefully up and down.
5. Incubate tubes for 20 min at room temperature to form the transfection mixture.
6. Remove medium from the wells of the 24-well plate containing cells and wash with PBS twice. Then add the transfection mixture to each well.
7. Incubate cells at 37°C in 5% CO_2 for 48 h.
8. If cell toxicity is observed after 24 h, replace the transfection medium with complete medium and continue incubation.

3.3. Luciferase Activity Assay

1. After 48 h, remove growth medium from wells and rinse cells with PBS twice.
2. Dispense 200 μL of 1× PLB into each well.
3. Gently shake the 24-well plate for 15 min at room temperature. Transfer lysate to a 1.5 mL tube. If residual cell debris is presented in the lysate, clear the lysate by centrifuge at 13,500 rpm for 15 min at 4°C and transfer the supernatant to a new tube.
4. Pre-dispense 100 μL of LAR II into 1.5 mL clear Eppendorf tubes.
5. Program the luminometer to perform a 2-s pre-measurement delay, followed by a 10-s measurement period for each reporter assay.
6. Carefully transfer 20 μL of cell lysate into the tube containing LAR II; mix by pipetting 2 or 3 times. Place the tube in the luminometer and initiate reading. Do not vortex, and avoid bubbles which can interfere with the mixing of Stop & Glo Reagent added in the next step, and affect the final reading.
7. Record the firefly luciferase activity measurement.
8. Remove the tube from the luminometer, add 50 μL of Stop & Glo Reagent and mix gently. Replace the sample in the luminometer, and initiate reading.
9. Record the Renilla luciferase activity measurement.
10. Calculate the ratio of firefly luciferase activity to Renilla luciferase activity. This is the normalized luciferase activity.

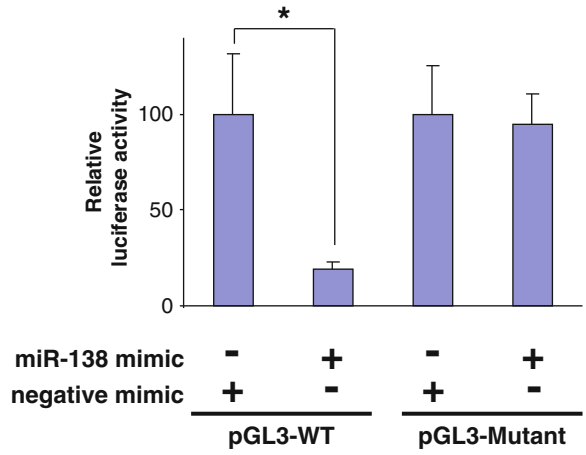

Fig. 2. MicroRNA-138 targeting the RhoC mRNA through a targeting sequence located at 3′-UTR. Dual-luciferase reporter assays were performed to test the interaction of hsa-miR-138 and its targeting sequence in the RhoC 3′-UTR using constructs containing the predicted targeting sequence (pGL3-WT) and mutated targeting sequence (pGL3-Mutant) cloned into the 3′-UTR of the reporter gene. Data represent three independent experiments with triplicate measurements. * indicates $p < 0.05$.

11. The effect of miRNA on the luciferase gene expression can be shown as changes in relative luciferase activity (Fig. 2). This is achieved by setting the normalized luciferase activity of **negative mimic + pGL3-WT + pRL-TK** sample to 100%, and the reading of **miR-138 mimic + pGL3-WT + pRL-TK** sample is shown as the percentage of **negative mimic + pGL3-WT + pRL-TK**. Similarly, for the mutants, relative luciferase activity is calculated by setting the normalized luciferase activity of **negative mimic + pGL3**-Mutant **+ pRL-TK** sample to 100%, and the reading of **miR-138 mimic + pGL3**-Mutant **+ pRL-TK** sample is shown as the percentage of **negative mimic + pGL3**-Mutant **+ pRL-TK** (see Note 5).

4. Notes

1. As a negative control, we created a reporter construct containing the mutated hsa-miR-138 targeting site by replacing the seed region of the targeting site with $T_{(7)}$. Other strategies for creating mutant reporter constructs are also being used in various studies, including mutating only the bases complementary to the miRNA sequence, mutating the entire miRNA targeting site (e.g., using reversed strand), or deleting the seed region.
2. Since the XbaI site is the only available restriction enzyme cutting site on 3′-UTR of pGL3-Control vector, the insert could have two orientations in pGL3-insert plasmid: the forward

insert sequence and reversed insert sequence (theoretically a 50/50 chance for each orientation). It may be worth the effort to create additional restriction enzyme cutting site on 3′-UTR of pGL3-Control vector. Doing so will allow different enzyme sites to be appended to the ends of the synthesized oligos and ensure that the ligation will be in the correct orientation. This can be achieved by simply inserting a short oligo containing multiple restriction enzyme sites into the XbaI site.

3. Since we only use one restriction enzyme (XbaI) for the cloning, self-ligation is expected. A negative control reaction may be incorporated in the ligation reactions, for controlling the self-ligation. Add all the components in the reaction except insertion DNA. We expect to observe no or very few colonies from the control reaction. The number of colonies to pick from "experimental" plate depends on the number of colonies grown on the control plate.
4. The negative control of the miRNA mimic used in our experiment is a miRNA from *Caenorhabditis elegans* (cel-miR-67: UCACAACCUCCUAGAAAGAGUAGA). While this miRNA has been suggested by Thermo scientific-Dharmacon to have minimal sequence identity with known miRNAs in human, mouse, and rat, it is important to verify that there is no potential targeting site for this miRNA in the mRNA fragment of our interest. An alternative control (cel-miR-239b, UUGUACUACACAAAAGUACUG) is available from Thermo scientific-Dharmacon, if additional negative control miRNA mimic is needed.
5. While most studies have focused on miRNA targeting sites located in the 3′-UTR, a number of miRNA targeting sites have also been found in the coding region (and in the 5′-UTR to a lesser extent). Although our method presented here is for testing the miRNA targeting site located in the 3′-UTR of the targeted mRNA, it can be adapted to test miRNA targeting sites in the coding regions. This can be achieved by simply inserting the miRNA targeting sequence into the XbaI site at the 3′-UTR of the pGL3-Control vector. This approach has been used in a number of studies (17, 18).

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Chapter 11

Expression Pattern Analysis of MicroRNAs in *Caenorhabditis elegans*

Meltem Isik and Eugene Berezikov

Abstract

MicroRNAs (miRNAs) are ~22 nucleotide single-stranded RNA molecules that originate from hairpin precursors and regulate gene expression at the posttranscriptional level by basepairing with target messenger RNA and blocking its translation or inducing its degradation. miRNAs play important roles in a variety of biological processes, including development, proliferation, differentiation, cell fate determination, apoptosis, signal transduction, host–viral interactions, and tumorigenesis. Methodological advances in miRNA studies allowed identification of biological roles for many miRNAs, and establishing the spatiotemporal expression patterns of miRNAs is one of the approaches to elucidate their biological functions. Expression pattern analysis of miRNAs helps to identify potential genetic interactors that exhibit similar expression patterns and this, combined with further supporting experiments, helps to identify the genetic pathways in which the specific miRNAs are involved. In this chapter, we describe a detailed protocol for the analysis of miRNA expression patterns in *Caenorhabditis elegans*.

Key words: microRNA, *Caenorhabditis elegans*, Expression patterns, Functional analysis of miRNAs

1. Introduction

MicroRNAs (miRNAs) are ~22 nucleotide single-stranded RNA molecules that originate from hairpin precursors and regulate gene expression at the posttranscriptional level by basepairing with target messenger RNA (mRNA) and blocking its translation or inducing its degradation (reviewed in ref. (1)). In specific cases, miRNAs can also stabilize target mRNAs (2) or even activate their translation (3).

A simple anatomy, invariant cell lineage, transparent body, and complete genome sequence make the nematode *Caenorhabditis elegans* a highly suitable model to study the functions of miRNAs. Conservation of many biological processes between nematodes and

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higher organisms potentiates the applicability of miRNA function in *C. elegans* to other animals (4). There are currently 155 annotated *C. elegans* miRNA genes (miRBase v.13), of which 103 reside in intergenic regions, 31 are embedded within an intron of a protein-coding gene in a sense orientation, and 21 are antisense intronic miRNAs.

The in vivo functions of a few miRNAs have been established. The functions of some miRNAs were identified by loss- and gain-of-function (forward) genetic screens. For instance, in *C. elegans*, the *lin-4* miRNA and the *let-7* family of miRNAs act in heterochronic pathway to control the timing of aspects of larval development (reviewed in ref. (5)). The *lsy-6* miRNA acts in the asymmetric differentiation of the left and right ASE chemosensory neurons by targeting the *cog-1* mRNA (reviewed in ref. (5)).

Reverse genetic approaches, which include miRNA knockout and miRNA overexpression studies, can also be used to determine miRNA functions. Recently, it was demonstrated that neither single null nor compound mutant worms results in an easily detectable phenotype for most *C. elegans* miRNA genes (6). Another study revealed the mutant phenotypes for 25 miRNAs in genetic backgrounds with reduced processing and activity of all miRNAs or with reduced activity of a wide array of regulatory pathways (7). These findings suggest that there is significant redundancy within genetic networks involving individual miRNAs, miRNA families, and transcription factors. Additionally, action of miRNAs seems to require genetic perturbations such as stress conditions and sensitized genetic backgrounds.

Another approach for miRNA functional analysis involves studying miRNA expression profiles via in situ hybridization (8, 9), Northern blotting (10, 11), or small RNA library sequencing from enriched tissues (12, 13). These methods allow identification of miRNA expression patterns in various organisms, which can suggest functions for specific miRNAs. The drawbacks of these methods are the relatively low sensitivity and difficulty to analyze spatiotemporal expression patterns in live animals, as the methods depend on animal fixation (in situ hybridization) or RNA purification (Northern blotting, sequencing). Spatiotemporal expression patterns for most genes (more than 350 transcription factors and ~1,800 other protein-coding genes) were identified in live *C. elegans* by reporter genes, e.g., fusions of promoters with green fluorescent protein (GFP) (14, 15). Although this approach reflects known gene expression in most of the cases (15), *promoter*::*gfp* fusions can only approximate the expression patterns of the genes and actual expression can be different due to a number of factors, including incompleteness of *cis*-regulatory elements used in the reporters, genomic context, copy number, and posttranscriptional regulation (16).

A collection of 73 transgenic *C. elegans* strains, each containing an miRNA *promoter*::*gfp* fusion construct, were analyzed for the spatiotemporal expression patterns of miRNAs (4). The data and transgenic lines provided a platform for functional miRNA studies

to delineate their roles in the development of the animal and to understand their functions in gene regulatory networks. Furthermore, posttranscriptional regulation of pri-miRNAs was shown to provide an additional layer of differential miRNA expression in nematodes. However, this study excluded the intronic miRNAs that are in sense orientation, except for two of them (*mir-58* and *mir-82*). While miRNAs residing in introns of genes in antisense orientation are, by definition, transcribed independently from the host gene, it has been assumed that sense-oriented intronic miRNAs are produced from the common transcript with their host genes (i.e., rely on the host gene promoters for their transcription (1)) and thus the expression of such miRNAs can be deduced from the expression patterns of the host genes. Although substantial experimental data exist to support the "common transcript" model of biogenesis of intronic miRNAs, there is growing evidence that many sense-strand intronic miRNAs are transcribed independently from their host genes. It was shown in *Drosophila* (for *mir-7* (8) and *mir-281* (17)), humans (18–20), and *C. elegans* (4) that sense-oriented intronic miRNAs have promoter regions independent of their host genes. Recently, we have demonstrated by *promoter*::*gfp* fusions that all intronic miRNAs that have conserved upstream sequences can be transcribed from their own promoters and have specific and distinct expression patterns that differ from expression patterns of host gene promoters (21). In our study, we used 5′ upstream sequences that show high conservation patterns between different *Caenorhabditis* species, whereas fixed 2 kb upstream sequences were used as promoters in the previous study (4). This allowed us to establish the expression pattern for *mir-58*, which could not be identified in previous studies; furthermore, the obtained expression patterns were corroborated by the published small RNA sequencing data.

In this chapter, we describe a protocol to analyze expression patterns of miRNAs in *C. elegans*. We provide the methodology for selecting the promoter regions of miRNAs, cloning of these promoters in reporter plasmids, generation of transgenic lines by microinjections or bombardments, and finally, the analysis of expression patterns by fluorescence microscopy.

2. Materials

2.1. C. elegans Strains and Culture Conditions

1. N2 and HT1593 (*unc-119(ed3)*) worms should be maintained on NGM/OP50 plates at 15–20°C.
2. NGM plates (NaCl, Bacto-agar, bacto-peptone, cholesterol (5 mg/mL in ethanol), dH_2O, 1 M $CaCl_2$, 1 M $MgSO_4$, 1 M KPO_4 (pH 6)).

3. OP50 *Escherichia coli*.
4. LB (10 g Bacto-tryptone, 5 g Bacto-Yeast Extract, 5 g NaCl, bring volume to 1 L, autoclave).
5. Egg plates: Ten chicken eggs, LB and 60 NGM plates (described in Subheading 3).
6. Egg salts buffer: 6.9 g NaCl, 3.6 g KCl, 1 L H_2O. Autoclave.

2.2. Preparation of miRNA Promoter::gfp Constructs

1. A vector with *gfp*::*3′ UTR* (see Note 1).
2. PCR reagents (a DNA polymerase with proofreading activity (i.e., Pfu Turbo DNA polymerase), PCR buffer, dNTPs, N2 genomic DNA).
3. Primers with flanking RE sites to amplify miRNA promoters.
4. Two restriction enzymes suitable for cutting both the vector and PCR products.
5. T4 DNA ligase and ligation buffer.

2.3. Preparation of DNA for Microinjections and Biolistic Transformation

2.3.1. Microinjections

1. Plasmid DNA can be isolated by standard alkaline lysis protocols and plasmid purification kits. However, residual contaminants can prevent transformation and extra washes in the commercial column-based procedures and/or further purification of DNA by phenol chloroform extraction, G-50 spin column, chloroform extraction, and ethanol precipitation is recommended.
2. A 20 μL solution of miRNA *promoter*::*gfp* vector (10–20 μg/mL final concentration) with pRF4 marker plasmid (*rol-6* gene) (100 μg/mL) should be prepared with sterile ddH_2O (see Note 2).

2.3.2. Biolistic Transformation

1. Plasmid DNA can be isolated by a plasmid purification kit or a standard alkaline lysis protocol. 5 μg of DNA is necessary per bombardment. If the cotransformation/selection marker and the construct of interest are not in the same plasmid, 5 μg of each plasmid should be added.
2. Restriction endonuclease of choice for plasmid linearization outside the selection marker and target regions.

2.4. Microinjection Components

1. Equipment: injection table, inverted DIC microscope, micromanipulator, pressurized injection system with needle holder, needle puller, a standard dissecting microscope.
2. Microinjection needles: FLG10 from Dagan Corp., Minneapolis MN; or equivalent from World Precision Inst., Sarasota FL or Clark Inst., Reading, England.
3. Injection pads: boil 2% agarose in 30 mL water. Place a drop (~100 μL) of hot agarose onto a 50 × 22-mm glass coverslip by

using a cut-off P200 tip. Put a second coverslip on top of the droplet and tap it gently. Slide coverslips apart after the agarose is solidified, and bake the coverslip with agarose pad in an oven at 80°C for 1 h to overnight. Worms do not stick well to the agarose pad if it is humid and it may be necessary to bake the pads once more before use.

4. Injection oil: Heavy Paraffin Oil (BDH Chemicals, Poole, England; Gallard-Schlesinger, Carle Place, NY).
5. Worm pick: a worm pick with a hair attached at its tip.
6. Worms: N2 worms should be well-fed and non-starved for several generations. Hermaphrodites with ~10 eggs inside are best for injections.
7. Needle-loading pipettes.

2.5. Microparticle Bombardment Components

1. Bio-Rad Biolistic® PDS-1000/He Hepta system (includes Hepta adapter and five macrocarrier holders).
2. Rupture discs (1350 psi) Bio-Rad.
3. Macrocarriers Bio-Rad.
4. Stopping screens Bio-Rad.
5. Hepta adapter Bio-Rad.
6. Gold bead microcarriers (ChemPur 0.3–3 μm or Bio-Rad 1 μm beads).
7. 0.1 M spermidine (Sigma S-4139 free base, tissue culture grade; filter-sterilized; store frozen at –20°C).
8. 2.5 M $CaCl_2$ (filter-sterilized).
9. Dehydrated ethanol (70 and 100%). Do not use old ethanol that will have absorbed a lot of water from the air.
10. 50% glycerol (filter-sterilized).

2.6. Fluorescence Microscopy

1. Agarose pads: 0.6 g low-melting agarose, 30 mL H_2O, a 50 mL Erlenmeyer flask, pipette tips, slides, and coverslips, 1 M sodium azide.
2. Fluorescence microscope.

3. Methods

3.1. Determination of Promoter Regions of miRNAs

1. (Optional) Prepare customs tracks file for the predicted stem-loop sequence of miRNAs for which the promoter regions will be selected (see Note 3). Open UCSC genome browser for *C. elegans* and press “add custom tracks.” Upload the custom tracks file.

2. Select the regions upstream of predicted stem-loop sequence of miRNAs depending on the conservation between different *Caenorhabditis* species (see Note 4).
3. Retrieve the DNA sequences for the selected regions and design primers with flanking restriction enzyme cut sites.

3.2. Cloning of miRNA Promoters Upstream of gfp Gene

1. Determine two restriction enzymes that are suitable for cutting both the vector with *gfp::3′ UTR* and the amplified miRNA promoter sequence (see Note 5).
2. Digest the vector with the selected restriction enzymes at upstream region of *gfp::3′ UTR* sequence. Purify the required band by 1% agarose gel electrophoresis.
3. Amplify the promoter regions of miRNAs from the N2 genomic DNA using primers with flanking restriction enzyme sites and digest the amplified PCR products with selected restriction enzymes.
4. Ligate the vector and insert in 1:3 ratio 4 h or overnight at room temperature.
5. Transform bacteria with the ligation product.
6. Grow 3–4 colonies from the ampicillin plates and check by restriction enzyme digestion for the correct inserts. The inserts can be further verified by Sanger sequencing.

3.3. Preparation of Transgenic Lines

3.3.1. Microinjections

1. Mix miRNA *promoter::gfp* vectors (10–20 μg/mL final concentration) with pRF4 (100 μg/mL final concentration). Dilute this mixture with sterile water to a final volume of 20 μL.
2. Inject 15–50 gonads for each DNA mix as described in ref. (22) and put each injected worm to a separate, seeded NGM plate. Grow at 20°C.
3. Pick roller F1 progeny to new plates.
4. Keep the plates that produce F2 rollers and throw the rest of the plates. F2 roller progeny generate lines that continue to transmit their array at a consistent frequency.

3.3.2. Biolistic Transformation

Preparation of NGM Plates

1. Mix 3 g NaCl, 21 g bacto-agar, 7.5 g bacto-peptone (see Note 6) and make up the volume to 1 L with dH_2O. Autoclave.
2. Cool to 55°C, and add (using sterile technique and swirling) 1 mL cholesterol (5 mg/mL in ethanol), 1 mL 1 M $CaCl_2$, 1 mL 1 M $MgSO_4$, and 25 mL 1 M KH_2PO_4 (pH 6.0).
3. Pour 9 cm plates about half-full, and flame the agar surface to remove air bubbles (or worms will burrow). Let it dry at least 1 day prior to seeding with OP50 *E. coli*.

Preparation of Egg Plates

Day 1

1. Prepare LB (400 mL), ten eggs, sterile bottle (500 mL), and 60 NGM plates.
2. Grow 40 mL of OP50 culture overnight.

Day 2

3. Separate yolks of ten chicken eggs into 500 mL sterile bottle. Shake well until the yolks form a homogenous solution. Bring volume to 400 mL with LB medium. Shake well.
4. Incubate at 60°C for 1 h to deactivate the enzymes present in the yolk. Cool to room temperature.
5. Add 40 mL of overnight OP50. Shake well. Distribute 5–8 mL of mixture per NGM plate. Allow to settle overnight.

Day 3

6. Gently pour off remaining liquid from plates. Allow to dry one more day.
7. Wrap plates and store at 4°C (see Note 7).

Growing *unc-119(ed3)* Strain for Transformation

1. Prepare several clean master plates of *unc-119(ed3)* worms by bleaching the worms and seeding the eggs on 9 cm OP50 plates. Once the plates are almost starving, transfer chunks on egg plates (see Note 8).
2. Place egg plates (lid on top) inside a clean box. Parafilm can be used to avoid contamination (see Note 9).

Preparation of Gold Particles

1. Weigh 60 mg of gold particles (0.3–3 μm, ChemPur) into a 2 mL tube.
2. Add 2 mL 70% EtOH, vortex 5 min, allow the particles to settle for 15 min, spin 3–5 s, and discard supernatant.
3. Add 2 mL deionized water, vortex for 1 min, allow the particles to settle for 1 min, spin 3–5 s, and discard supernatant. Repeat this washing step for two more times.
4. Resuspend the gold particles in 1 mL of 50% sterile glycerol. The final concentration of gold is 60 mg/mL and is sufficient for ten bombardments. Prepared suspension can be stored for 1–2 month at 4°C or room temperature.

Coating of Gold Particles with DNA

1. Linearize 5 μg of plasmid DNA by digesting the plasmid with a restriction enzyme that does not cut inside the transgene of interest and the *unc-119* gene. Cleaning of DNA after restriction digestion is not necessary. Final reaction volume should be 50 μL.

2. Vortex gold beads (60 mg/mL) for 5 min to disperse clumps (see Note 10), take 100 μL into 1.5 mL tube, add 50 μL of linearized DNA, and vortex 1 min at a moderate speed (see Note 11).
3. Add 150 μL 2.5 M $CaCl_2$ and vortex at a moderate speed for 1 min.
4. Add 60 μL of 0.1 M spermidine (see Note 12), vortex 3–5 min, settle 1 min, spin for 3 s, and remove supernatant.
5. Add 300 μL 70% EtOH, vortex briefly, settle, spin for 3 s, and remove supernatant.
6. Add 500 μL absolute EtOH, vortex briefly, settle, spin for 3 s, and remove supernatant.
7. Add 140 μL absolute EtOH, vortex briefly, and proceed to loading DNA-coated gold particles onto macrocarriers (see Note 13). By this point plate with worms should be nearly ready for bombardment.

Preparation of Worms

1. Put one 9 cm NGM plate to 37°C for 1 h. The plate should be dry enough to quickly absorb all liquid from a pellet of worms. Place the dried plate on ice to cool down.
2. Wash worms with egg salts buffer from 5 to 6 egg plates into a 50 mL tube (see Note 14).
3. Allow worms to settle for 5–10 min. Remove supernatant containing younger animals and most of the bacteria.
4. Resuspend worms in 30 mL of egg salts buffer. Allow worms to settle and remove supernatant. Repeat the procedure several times. The goal is to obtain 2 mL sediment of worms clean from large pieces of debris and L1s.
5. Remove the supernatant and transfer 2 mL of worms to the prechilled dry NGM plate. Allow the worm liquid to distribute uniformly on the plate. Keep the plate on ice (see Note 15). Once the liquid is absorbed by agar, a uniform layer of worms would form. While plate with worms is drying, proceed to loading of macrocarriers with DNA-coated gold particles (see Note 16).

Bombardment

1. Read the manufacturer's instructions to set up the Bio-Rad Biolistic PDS-1000/He particle delivery system with the Hepta adapter (23).
2. Macrocarrier holder, Hepta adapter, and stopping screens should be sterilized in EtOH or autoclaved regularly. Clean the bombardment chamber with 70% ethanol before each use.
3. Rinse seven macrocarriers in 100% ethanol and allow them to dry on a tissue paper.

4. Place macrocarriers into Hepta adapter holder using the seating tool. A pipette tip can be used to ensure that the macrocarriers are fastened into the holder.
5. Spread 20 μL of DNA-coated gold beads onto each macrocarrier and let dry (see Note 17).
6. Assemble stopping screen and macrocarrier holder together.
7. Soak a 1350 psi rupture disc in isopropanol for 3–5 s, place in the retaining cap of the Hepta adapter, and tighten the adapter onto the helium gas acceleration tube.
8. Place the assembled macrocarrier holder into PDS-1000 chamber on the third shelf from the top.
9. Place the plate holder into PDS-1000 chamber on the fourth shelf from the top and place the open NGM plate with worms in the center of the plate holder.
10. Close the chamber and vacuum to 27 in. Hg, press "fire" button and hold until disc ruptures.
11. Release vacuum, remove the plate, and shut down the bombardment machine according to manufacturer's instructions.

Post-bombardment Care of Worms

1. Allow worms to recover for 30 min.
2. Wash the worms off the plates with 11 mL of egg salts buffer and distribute to 20 9 cm NGM plates seeded with OP50.
3. Incubate plates at appropriate temperature depending on the transgene used.
4. Allow worms to grow and starve for 10–14 days at 20°C (see Note 18).
5. Stable transformants with wild-type phenotype are easily identified on starved plates. Single several worms from each plate for further analysis (see Note 19).

3.4. Fluorescence Microscopy

3.4.1. Preparation of Agarose Pads for Microscopy

1. Add 0.6 g agarose to 30 mL H_2O in a 50 mL Erlenmeyer flask.
2. Melt carefully in a microwave without boiling over.
3. Add 1 mL of 1 M sodium azide solution to 30 mL of melted 2% agarose.
4. Place slides on a clean bench. Use a Pasteur pipette or a 200 μL pipette tip that was cut by a scissor to place two drops of agarose on each slide, quickly covering with a second slide before agarose solidifies.
5. After the agarose pads congeal and become cloudy, separate the slides.

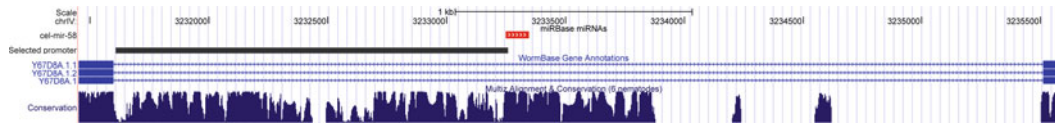

Fig. 1. An example of miRNA promoter region. Pre-miRNA sequence of *mir*-58 is shown in *red*, conserved region upstream of the miRNA and up to the exon border, shown in *black*, was selected as a promoter region for expression studies (21).

3.4.2. Preparation of Worms for Fluorescence Microscopy

1. Grow worms on NGM plates with OP50 at 20°C (see Note 20).
2. Wash worms off the plates with 2 mL M9 solution into a 2 mL tube and let them sink for ~3 min.
3. Wash the worms several times until the solution becomes clear. Take 2 μL of the worm pellet and put it on the agarose pad.
4. Use a coverslip to cover the agarose pad before the droplet dries out.

4. Notes

1. The vector should have *gfp* gene with a commonly used 3′ UTR (i.e., *unc-54* or *let-858* 3′ UTR). It may have *unc-119* selection marker gene if the selected method of transformation is bombardment. However, a vector with *unc-119* gene can be bombarded separately as well. For microinjections, *rol-6* (i.e., pRF4 plasmid) is the preferred marker gene.
2. miRNA *promoter*::*gfp* construct can be injected without the vector backbone by cutting it out by restriction enzymes or amplifying by PCR. This was shown to increase the transformation efficiency (24).
3. This step is optional; however, custom tracks may be useful for keeping track of your promoter selections and further analysis of your data. Check the webpage http://genome.ucsc.edu/goldenPath/help/customTrack.html for information on how to prepare custom tracks.
4. The 5′ upstream sequences (promoters) should be selected until the end of the conservation region. If conservation regions coincide with other genes 3′ UTR, the sequence should be selected until the annotated end of the 3′ UTR. For intronic miRNAs, promoters should be selected in the same way, by considering the conservation regions within the introns and by excluding exonic sequences. An example of miRNA promoter region is shown in Fig. 1.
5. If you plan to clone several miRNA promoters, best is to select two restriction enzymes that are suitable for cutting the vector containing *gfp*::*3′ UTR* and all of the amplified PCR products

(promoters). Make sure that these restriction enzymes do not cut within the promoter regions but only the flanking restriction enzyme cut sites.

6. Rich 2% NGM plates supports long-term bacterial growth and less burrowing of worms into the agar.
7. It takes several days for egg plates to dry sufficiently. Do not turn egg plates upside down until they are dry, since this can lead to leakage and contamination at the sides of the plates. We generally keep them lid up even after seeding the plates with worms.
8. Egg plates can be seeded by using bleached eggs or chunking from a clean master plate. Since *unc-119(ed3)* is a slow growing strain, it is handy to maintain master plates. Never seed egg plates by washing or chunking worms from previous egg plate!—it would inevitably lead to contamination.
9. It is essential that the beads do not clump. If beads clump, use additional vortexing, pipetting, or sonication until all large clumps are broken up.
10. Using parafilm to cover the seeded egg plates can sometimes (depending on the preparation of egg plates) lead to sickness or death of worms because of hypoxic conditions. The state of the worms is important for successful transformations, therefore it is better to keep seeded egg plates in clean boxes rather than using parafilm.
11. Do not vortex at high speeds to avoid shearing of DNA.
12. Since spermidine is a labile molecule, it is important to store it in small aliquots at −20°C, and thaw the solution right before performing the bombardment. Protamine, which is a powder and more stable at room temperature, can also be used instead of spermidine to deliver foreign DNA (25).
13. Since the beads tend to form clumps at this point, transfer the microparticles onto the macrocarriers as soon as possible.
14. The best time for harvesting worms is when the food in the egg plate is almost finished. Young adults transform most efficiently.
15. If the plate is not kept on ice, worms would start to crawl and form clumps.
16. The timing of the procedure is important for the end result. The order that should be followed during the procedure is.
    (a) Prepare gold particles and linearize DNA.
    (b) Put an NGM plate to 37°C for dehydration.
    (c) Harvest worms with egg salts buffer.
    (d) Start coating gold particles with DNA and continue washing the worms in the meantime.
    (e) After addition of spermidine, start sterilizing the macrocarriers.

(f) Put NGM plate on ice.

(g) Put worm solution on prechilled NGM agar.

(h) Place macrocarriers into the macrocarrier holder and load-coated gold particles.

(i) After the gold particles are dried and the worm solution forms a viscous layer on top of the NGM plate, bombard.

17. Use a pipette tip to ensure that the macrocarrier is fastened into the holder. The DNA suspension should be added after macrocarrier is fastened to the macrocarrier holder. Trying to fasten the macrocarrier after DNA suspension is dry may lead to the loss of gold particles.
18. Usually, transformants can be identified already in the F1 progeny within 5 days after bombardment but most of them would be transient. Therefore, it is necessary to wait until *unc-119(ed3)* worms die and wild-type moving worms outgrow the plate. This also makes the selection easier.
19. In some cases, it is possible to identify two or more independent transformants per plate. Therefore, it is necessary to single several worms per plate.
20. The conditions of growth (i.e., temperature, starvation) may affect the promoter activities. Therefore, it may be useful to try different conditions of growth to find out whether the expression patterns of experimented miRNAs are sensitive to these changes.

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Chapter 12

Use of Viral Systems to Study miRNA-Mediated Regulation of Gene Expression in Human Cells

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Abstract

MicroRNAs (miRNAs) are a class of small ~22 nt regulatory RNAs that modulate mRNA expression in all multicellular eukaryotic organisms. Interestingly, viruses also encode miRNAs and these viral miRNAs target cellular and viral mRNAs to regulate virus replication and latent infection. In particular, herpesviruses encode a large number of miRNAs. Herpesvirus infection also changes the normal expression profile of cellular miRNAs. New genetic tools have recently been generated to study the function of viral and cellular miRNAs in virus-infected cells. The creation of these reagents and use in Epstein-Barr virus-infected lymphoblastoid cell lines are discussed as a model viral system for the investigation of miRNA function.

Key words: miRNA, Epstein-Barr virus, Sponge, mRNA target, Virus

1. Introduction

MicroRNAs (miRNAs) are small ~22 nt RNAs that regulate mRNA expression of hundreds of genes in organisms ranging from *Caenorhabditis elegans* to humans. Interestingly, viruses also encode miRNAs as well as alter the expression and function of cellular miRNAs (1). In order to study the role of viral or cellular miRNAs in infected cells, reagents must be designed to interrogate their function. In this chapter we describe methods to (1) generate and use miRNA sponges to antagonize miRNA function, (2) generate luciferase indicator assays to study miRNA function, and (3) study miRNA target interactions using 3′ UTR indicators, western blotting, and quantitative RT-PCR assays. These assays are useful in the study of both viral and cellular miRNAs. The model system that we use to perform these studies is the latently Epstein-Barr virus-infected lymphoblastoid cell lines. However, these reagents could be applied to any viral infection system in principle.

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The development of miRNA sponges by the Sharp laboratory has provided a tool to stably impair the function of a specific miRNA or set of miRNAs in a cell line or animal (2, 3). This approach has advantages over miRNA antagomirs (4) in that sponges can be regulated by a RNA polymerase II-driven promoter and expressed from a lentiviral or retroviral backbone. Therefore, expression of miRNA sponges is stable due to integration and sorting or selection of transduced cells. Furthermore, regulated expression of sponges is possible with tetracycline-regulated promoters (5). Finally, multiple miRNA seed families can be targeted simultaneously because of the modular nature of the miRNA binding site cassette.

In addition to sponges, several core tools have emerged for the functional analysis of miRNA-mediated repression of mRNA expression. Indicator assays can directly assess viral or cellular miRNA function by tethering perfectly complementary miRNA binding sites to the 3′ UTR of the luciferase gene (6). To directly address miRNA targeting of specific mRNAs, 3′ UTR regions containing endogenous miRNA binding sites and context can be appended to luciferase in a similar manner and indicator assays performed. Finally, the effects of miRNAs on endogenous target mRNA levels and protein output can be assessed by quantitative RT-PCR and Western blotting in cells either expressing a given miRNA heterologously or expressing a sponge construct ablating the function of a given miRNA. The protocols described herein provide practical information regarding the construction of the aforementioned reagents and their use in interrogating miRNA function in virus-infected cells.

2. Materials

2.1. Molecular Biology

1. Annealing buffer: 30 mM Hepes, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate.
2. Restriction enzymes: XbaI, EcoRI, NheI, HpaI, and ClaI (New England Biolabs (NEB), Ipswich, MA).
3. Klenow fragment (NEB).
4. Quick Ligation kit (NEB).
5. Luria-Bertani Agar plates with 100 μg/mL Ampicillin.
6. QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA).
7. MSCV Retroviral Expression System (Clontech, Mountain View, CA).
8. Gel Extraction Kit (QIAEXII, Qiagen).
9. QIAquick Nucleotide Removal Kit (Qiagen).

10. MAX Efficiency Stbl2 competent cells (Invitrogen).
11. Amicon 100k cutoff spin columns (Amicon Ultra-15 Centrifugal Filter Units-Millipore, Danvers, MA).

2.2. Cell Culture

1. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (Gemini Bio-Products).
2. Roswell Park Memorial Institute (RPMI) 1640 Medium (Invitrogen) supplemented with 10% fetal calf serum (Gemini Bio-Products).
3. 10,000 U/mL Penicillin plus Streptomycin (Invitrogen).
4. 200 mM L-Glutamine (Invitrogen).
5. Phosphate-buffered saline (PBS) (1× PBS, Invitrogen).
6. Polyethylenimine (PEI), make 1 mg/mL stock in distilled H_2O (PEI, linear, MW 25,000 from Polysciences, Inc available from Fisher Scientific).
7. BD Diva or other 488 exciting laser cell sorter.

2.3. Quantitative RT-PCR

1. mirVana miRNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX).
2. BioPhotometer spectrophotometer (Eppendorf North America, Hauppauge, NY).
3. TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).
4. RT-specific primers and TaqMan probes for mature miRNAs (miR34a: Cat# 4427975) and small nucleolar RNA (RNU48: Cat# 4427975) as internal control (Applied Biosystems).
5. Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL) (Applied Biosystems) Cat# 4346906.
6. MicroAmp Optical Adhesive Film (Cat# 4346906, Applied Biosystems).
7. StepOnePlus Real Time PCR System (Applied Biosystems).

2.4. Luciferase Assay

1. Dual-Luciferase Reporter Assay System (Promega Corporation, WI).
2. Constructs used: pNL-SIN-CMV-FLuc with or without the miRNA binding site and pNL-SIN-CMV-RLuc.
3. Packaging system: pMDLg/pRRE (gag/pol elements), pRSV-REV, and pMD.G (env elements) (7).
4. Polybrene (Millipore).

2.5. Western Blotting

1. Triton lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate, 20 μM NaF,

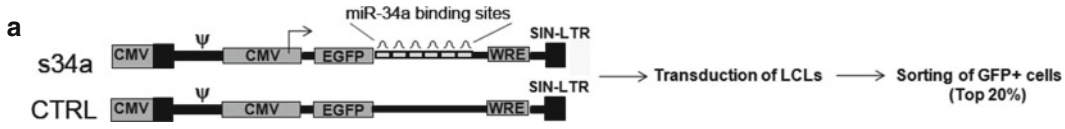
10 μM pyrophosphate, and 1× Complete EDTA-free protease inhibitors (Roche)).

2. Non-fat dry milk (NFDM).
3. NuPAGE® Bis-Tris Gels (Invitrogen).
4. NuPAGE Gel system (Invitrogen).
5. ECL detection system (Amersham).

3. Methods

3.1. Generation of miRNA Sponges

Lentiviral sponges in the pLCE backbone generate GFP-marked stable transductants that block miRNA function and decrease their stability (2, 3). This highly versatile approach has now been widely accepted in the field (3, 5, 8,12). A schematic of the approach used in EBV-transformed cells is shown in Fig. 1a.



b

Example flow of miRNA sponge oligo construction:

hsa-miR-34a mature sequence:
UGGCAGUGUCUUAGCUGGUUGUU

After U to T conversion (RNA to DNA):
TGGCAGTGTCTTAGCTGGTTGTT

Reverse complement of miRNA sequence:
AACAACCAGCTAAGACACTGCCA

Introduction of bulge in miRNA sequence (bold) = miRNA site in sponge
AACAACCAG**GAT**AGACACTGCCA

Fig. 1. Generation of miRNA-targeting sponges to study the role of miRNAs in virus-infected cells. (**A**) A schematic diagram of the lentivirus-based miR34a sponge (s34a) and control (CTRL). The GFP gene is driven by a CMV promoter, while the 3′ UTR of this construct contains multiple imperfect binding sites for miR-34a (or your miRNA of choice, including multiple miRNA seed families). Following production of this lentivirus with appropriate packaging and pseudotyping constructs in 293 cells, the sponge and control can be introduced separately into your cells of choice (EBV-infected lymphoblastoid cell lines, or LCLs in our case). Following transduction, sorting for the highest 20% GFP-positive cells gives the most robust sponge effect, which is manifested in decreased miRNA activity on endogenous or experimental targets as well as destabilization of the mature miRNA, as evidenced by reduced expression by qRT-PCR or primer extension. (**B**) An example of the work flow going from mature miRNA sequence (miR-34a in this case) through conversion to a DNA template, reverse complementing the sequence, and then introducing a requisite bulge to design the oligos used in cloning the sponge.

3.1.1. pLCE-Derived miRNA Sponges

1. Design the oligonucleotides encoding for the sponge as follows:

   The forward primer corresponds to three copies of the reverse complement of the miRNA sequence with the insertion of a bulge at positions 10–12 (Fig. 1b). For cloning the sponge in pLCE vector, a modified XbaI site (CTAG**C**) is added at the 5′ end of the oligonucleotide and another XbaI followed by an EcoRI (TCTAGATTTG) are added to the primer at the 3′ end. The first XbaI site is modified in order to destroy it after ligation to the vector and to allow the addition of multiple copies of the sponge. The reverse primer is the reverse complement of the forward primer with the addition of an AATT site to 5′ end and with only a G for the XbaI site.

   Example primers:

   Spng miR34 fw primer (targets underlined): 5′CTAGC<u>AACAA C C A G</u> G A T <u>A G A C A C T G C C A</u> G T T T T G <u>A A C A A C C A G</u> G A T <u>A G A C A C T G C C A</u> G T T T T G <u>A A C A A C C A G</u> G A T <u>A G A C A C T G C C A</u> T C T A G A T T G 3′

   Spng miR34 rv primer: 5′AATTCAAATCTAGATGGCAGT GTCTATCCTGGTTGTTCAAAACTGGCAGTGT C T A T C C T G G T T G T T C A A A A C T G G C A G T GTCTATCCTGGTTGTTG 3′

2. Anneal the forward and the reverse primers by mixing equal amount of the oligonucleotides (2 μL of 10 μM stocks) in 46 μL annealing buffer. Heat at 95°C for 5 min. Cool to 37°C for 60 min.
3. Digest 3 μg of pLCE with XbaI/EcoRI and purify the linear plasmid by QIAEX II gel extraction kit.
4. Ligate 150 ng of XbaI/EcoRI digested pLCE with the annealed oligonucleotides (1 μL of mixture) using the Quick Ligation kit as recommended (NEB).
5. Transform 5 μL of the ligation reaction in competent DH5α (100 μL). Incubate on ice for 30′, heat shock for 45 s at 42°C, and leave for 2′ on ice. Add 400 μL of LB (Luria Broth) and incubate at 37°C for 30 min shaking. Plate on LB-Agar plates with 100 μg/mL ampicillin. Incubate at 37°C overnight. Inoculate singular colonies in 3 mL of LB-100 μg/mL Ampicillin/each overnight at 37°C shaking. Purify the DNA by miniprep (we use QIAprep Spin Miniprep Kit). Screen for positive clones by ClaI/XbaI restriction enzyme digestion and sequencing the plasmid using the following oligo (5′ TTGTACCTAGTGGAACCGG 3′).
6. Repeat the same process for inserting further 3× miRNA binding site (sponge) sequences. Digest the single 3×-miRNA binding site containing sponge with XbaI/EcoRi and clone the annealed oligos again to produce a 6×-site containing

sponge. Finally, repeat this procedure cloning the oligos into the 6× sponge to create the 9×-site containing sponge (see Note 1).

3.1.2. MSCV-miRNA Sponges

The retroviral MSCV-based sponge has the advantage of including a puromycin resistance gene that can be used for selecting cells transduced with the sponge construct. The construct can be easily generated by removing the DNA fragment containing *gfp* and the miRNA binding sites from the pLCE-based sponge and inserting into MSCV-puro (Clontech, catalog no. 634401).

1. Digest 7.5 μg pLCE-miRNA sponge with NheI and blunt it using Klenow fragment and standard procedures. Purify the linear DNA using a QIAquick PCR purification kit and digest with EcoRI. Run a 2% agarose gel and purify the ~1 kb sponge fragment using the QIAEX II Purification kit.
2. Digest pMSCV-Puro with HpaI and purify using QIAquick PCR purification kit. Digest DNA with EcoRI and purify vector following running into 2% agarose gel using the QIAEX II Purification kit.
3. Ligate insert/vector with a molar ratio 3 to 1 and following the same protocol reported for pLCE-miRNA sponge cloning. Transform ligation into Stbl2 competent bacteria and plate on ampicillin LB plates.
4. Screen minipreps of colonies by digestion with BglII/EcoRI looking for the ~1 kb insert.

3.2. Generation of miRNA Sponge Expressing Virus-Infected Cells

For the preparation of the virus in 150 mm × 25 mm dish:

1. Plate HEK 293T in DMEM media supplemented with 10% FBS, 100 U/mL Penicillin, and 2 mM L-Glutamine (D10) in order to have a confluence of approximately 70% the next day. Incubate at 37°C for at least 18 h.
2. Replace the media with 20 mL of serum-free DMEM.
3. Transfect HEK 293T with miRNA sponge constructs using PEI.

   For pLCE constructs: mix 19.4 μg of lentiviral vector (pLCE or pLCE-sponge) with the packaging system consisting of 4.8 μg each of pMDLg/pRRE (gag/pol elements), pRSV-REV, and pMD.G (envelope/pseudotyping elements) (7).

   For MSCV-puro constructs: mix 19.4 μg of retroviral vector (pMSCV-Puro-egfp or pMSCV-Puro-egfp-sponge), 4.8 μg of pMSCV-Gag/Pol, and 1 μg of pHIT-G.
4. Mix the DNA with 675 μL of 150 mM NaCl. Vortex and add 100 μL of 1 mg/mL PEI (linear, MW-25,000 from Polysciences, Inc.). Vortex the DNA-PEI mix and incubate for 10 min at room temperature.

5. Vortex again and add it to the cells drop by drop. Leave at 37°C for 6 h and then replace the media with 2% FBS supplemented RPMI 1640 media containing 100 U/mL Penicillin and 2 mM L-Glutamine (R2). Incubate at 37°C for 48 h.
6. Harvest the supernatant, centrifuge it at $330 \times g$ for 5 min to remove any detached cells and filter through 0.45 μm filter. Concentrate the supernatant 100× using 100 kDa cutoff spin columns (Amicon Ultra-15 centrifugal filter units, Millipore).
7. Titer the viral particles in BJAB cells for GFP levels (see Note 2). Plate 1×10^5 cells in 24-well plates in a volume of 0.5 mL of RPMI 1640 media supplemented with 10% FBS, 100 U/mL Penicillin, and 2 mM L-Glutamine (R10). Transduce with increasing amounts of concentrated virus (0.2, 0.5, 1 and 5 μL) and incubate for 24 h at 37°C. The following day, measure the % of GFP-positive cells by FACS or fluorescent microscopic analysis to calculate the titer of transducing units (TU)/mL. The number of transducing units is the number of particles necessary to turn 1 cell GFP-positive. For example, if 10^5 cells become 60% GFP-positive using 100 μL of virus supernatant, then 6×10^4 TU are present in the 100 μL stock giving a titer of 6×10^5 per mL.
8. For LCL infection, transduce at least 1×10^6 LCLs (5×10^5 cells/mL) at a multiplicity of infection (MOI) of 2 in the presence of polybrene (5 μg/mL) in R10. Incubate at 37°C for 48 h. Replace the media with R10 and incubate the cells another 1–4 days at 37°C.
9. For the cells transduced with lentivirus (pLCE and pLCE-*sponged* cells), sort the GFP-positive cells by FACS (BD Diva or other 488 exciting laser cell sorter) using the same mean fluorescence intensities (MFI). Since cells that express high levels of GFP are those with higher loss of miRNA function, we usually sort the top 10–20% GFP-positive cells (see Note 3).
10. For retroviral transduction of LCLs, use the same conditions reported at step 7. However, differently from LCLs transduced with lentivirus, after 72 h post-infection put the cells in selection with puromycin (1.25 μg/mL for LCLs) and expand for 2 weeks before sorting.

3.3. Viral or Cellular miRNA Indicator Assay

To demonstrate that the sponge expressing cells have a reduction in miRNA activities, we use indicator assays by inserting perfect miRNA binding sites at the 3′ UTR of the firefly luciferase (FLuc) gene as initially described by Gottwein et al. (6). pNL-SIN-CMV-FLuc and pNL-SIN-CMV-RLuc (Renilla luciferase control) are the constructs used in our laboratory for these assays.

3.3.1. Construction of miRNA Indicator Constructs

1. Design the oligonucleotides encoding the indicator following the same strategy used for cloning the sponge. The forward primer corresponds to two copies of sequences with perfect complementarity to a given miRNA with the addition at the 5′ end of a modified ClaI site (CGgg) and a second ClaI site upstream of XbaI such that aATCGATGATCT is appended to the 3′ end. The reverse primer is the reverse complement of the forward primer with the addition of CTAG to 5′ end and with CC at the 3′ end (6). Anneal the oligonucleotides as already described.

   Example primers (target sequences underlined):

   pNL-miR34-sense: 5′ CGGGAACAACCAGCTAAGACACTGCCA

   GTTTTGAACAACCAGCTAAGACACTGCCAAATCGATGATCT 3′

   pNL-miR34-antisense: 5′ CTAGAGATCATCGATTTGGCAGTGTCTTAGCTGGTTGTT

   CAAAACTGGCAGTGTCTTAGCTGGTTGTTCC 3′

2. Digest 3 μg of pNL-SIN-CMV-FLuc with ClaI/XbaI and purify the plasmid using QIAquick PCR purification kit.
3. Ligate the digested vector (150 ng) with the annealed oligonucleotides (1 μL) with Quick ligation kit as recommended (NEB).
4. Follow the same protocol reported starting for pLCE-miRNA sponge cloning for inserting the oligo containing the two additional binding sites.

3.3.2. Luciferase Indicator Assay

1. Plate HEK 293T in D10 in order to have a confluence of approximately 70% the next day. Incubate at 37°C for at least 18 h.
2. Replace the media with 20 mL of serum-free DMEM.
3. Transfect HEK 293T with the miRNA indicator construct and controls with PEI. Mix 19.4 μg of lentiviral vector (pNL-SIN-CMV-FLuc with or without the miRNA binding site) and pNL-SIN-CMV-RLuc with the packaging system consisting of 4.8 μg each of pMDLg/pRRE (gag/pol elements), pRSV-REV, and pMD.G (env elements) (7). Follow the same protocol already described above without performing the titration, as these constructs are not GFP+. Assume all the constructs having the same TU/mL and prepare two mixes using the same amount of concentrated viruses: FLuc-miRNA Indicator/RLuc and FLuc/RLuc.
4. Plate the cells to be transduced the day before. For LCLs plate 5×10^5 cells in 24-well plate in 1 mL of R10. Add the virus mix (25 μL) and Polybrene 5 μg/mL to the cells. As controls use a

cell line that does not express the miRNA of interest and a cell line expressing it. Incubate 24 h at 37°C.

5. Harvest the cells by centrifugation (330×*g*, 5 min at room temperature). Wash with PBS 1× and lyse the cells in 50 μL of 1× passive buffer (Promega, E154).
6. Perform a dual-luciferase assay (Dual-luciferase Reporter assay Promega, E151A) to measure the FLuc and RLuc values. The ratio of Firefly to Renilla activity indicates the miRNA activity (lower ratios indicate higher miRNA repression/activity) and is normalized to cells not expressing the selected miRNAs.

3.4. Quantitative RT-PCR Analysis to Detect Decrease in miRNA Levels in Sponged Cells

Our empirical data suggests that miRNA sponges decrease the stability of the targeted mature miRNA. Recently, a report suggests the existence of a physiological pathway whereby miRNA destabilization occurs in Drosophila (9). Despite not having a clear mechanism by which miRNA sponges destabilize miRNAs in mammalian cells, our data suggest that this is a good and easy proxy for miRNA sponge function.

1. Purify total RNA from transduced and sorted cells using mirVana miRNA Isolation kit (Ambion) as instructed (see Note 4).
2. Determine RNA concentration by spectrophotometer (Eppendorf BioPhotometer).
3. Reverse transcribe the relevant miRNA by using the Applied Biosystems individual stem-loop primers designed to only detect mature miRNA (TaqMan MicroRNA Assays-ABI) and the small nucleolar RNA (RNU48) that will be the internal control using the TaqMan MicroRNA Reverse Transcription kit (ABI). Prepare the following mix on ice: 0.15 μL dNTP mix (100 mM total), 1.5 μL 10× RT buffer, 0.19 μL RNase inhibitor (20 U/μL), 1 μL Multiscribe RT enzyme (50 U/μL), and 10 ng of RNA (2 ng/μL). Mix gently and incubate 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Spin down briefly and keep the samples on ice.
4. Assemble the QPCR reaction in 96-well plate. Each sample should be run in triplicate. Prepare a mix with an excess of volume. For each reaction mix 10 μL of TaqMan Gene expression master mix (2×), 1 μL of TaqMan Gene Expression Assay (20×), and 2 μL of cDNA and 7 μL of H_2O.
5. Centrifuge the plate briefly at 200×*g* and run the following program on an Applied Biosystems StepOne Plus (any QPCR machine will suffice): 2 min at 50°C, 10 min at 95°C for the enzyme activation and 40 cycles consisting of 15 s of denaturation at 95°C and 1 min at 60°C for the annealing and extension.

6. The cycle number, or C_T, for the samples is normalized relative to the C_T of RNU48 in each cell line. Normalized values from sponged cells are then compared to control cells that are set equal to 100%.

3.5. Analysis of miRNA-Mediated Target Regulation

3.5.1. 3′ UTR Luciferase Assays

This section describes cloning of the full-length 3′ UTRs of putative targets downstream of Firefly luciferase in the pL/SV40/GL3 vector. For 3′ UTR longer than 1 kb, it is reasonable to clone 500 bp upstream and 500 bp downstream of the predicted miRNA binding site. Primers can be designed using Primer Express (Applied Biosystems) or other algorithms for optimizing annealing temperature vs. product length.

1. Reverse transcribe 500 ng of RNA from cells expressing the miRNA target with the High capacity cDNA reverse transcription kit (Applied Biosystems). Prepare the following mix on ice: 1 μL oligo(dT)20 primer (50 μM), 2 μL 10× RT buffer, 0.8 μL 25× dNTP mix, 2 μL 10× RT Random primers, 1 μL of Multiscribe Reverse transcriptase, 1 μL RNase, 0.8 μL dNTP mix (100 mM total), 5.2 μL of H_2O, and 10 μL of RNA (50 ng/μL).
2. Mix gently and incubate 10 min at 25°C, 120 min at 37°C, and 5 s at 85°C. Spin down briefly and keep the samples on ice.
3. Perform the PCR amplification of the 3′ UTR fragment. Mix 100 ng of cDNA with 10 μL of 10× Pfu buffer, 8 μL of dNTP (2.5 mM), 2.5 μL each of specific forward and reverse primers (inserting restriction sites at 5′ and 3′ end), 2 μL of Pfu Turbo, and 71 μL H_2O.
4. Mix gently and run the following program:
   (a) 1 cycle: 2 min at 95°C.
   (b) 35 cycles: 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C
   (c) 1 cycle: 10 min at 72°C.
   (*) Note that the annealing temperature and the extension time should be adjusted with respect to the primers and the length of the fragments.
5. Purify the ~1 kb fragment by 2% agarose gel (QIAquick) and digest it with the same enzyme used for cutting the vector in Subheading 3.3.1.
6. Ligate insert/vector with a molar ratio 3 to 1 and follow the protocol as described in Subheading 3.1.1 for pLCE-miRNA sponge cloning.
7. Plate HEK 293T cells the day before the transfection in order to obtain 70% of confluence the following day, typically ~10^5 cells/well in a 24-well plate.

8. Co-transfect the Fluc construct carrying the target 3′ UTR or the empty vector (5 ng), an RLuc-based internal control vector (pL/SV40/RLuc) (5 ng), and an miRNA expression vector (or sponge) (see Note 5) (0.5 μg) in each well of a 24-well plate (10) using PEI scaled down to an appropriate amount for a 24-well. Incubate 48 h at 37°C.
9. Harvest the cells by centrifugation (330 × *g*, 5 min at room temperature). Wash the cells with PBS and perform the lysis in 50 μL of 1× passive buffer (Promega).
10. Perform a dual-luciferase assay (Dual-luciferase Reporter assay Promega) to measure FLuc and RLuc values.
11. Compare FLuc/RLuc ratio of cells with or without expression of the given miRNA (see Note 6).

3.5.2. Western Blot Analysis

Prepare the protein extract of cells overexpressing the relevant miRNA *sponged* cells and the respective controls as follows:

1. Plate the cells in order to have at least 2×10^6 cells the following day.
2. Wash the cells with ice-cold PBS.
3. Incubate with 30–50 μL of Triton lysis buffer for 30 min on ice.
4. Centrifuge at 21,000 × *g* for 30 min at 4°C and save the supernatant in a new tube.
5. Calculate protein concentration by Bradford method assay (BIO-RAD).
6. Resolve 10–50 μg of protein extract on NuPAGE® Bis-Tris Gels (Invitrogen). Gel percentage depends on the size of the protein of interest.
7. Transfer the protein on PVDF membrane and block for 30 min in 5% NFDM, TBS, 0.05% Tween20.
8. Incubate with specific antibody directed against the miRNA target.
9. Wash three times with TBS, 0.05% Tween20 (TBST).
10. Incubate with the corresponding horseradish peroxidase-conjugated secondary antibodies (dilution 1/3,000) (Pierce) for 1 h at RT.
11. Wash three times with TBST.
12. Develop with ECL detection system (Amersham).
13. Use anti-GAPDH (Santa-Cruz) as internal control.

3.5.3. Quantitative RT-PCR of miRNA Target mRNAs

1. Plate the cells (we use EBV-transformed LCLs) in order to have at least 2×10^6 cells the following day.
2. Wash the cells in PBS 1× and extract RNA using RNeasy Mini Kit (Qiagen). Use the protocol including DNase digestion recommended by the manufacturer.

3. Determine RNA concentration by spectrophotometer (Eppendorf BioPhotometer).
4. Reverse transcribe 500 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Prepare the following mix on ice: 2 μL 10× RT buffer, 0.8 μL 25× dNTP mix (100 mM), 2 μL 10× RT Random primers, 1 μL Multiscribe Reverse transcriptase, 1 μL RNase inhibitor, 3.2 μL of H_2O, and 10 μL of RNA (50 ng/μL) (see Note 7).
5. Mix gently and incubate 10 min at 25°C, 120 min at 37°C, and 5 s at 85°C. Spin down briefly and keep the samples on ice.
6. Dilute the cDNA to 9 ng/μL and use 5 μL/reaction (45 ng total).
7. Assemble the QPCR reaction in 96-well plate in a total volume of 25 μL. Each sample should be run in triplicates without forgetting the H_2O control. Prepare a mix with an excess of volume. For each reaction mix, use 12.5 μL of Power SYBR Green PCR master mix 2× (Applied Biosystems), 1 μL each of target-specific forward and reverse primers (10 μM) 5 μL of cDNA and 5.5 μL of H_2O. As a control use specific GAPDH primers.
8. Centrifuge briefly the plate and run the following program: 10 min at 95°C for the enzyme activation and 40 cycles consisting in 15 s at 95°C and 1 min at 60°C. Add a melting curve at the end of the amplification.
9. Normalize target gene C_T values vs. internal control (in our case GAPDH). Compare normalized values of cells with depleted miRNA or overexpressing it with values obtained by the control cells that are set equal to 100%.

4. Notes

1. We empirically determined that 6× sponge sequences were sufficient to knock-down miRNA levels and activity by ~80%. Furthermore, it is possible to perturb more than one miRNA by adding 3× sponge cassettes targeting different miRNAs to an existing sponge. This has been done empirically with up to 27 miRNA binding sites.
2. Determining the titer of GFP-expressing sponge could be done on any cell type. We generally use BJAB cells for ease of access. However, 293 or HeLa could be used as well and generally have a 5–10-fold increased susceptibility to infection by VSV G-pseudotyped lentivirus.
3. We empirically determined for the miR-34a sponge that the top 20% GFP-positive cells had a 4–5-fold reduction of miR-34a levels and function. In contrast, the top 50% GFP-positive cells only

had a ~2-fold reduction. However, for other miRNAs it may be the case that the bulk GFP-positive population is sufficient for more robust miRNA ablation and phenotypic analysis (5).

4. We routinely sorted ~10^6 cells for phenotypic assays and analysis using qRT-PCR of miRNAs. This will vary depending upon the expression level of the given mature miRNA.
5. The cloning and expression of viral and cellular miRNAs in the 3′ UTR of GFP and other cDNAs has been described previously in a comprehensive methods chapter (11).
6. 3′ UTR activity is determined by comparing the Fluc + target 3′ UTR/Rluc-control values to Fluc-control/Rluc-control values in the presence or absence of the given miRNA. If the miRNA is targeting the 3′ UTR, then the Fluc-3′ UTR/Rluc-control value will be smaller than the Fluc-control/Rluc-control value when the miRNA is expressed. Conversely in sponge expressing cells, this value will increase relative to control cells indicating derepression of the target due to abrogation of miRNA activity.
7. Set up a control reaction lacking the reverse transcriptase enzyme.

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Chapter 13

Cloning, Expression, and Functional Analysis of Genomic miRNA Using Retroviral System in Cancer Cells

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Abstract

MicroRNAs have emerged as important post-transcriptional regulators of gene expression. Identification of cancer-regulated microRNAs or other classes of endogenous small RNAs have advanced our knowledge in cancer progression and metastasis. Among many tools, small RNA cloning is a powerful method to identify new microRNAs (miRNAs) and to profile miRNA expression and function. Retroviral system is also the minimum requirement for the studying of miRNAs in a highly stable population of cancer cells or other primary cell types with high expression. This chapter describes a step-by-step protocol that is optimized to clone directly one of the miRNA miR-145, as an example, from genomic DNA into retroviral vector to yield ultimate overexpression for functional study in prostate cancer cells. The small RNAs cloned by this protocol will have an easy and simple way of cloning from genomic DNA to maintain the necessary motifs of native enhancer for enhancement of mature miRNA expression. Furthermore, the procedure eliminates miRNA extraction and cDNA synthesis before cloning and sequential cloning of more than one miRNA makes this protocol cost- and time-effective to eliminate many frequent cell culturing.

Key words: MicroRNA, miRNA, Noncoding RNA, Retroviral vector, Cloning, Post-transcriptional gene regulation, Prostate cancer

1. Introduction

MicroRNAs are small RNA molecules of 20–23-nucleotide long that regulate mRNA expression at the post-transcriptional level in cells (1). Mature microRNAs (miRNAs) are produced from primary precursor transcripts (pri-miRNAs) that are encoded by miRNA genes in the genome. This production happens through two endonucleolytic cleavage steps: the first step is carried out by Drosha, an RNase III type protein, which liberates a 70-nucleotide stem-loop structure (termed pre-miRNA) from the pri-miRNA

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transcript. The pre-miRNA is then transported to the cytoplasm by Exportin-5 and further processed by Dicer to produce the mature miRNA (2, 3). As pre- and pri-miRNAs do not share any common sequence motif, it is thought that the pre-miRNA hairpin structures themselves represent the primary signal that initiates Drosha processing. Thus, how to conserve such common sequence motif and be able to distinguish between the three types of RNA molecules for subsequent in vitro and in vivo analysis of miRNA is important to create an optimal protocol.

The first miRNA was discovered by genetic analysis in 1993 in *Caenorhabditis elegans* (4). Since 2001, most of the other discovered miRNAs have been made using the protocol of small RNA cloning and also using the recently developed commercial ones (5). Most traditional miRNA cloning are based on designing 5′ and 3′ linker at two ends of small RNAs using T4 RNA ligase, and after gel purification and reverse transcription, the resulted cDNA is amplified for cloning by PCR with primers having 5′ restriction enzyme sites. The purpose of this method is to reduce the possibility of microRNA loss in the digestion set and partially offsets the ligation bias of T4 RNA ligase. The disadvantage of this strategy is the limitation of the cell numbers and the available RNA quantity. Furthermore, T4 RNA ligase efficiency is often a problem for small RNA cloning due to the significant amount of side products that can be accumulated (6) and the miRNAs containing the restriction site is cut and thus cannot be cloned.

The protocol we describe here is modified from many protocols that so far have been established. But our procedure is more cost-and time-efficient by using extraction of genomic DNA and direct cloning of stem-loop sequence enriched with essential elements for enhancement of mature miRNA expression. The procedure eliminates RNA extraction and cDNA synthesis before cloning. Furthermore, our modified protocol saves time for upstream expression and functional analysis with established retroviral vector to handle expression for more than one miRNAs which they are sequentially cloned in multiple cloning sites. The advantages of this modification greatly decrease the chance of self-ligation between linkers which happens commonly in miRNA cloning approach. The resulted miRNA clones can be sequenced using traditional method or subjected to direct high-throughput pyrosequencing or used in stable transfection in cancer cells for expression and functional analysis.

2. Materials

2.1. DNA Preparations

1. Tissue DNA Extraction Kit.
2. 1× PBS buffer, diluted from 10× PBS.
3. Absolute 100% ethanol.
4. Sterile water.
5. Sterile microfuge tubes.

2.2. Cloning of miRNA Precursor

1. DNA polymerase.
2. 10× polymerase buffer.
3. dNTP mix.
4. Glycerol (sterile).
5. Restriction enzymes: *Bgl*II, *Xho*I, and *Hpa*I.
6. CIP.
7. Enzyme T4.
8. Sterile water.
9. Sterile PCR tubes.
10. DNA loading buffer (6×))30% glycerol, 0.02% (w/v) xylene cyanol, 0.02% (w/v) bromophenol blue].
11. 1 kb DNA Ladder.

2.3. Primers for Cloning of miR-145

1. 5′ PCR primer with *Bgl*II restriction enzyme site; 5′-GAA GATCTTCCCAGAGGGTTTCCGGTACTT-3′.
2. 3′ PCR primer with *Xho*I restriction enzyme site; 5′-CGCCG CTCGAGCGGAACGCAAAGGTTTGGAACAT-3′.

2.4. Plasmid

1. MSCV-PIG viral vector (see Fig. 1a for map).
2. pGag-Pol.
3. pVSV-G.

2.5. Transformation and DNA Isolation

1. Gel Extraction kit.
2. DH5α competent cells.
3. LB ampicillin plates (50 μg/mL).
4. Mini-Prep Kit.
5. LB growth medium including ampicillin (100 μg/mL).
6. ExoSAP-IT (VMR).
7. Gene Pure LE quick dissolve agarose (GeneMate) 1–2% gels.
8. TAE buffer (1×): 40 mM Tris-acetate (pH 8.4), 1 mM EDTA (pH 8.0).
9. 100 bp DNA ladder.

2.6. Creating Stable Cell Line and Transfection

1. HEK 293T (Human Embryonic Kidney cell line).
2. 2 M $CaCl_2$.

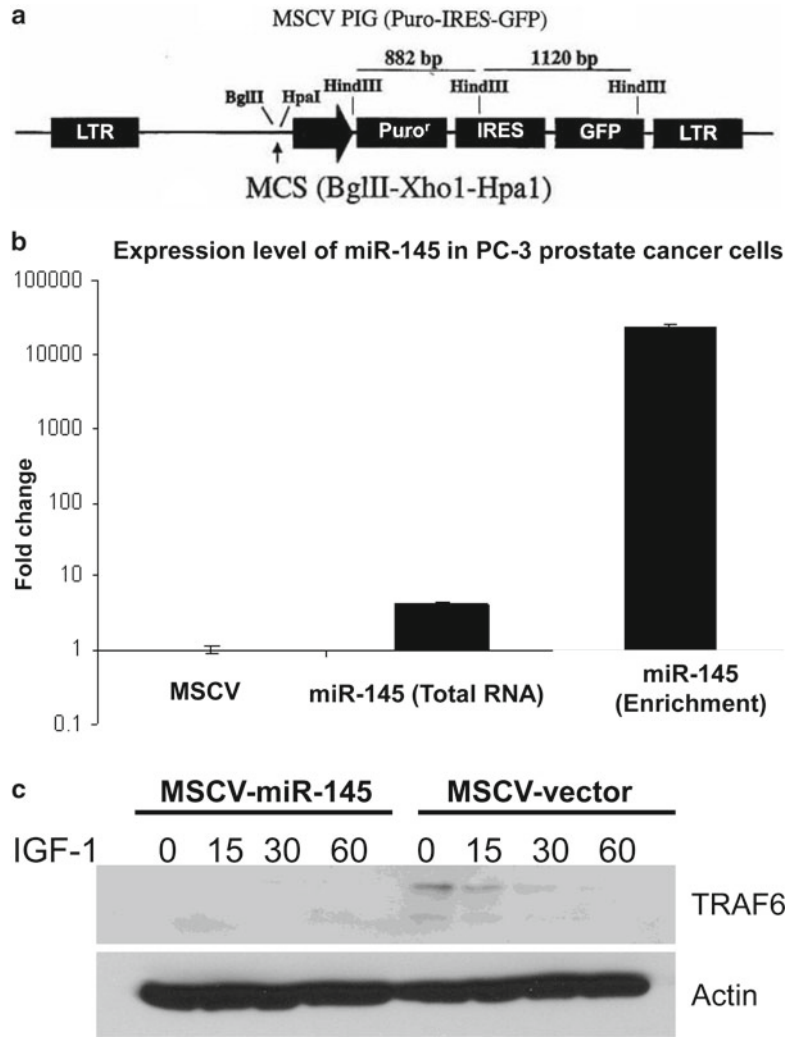

Fig. 1. (**a**) Map of MSCV-PIG vector used for cloning of miRNA. (**b**) Validation of miR-145 expression in prostate cancer cells (PC3). PC3 cells were infected with MSCV retroviral vector (alone) and miR-145. Total RNA and enriched miRNA from infected and stable cancer cell line were used to synthesize cDNA of miR-145 for quantitative RT-PCR analysis. (**c**) Functional analysis of miR-145 using Western blot analysis. DU145 prostate cancer cells infected with MSCV-vector or MSCV-miR-145 were serum-starved for 1 day, treated with 50 ng/mL IGF-1 for various time points. TRAF6 protein was targeted by miR-145 and was downregulated. Actin was used as a loading control.

3. 2× HBSS: 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM Dextrose, 50 mM HEPES; pH 7.05.
4. Sterile water.
5. 15 and 50 mL polypropylene tubes.

2.7. Infection

1. 0.45 μM filters.
2. 8 μg/mL polybrene.

2.8. Cell Culture

1. Dulbecco's Modified Eagle Medium (DMEM).
2. Fetal Bovine Serum.
3. Penicillin (10,000 U/mL).
4. Streptomycin (10,000 U/mL).
5. 4 μg/mL puromycin (selection marker).

2.9. Isolation and Preparation of RNA

1. mirVana™ RNA Isolation kit (Ambion).
2. TRIzol Reagent (Invitrogen).
3. Chloroform.
4. Isopropanol.
5. Ethanol (80%).
6. Elution solution (0.3 M NaCl).
7. Glycogen (20 μg/μL; Invitrogen).
8. 1 M Tris–HCl, pH 7.8 (RNase-free).
9. 1 M $MgCl_2$ (RNase-free).
10. 10 mM ATP (RNase-free).
11. 50 mg/mL ultrapure BSA (RNase-free).
12. Deionized formamide solution (50%, in Milli-Q water).
13. DEPC-treated water or RNase-free Milli-Q pure water, sterile.
14. TBE buffer (0.5×): 50 mM Tris, 50 mM boric acid, 1 mM EDTA.
15. Sterile RNase-free microfuge tubes.
16. Electrophoresis chamber to cast and run of gel.
17. Rotating shaker for microcentrifuge tubes.
18. Safe Imager blue-light transilluminator.

Quantitation of miRNA

2.10. Reverse Transcription-SYBR-Based miRNA Assay

1. TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems).
2. miR-145 RT primer (50 nt, DNA oligonucleotide): 5′-GTT GGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGA GCCAACAGGGAT-3′.
3. 5S RT primer: 5′-GTTGGCTCTGGTGCAGGGTCCGAGG TATTCGCACCAGAGCCAACAAAGCC-3′.
4. First-strand buffer (5×).
5. 0.1 M DTT (RNase-free).
6. dNTP solution (2.5 mM).
7. KOH (150 mM)/20 mM Tris base solution.
8. HCl solution (150 mM).

2.11. Reverser Transcription-TaqMan® MicroRNA Assay

1. TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems).
2. 5× Stem-loop RT Primer.
3. Nuclease-free water.

2.12. Real-Time PCR-SYBR-Based miRNA Assay

1. SYBR® Green PCR Master Mix (Applied Biosystems).
2. miR-145 forward Primer (23 nt, DNA oligonucleotide): 5′-CGAGGCGTCCAGTTTTCCCAGGA-3′.
3. miR-145 reverse primer (23 nt, DNA oligonucleotide): 5′-TGCAGGGTCCGAGGT-3′.
4. 5S forward primer; 5′-CGCCTGGGAATACCGGGTG-3′.
5. 5S reverse primer (23 nt, DNA oligonucleotide): 5′-TGCAGGGTCCGAGGT-3′.
6. Thermal cycler Applied Biosystems; StepOnePlus.
7. Nuclease-free water.
8. 96-Well reaction plates.

2.13. Real-Time PCR-Taqman MicroRNA Assay

1. TaqMan® Universal PCR Master Mix II (2×).
2. TaqMan® Small RNA Assay (20×).
3. Nuclease-free water.
4. 96-Well reaction plates.

3. Methods

Cloning and Expression of miRNA

3.1. Designing of Primers

We usually design our specific primers for each stem-loop sequence of target miRNA using primer3 software (http://frodo.wi.mit.edu/primer3).

1. Extract stem-loop sequence using mirbase data base (http://mirbase.org).
2. Blast the sequence in NCBI (http://www.ncbi.nlm.nih.gov/) and find upstream and downstream sequence (approximately ±200 bp). We usually validate the results using UCSC Genome Browser (http://genome.cse.ucsc.edu) (see Note 1).
3. Upload extracted genomic sequence in primer3 software to get ideal annealing temperature with minimum secondary stucture and primer dimers.
4. Design restriction enzyme sites (*Bgl*II, *Xho*I, *Hpa*I) in the upstream of primers. Follow the principal rules in the designing of primers for cloning.

5. To use specific primers in the SYBR-based miRNA assay system, design forward primer for the sequence exactly on mature miRNA located in the stem-loop structure, and then design reverse primer on the universal RT primer, which can be commercially synthesized (Applied Biosystems). Add or remove nucleotide(s) from 5′ site of forward primer to have optimal match with reverse primer to eliminate any secondary structure and also nonspecific signal after PCR amplification.

3.2. Extraction of Genomic DNA

Purify genomic DNA with Phenol/chloroform reagent following the principal direction in public protocol or using commercially available tissue DNA extraction kit, following their protocols (7). We typically extract DNA from 293T cells cultured in one 60-mm plate. During DNA extraction, low-retention micro-centrifuge tubes should be used in all steps and the DNA pellet should be washed with cold 70% ethanol and dried well (see Note 2).

3.3. Touch Down PCR

1. Set up the following PCR mix in two tubes: one containing genomic DNA and other without it (negative control).
   (a) 10× DNA Polymerase buffer 2.5 μL
   (b) 50 mM $MgCl_2$ 0.75 μL (1.5 mM in final concentration)
   (c) 10 mM dNTPs 0.5 μL (each dNTP 0.2 mM in final concentration)
   (d) Glycerol 0.25 μL
   (e) Water 17 μL
   (f) Forward primer 1 μL (10 μM)
   (g) Reverse primer 1 μL (10 μM)
   (h) Genomic DNA 1 μL (100 ng/μL)
   (i) DNA Polymerase 1 μL
   (j) Final volume 25 μL
2. Place mixture in the thermal cycler with a heated lid.
3. Run the reaction in the following Touchdown PCR program:
   (a) 95°C, 6 min
   (b) 94°C, 30 s
   (c) 65°C, 30 s [— 1.0 +0.00---R=3.0^x/s +0.0^x/s---G=0.0]
   (d) 72°C, 45 s
   (e) GOTO 2 REP 10
   (f) 94°C, 30 s
   (g) 55°C, 30 s
   (h) 72°C, 45 s
   (i) GOTO 6 REP 15
   (j) 72°C, 15 min

HOLD 10°C ENTER

END

4. Resolve the PCR products in a 2% agarose gel.
5. Determine the optimal cycle number to obtain optimal product amplification.
6. Repeat PCR with selected number of cycles using 50 μL reaction volumes.

3.4. Gel Isolation and Purification of DNA Fragment

Use the 1% agarose gel (Agarose DNA fragments 50–1,000 bp) and purify the bands with any commercially available gel extraction kit. (see Note 3).

3.5. Restriction Enzyme Digestion of PCR Product and Vector

1. Before adding enzymes, set aside 5 μL of all samples for gel analysis.
2. Add 10 U of enzyme (*Bgl*II, *Xho*I) accordingly and incubate for 2–16 h at 37°C.
3. To prevent the product from self-ligation if using single restriction enzyme digestion, incubate the vector plasmid with CIP at 37°C for 60 min. Purify the digestion products by gel extraction kit once again.
4. Run digested PCR product and vector in 1% TAE gel.
5. Cut out desired region of gel under blue-light transilluminator immediately after exposure.
6. Follow gel purification described at Subheading 3.4.

3.6. Cloning

MSCV-PIG retroviral plasmid vector used in our protocol has Open Reading Frame (ORF) including *Bgl*II, *Xho*I, *and Hpa*I. These restriction enzyme sites have the capability to deliver two different insert DNA (miRNA), in which they can be cloned individually or sequentially. We recommend using a 1:3 molar ratio of vector:insert DNA when cloning a fragment into a retroviral plasmid vector. The following example illustrates the conversion of molar ratio to mass ratio for a 3.0 kb and a 0.5 kb insert DNA fragment:

```
[(ng of vector × kb size of insert)/kb size of
vector] × molar ratio of (insert/vector)=ng of
insert
```

1. For each reaction contains: Vector DNA 100 ng; Insert DNA 17 ng; Ligase 10× Buffer 1 μL; T4 DNA Ligase (weiss units) 0.1–1 U, and Nuclease-free water to final volume of 10 μL.
2. Mix the reaction gently and incubate the mixture at room temperature for around 4 h or overnight at 16°C.
3. Thaw DH5α competent cells on ice, warm LB medium, and warm LB-Amp plates.

4. Transfer 10 μL of ligation reaction into a vial of competent cells and mix gently. Do not mix by pipetting up and down.
5. Keep 30 min on ice, heat shock for 45 s at 42°C, and immediately recover for 2–3 min on ice.
6. Plate 50 μL, 150 μL of transformation mix per each LB plate inoculated with 50 μg/mL ampicillin at 37°C overnight.
7. Incubate overnight at 37°C.
8. Isolate plasmid DNA from 5 to 10 clones from each library for plasmid screening and sequencing.

3.7. Plasmid Screening, Extraction, and Sequencing

1. Pick up colonies into 20 μL standard PCR mix, using forward and reverse designed primers (see Note 4). Care should be taken to only touch the middle of the colony to avoid contamination. PCR condition: 94°C 8 min; 94°C 20 s, 60°C 30 s, 72°C 90 s, 30 cycles; 72°C 5 min; 4°C forever.
2. Run 5 μL on 2% agarose gel using 100 bp ladder as a size marker.
3. Extract plasmid DNA from 5 to 10 clones by using commercially available Mini-Prep Kit.
4. Before sending the amplicon for sequencing, the universal primer in the PCR reaction has to be determined.
5. Take 5 μL of PCR product and add 2 μL ExoSAP-IT. Incubate at 37°C for 15 min.
6. Heat at 80°C for 15 min to inactivate ExoSAP-IT. The DNA is ready to be sequenced.

3.8. Retroviral Production and Infection

Day 1

1. Seed 293T cells in DMEM supplemented with 10% Fetal Bovine Serum and ampicillin and streptomycin in 100 mm^3-dish. Maintain at 37°C in 5% CO_2 environment (see Note 5).

Day 2

2. 16–24 h later, aspirate off the media and refresh cells with 10 mL medium. Cells should be around 50–60% confluent.
3. Transfect 293T cells with packing plasmids and target plasmid at the ratio listed in the table below:

	Ratio	μg (in 100 mm^3-dish)
Envelop plasmid (gag/pol)	3	7.5
Packing plasmid (VSV-G)	1	2.5
miRNA construct	4	10

Amount of reagents are used in different size of plate:

	293T/DMEM (mL)	DNA/H_2O	2× HBSS (μL)	2 M $CaCl_2$ (μL)
12-Well plate	0.3×10^6/0.8	1.6 μg/25 μL	40	5
6-Well plate	0.75×10^6/2	4 μg/87.5 μL	100	12.5
100 mm dish	4×10^6/10	20 μg/437.5 μL	500	62.5

Calcium Phosphate Transfection:

(a) Add DNA and H_2O to a polypropylene tube
(b) Add 2 M $CaCl_2$ slowly to mix the solution
(c) Add 2× HBSS drop by drop, vortex to mix
(d) Incubate solutions at room temperature for 30 min
(e) Slowly and evenly add to the cells
(f) Refresh cells with regular 10% FBS DMEM medium 6–8 h later
(g) Incubate for 48 h

Day 3

4. Seed your host cells for infection.

Day 4

5. 48 h after transfection, harvest the 10 mL virus-containing medium derived from day 2. Centrifuge supernatant at 1,200 rpm for 10 min and filter through the 0.45 μM filter (infection medium).
6. Add 10 mL regular medium into 293T cells, for the second infection.
7. Add 2 mL fresh medium into the 10 mL infection medium (12 mL in total) plus polybrene (8 μg/mL) with 1:1,000 medium: polybrene ratio.
8. Remove the medium from the host cells and replace it with the medium derived from Subheading 3.8, step 7 and centrifuge at 1,800 rpm for 45 min to optimize infection efficiency.

Day 5 or 6

9. Another 24–48 h, harvest virus-containing medium and infect the host cells one more time (repeat steps 5–8, Subheading 3.8).

Day 6 or 7

10. 24 h later, select your host cells with the selection marker (puromycin, 4 μg/mL).

Day 10–13

11. 4–7 days later, harvest the selected cells to check its overexpression efficiency (see Notes 6–10).

Quantitation of MicroRNA

After obtaining stable cell lines with miRNA overexpression, in order to further carry out functional studies of miRNAs, it is important to quantitate their expression levels in the stable cell lines. Because of their short sequences, detection and quantification of miRNA are more difficult. In the following sections, we will discuss in details the steps of miRNA quantitation, and we will provide two different methods for quantitation of microRNA: SYBR-based miRNA assay and TaqMan® MicroRNA Assay. Both methods are very commonly used because they offer high sensitivity since only small amounts of RNA are required, and as well as high specificity.

*****Every step procedure from here should be done in an RNase-free environment, and keep all lab equipment and materials RNase-free.

3.9. Total RNA Extraction

1. Aspirate medium from cells grown in dishes and wash with 1× PBS.
2. Purify total RNA with TRIzol reagent following steps 3–12, Subheading 3.9 (see Note 11).
3. In the fume hood, add 4 mL of TRIzol to the cells on 100-mm plate, and 2.5 mL on 60 mm plate; 1 mL on 35 mm plate, and 3 mL on 3D culture (35 mm plate).
4. Mix solution for 2–3 min until it is no longer viscous.
5. Transfer solution into a 15-mL polypropylene tube, and centrifuge the samples for 10 min at 9,000 rpm at 4°C and transfer supernatant into fresh tubes.
6. Add 20% of Chloroform for TRIzol® (i.e., 0.2 mL Chloroform for each 1 mL of Trizol®).
7. Close tubes firmly and shake and vortex vigorously for at least 15 s. Incubate at room temperature for 10 min.
8. Centrifuge for 15 min at 9,000 rpm at 4°C and transfer aqueous (upper) phase to fresh tube, avoid transfer of any interface.
9. Add 0.5 volume of Isopropanole (IPA) to the clean supernatant (i.e., 0.5 mL IPA for every mL of TRIzol added) and add 0.5 μL Glycogen for every mL of TRIzol added.
10. Mix immediately by inverting tubes 5–8 time and incubate at room temperature for 5–10 min.
11. Centrifuge at 9,000 rpm for 10 min, 4°C and discard supernatant.

12. Wash with 1 mL of 75% EtOH (RNase-free) to each tube. Resuspend dry pellet in nuclease-free water and keep on ice.
13. Determine RNA concentration and purity by UV absorbance using a Nanodrop apparatus.
14. Test the integrity of the RNA sample by resolving 1 μg of total RNA in a 1.5% agarose gel in 1× TAE buffer.
15. The starting amount of total RNA for the library should be in the range of 200–300 μg. Keep the concentration in the range of 3–5 μg/μL. Keep the RNA sample on ice at all times or store at −20°C until used. Proceed to next step.

3.10. Small RNA Enrichment from Total RNA Samples

The enrichment procedure for small RNAs described in mirVana™ miRNA isolation Kit can be used to enrich total RNA samples for the small RNA fraction (<200 nt). The total RNA sample is simply mixed with Lysis/Binding buffer and miRNA homogenate additive and is sequentially bound to two filters without phenol extraction. The end product is two fractions enriched either in large (first filter) or small (second filter) RNA species that can be used in independent experiments. Follow the mirVana™ miRNA isolation protocol provided by the kit.

Concentration of RNA

Small RNA < 200 nt = $33 \times A_{260}$ (μg/mL)

Total RNA: $40 \times$ dilution factor $\times A_{260}$ (μg/mL)

3.11. Reverse Transcription

For stem-loop reverse transcription using the TaqMan® MicroRNA Assay, the reverse transcription condition is the same; replace RT primer with the 5× RT primer supplied by the assay kit. Because of the high-specificity and sensitivity of stem-loop RT, 100 ng/μL of RNA is sufficient. Skip step 2, Subheading 3.11 if using TaqMan® MicroRNA Assay.

1. Mix 3 μL RT primer (1 μM) with 5 μL miRNA samples (500 ng/μL).
2. Heat at 80°C for 5 min, chill on ice for at least 1 min.
3. Prepare total amount of 7 μL RT master mix including: 0.15 μL dNTP mix (100 mM total), 1.0 μL Multiscribe™ RT enzyme (50 U/μL), 1.5 μL 10× RT Buffer, 19 μL RNase Inhibitor (20 U/μL), and 4.16 μL Nuclease-free water.
4. Combine RT master mix with mixture of primer and miRNA. Mix gently and centrifuge briefly.
5. Run reverse transcription using the following program; 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. cDNA synthesis reaction can be stored at −20°C or used for PCR immediately.

3.12. Quantitation of MicroRNA by Real-Time PCR-SYBR-Based miRNA Assay

1. Prepare the reaction as indicated below: SYBR® Green PCR Master Mix 7.5 μL; Universal reverse primer (20 μM), 0.5 μL; miRNA-specific forward primers (20 μM), 0.5 μL; DEPC-treated ddH_2O, 3.5 μL; 3 μL cDNA.
2. Perform quantitative PCR according to the following steps: 2 min at 50°C, 2 min at 95°C, followed by 40 cycles of 15 s at 95°C, 60 s at 60°C.
3. Amplify miRNA for 35 cycles at an annealing temperature of 60°C using miRNA-specific forward primers and reverse primer.
4. Use internal control of U6 (or 5S) as small nuclear RNA, because U6 is stably expressed in human tissues.
5. Separate the PCR products on 3% agarose gel.
6. Excise and purify the PCR bands and clone into pEASY-T1 vector (TransGen Biotech). Sequence the clones with PCR products for validation (Optional).
7. Carry out real-time PCR reaction using the Applied Biosystems thermal cycler for detection of miRNA expression.
8. Monitor melting curve for each PCR to avoid nonspecific amplification (Fig. 1b) (see Note 13).
9. Analyze the functions of miRNA by performing Western blot analysis (Fig. 1c) (see Note 14).

3.13. Quantitation of MicroRNA by Real-Time PCR-TaqMan® MicroRNA Assay

1. For each miRNA gene analyzed, three replicates of each PCR reaction are needed.
2. For each miRNA gene analyzed, an endogenous control (also in triplicates) is needed.
3. For each triplicate, prepare the following: 3.6 μL TaqMan® Small RNA Assay (20×); 4.8 μL RT reaction product; 36 μL TaqMan® Universal PCR Master Mix II (2×); 27.61 μL Nuclease-free water. Total volume: 72.01 μL, mix and centrifuge.
4. Transfer 20 μL of the PCR reaction mix to each of the three wells on a 96-well plate.
5. Seal the plate with the adhesive cover, centrifuge the plate briefly.
6. Load the reaction plate onto the real-time PCR instrument, use the following parameters: Run Mode: Standard; Sample Volume: 20 μL; Thermal Cycling Conditions: 95°C for 10 min; 95°C for 15 s, 60°C for 60 s; repeat steps for 40 cycles.
7. Analyze real-time PCR result (see Note 12).
8. Analyze the functions of miRNA by using Western blot analysis (see Note 15).

4. Notes

1. Computational miRNA prediction represents a valuable alternative which can be performed with comparably little technical effort. Target genes for each miRNA are predicted using miRBase Targets version 5 program (http://microrna.sanger.ac.uk/targets/v5/). A prediction of target genes for the known miRNAs is undertaken by software for miRNA targets version 5 and TargetScan 5.1.
2. To optimize cloning efficiency, extracted DNA is usually diluted to100 ng/μL. Generally 100 ng of genomic DNA template is recommended. Less DNA template should be used for amplification of vector (5–30 ng) as PCR target.
3. Care should be taken to avoid denaturing of DNA throughout the protocol except a few steps specified in the protocol. To avoid denaturing, the DNA pellet should never be dried out, and buffer rather than water.
4. In addition to sequencing from ligation product, directly amplify plasmid from colonies by using PCR and Mini-Prep Kit is another way for plasmid sequencing.
5. 293T is used as the packaging cell line to which both the transfer plasmid and helper plasmids are co-transfected for virus vector production. 293T cells are also used as target cells for in vitro efficacy testing of the vectors.
6. Stock of puromycin is 10,000× in medium stored at 4°C (40 mg/mL) and user stock is 100×. Final concentration is 4 μg/mL.
7. The final pH of 2× HBSS should be 7.05 ± 0.05. Filter through a 0.2 μm filter, aliquot, and store at −20°C. The quality of the HBSS is very important to the transfection efficiency. Try to avoid multiple freeze/thaw cycles. To thaw, warm to room temperature and invert or vortex the tube to achieve uniform mixing.
8. All materials that have been in contact with the retrovirus must be bleached thoroughly before discarding in the biohazard waste.
9. Efficiency of the vector systems was functionally demonstrated by development of a transgenic-enhanced green fluorescence protein (GFP) expressing cell line. Determine GFP expression profile of transfected cells 72 h or at indicated time points, by flow cytometry (FACSCaliber, BD BioSciences, USA).
10. For pseudotyping, Vesicular Stomatitis Virus envelope glycoprotein (VSVG) expression plasmid pMD.G (from Dr. Dos Sarbassov, MDACC) was used. The expression cassette con-

sists of CMV promoter, betaglobin intron, VSVG coding frame followed by poly-A sequence.

11. During RNA isolations, low-retention microcentrifuge tubes should be used during all steps and the RNA pellet should be washed with 80% ethanol (not 70% ethanol). Wear gloves when handling TRIzol, work RNase-free.
12. When using TaqMan® MicroRNA Assays for direct-RT method, the assay will provide 5× Stem-Loop RT Primers needed for specific miRNA. It is very important to specify exactly which miRNA is needed to analyze and purchase from Applied Biosystems website.
13. To calculate the fold change, an endogenous control and a reference sample are needed. The endogenous control can be 5S, U6, U47, RNU24, RNU6B for human samples, and snoRNA-202, snoRNA-234, and snoRNA-420 for mouse samples. The reference sample in this case will be the cell line that only expresses vector control, or mock. The ΔΔCt method will be used. First obtain the expression data for miRNAs and endogenous controls from the experimental samples (miRNA overexpressed cell lines) as well as the reference samples (mock overexpressed cell lines). Calculate the ΔCt for the experimental and reference samples using: $\Delta Ct = Ct_{\text{experimental/reference}} - Ct_{\text{endogenous control}}$. Then calculate ΔΔCt by using: $\Delta\Delta Ct = \Delta Ct_{\text{experimental}} - \Delta Ct_{\text{reference sample}}$. Then calculate the fold change using: $2^{-\Delta\Delta^{Ct}}$.
14. To check whether the miRNA of choice really represses the expression of its target genes, harvest the stable cell lines that overexpress the miRNA and subject the cells to Western blot analysis of the protein level of target gene.
15. In our protocol, the example data that we presented were obtained by using SYBR-based miRNA assay. We chose to also discuss TaqMan® MicroRNA Assay since it is another commonly used method to quantitate microRNA expression and offer the readers an alternative option for gauging microRNA expression.

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Chapter 14

Preparing Synaptoneurosomes from Adult Mouse Forebrain

Giovanni Lugli and Neil R. Smalheiser

Abstract

Many neuroscience studies involve subcellular fractionation to produce isolated or enriched synaptic fractions. Synaptosomes are prepared by flotation of synaptic membranes on sucrose or Percoll gradients. Alternatively, synaptoneurosomes are prepared by filtration of tissue homogenate through a series of filters to obtain a fraction that is enriched in pinched-off dendritic spines. Whereas the protocol for making synaptosomes is reasonably well standardized and well described in the literature, there is (to our knowledge) no detailed lab protocol for making synaptoneurosomes. Here, we give the methods used in our laboratory to produce synaptoneurosomes that are suitable for studying RNAs and proteins.

Key words: Synaptosomes, Synaptoneurosomes, microRNA, Subcellular fractionation

1. Introduction

A major technique in neuroscience is subcellular fractionation to produce isolated or enriched synaptic fractions. The most popular type of synaptic fraction is the *synaptosome* (*Syn*), which involves flotation of synaptic membranes on sucrose or Percoll gradients (1, 2). These are thought to consist largely of presynaptic axon terminals with adherent postsynaptic densities. Some associated glial end-feet and dendritic spine cytoplasm are also present. Alternatively, the *synaptoneurosome* (*SynN*) involves filtration of tissue homogenate through a series of filters to obtain a fraction that is enriched in pinched-off dendritic spines (3, 4). It should be noted that terminology is not uniform in this field, and in fact, the majority of published studies that discuss "synaptoneurosomes" are actually utilizing synaptosomes. Both types of synaptic fractions have their uses, and we have used both in our studies characterizing synaptic microRNAs, their precursor RNAs and the proteins involved in their biogenesis and function (5–7). Whereas the

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protocol for making synaptosomes is reasonably well standardized and well described in the literature, there is (to our knowledge) no detailed lab protocol for making synaptoneurosomes. Here, we give the methods used in our laboratory to produce synaptoneurosomes that are suitable for studying RNAs and proteins.

2. Materials

Before you start preparing any solution and working with RNA, clean up all lab bench work space and tools as described below and make sure to use RNase free solutions.

To destroy RNAses that may be associated with tools and water used for RNA studies, you can treat them with DEPC solution if they can be autoclaved, or with RNAsecure (Ambion) if not autoclavable. The treatment with DEPC of water or tools requires subsequent autoclaving to make sure the DEPC is fully inactivated. NEVER add fresh DEPC to solutions that you use during the preparation of SynN if not previously autoclaved. DEPC* will be indicated if it is to be freshly added to a solution.

1. Three adult mouse forebrains (cortex plus hippocampus).
2. 2× Homogenization buffer (HB).

   2× HB 100 mM HEPES pH = 7.5, 250 mM NaCl, 200 mM Sucrose, 4 mM K Acetate.

   Prepared with DEPC treated water (Ambion cat# AM9922) and filtered through 0.2 μm filter.
3. Protein inhibitors stock solutions: 0.5 M EDTA (in water), 0.2 M PMSF (in Methanol), 1 M NEM (in Methanol), 10 mg/mL Leupeptin, 1 mg/mL pepstatin A (in Methanol), 2 mg/mL Aprotinin. Prepare small aliquots of stock solutions with DEPC water (Ambion) or Methanol and store all of them at −20°C to avoid freeze-thaw (except the EDTA solution is stored at room temperature). Final concentrations: 10 mM EDTA, 2 mM PMSF, 10 mM NEM, 10 μg/mL Leupeptin, 1 μg/mL pepstatin A, 2 μg/mL Aprotinin.
4. RNase inhibitors: SuperaseIN (Ambion cat# AM2696) 20 U/μL, RNaseOUT (Invitrogen cat# 10777019) 40 U/mL.
   Final concentrations: SuperaseIN (Ambion) 160 U/mL, RNaseOUT (Invitrogen) 160 U/mL.
5. 10× PBS (bought pre-made).
6. RNAsecure (Ambion cat# AM7006).
7. Filters: Nylon mesh 150 (cat # CMN-0149-C), 60 (cat # CMN-0062-C), 30 (cat # CMN-0030-C) micron from Small Parts, Inc. Polypropylene filters 10 μm (cat#61757) from Pall Corporation.

8. Filter supports: Nalgene In-line filter holder (cat # 330-4000).
9. Luer-lok tip Syringes 30/50 mL.
10. DEPC.
11. Dounce tissue grinder 15 mL (cat# K885300-0015).
12. Centrifuge (with refrigeration).
13. Autoclave.
14. Instant Sealing Sterilization Pouch (Fisherbrand, cat# 01-812-54).
15. Trizol (Invitrogen cat# 15596026).
16. Glycogen, Glycoblue (Ambion cat# AM9516).
17. Ultracentrifuge tubes (1.5 mL) Rnase and Dnase free.
18. Turbo DNA-free (Ambion cat# AM1907).
19. DEPC-treated water (pre-made, Ambion cat# AM9922).
20. RNAzap (Ambion cat # AM 9780).
21. RNAlater (Ambion cat# AM7024).

3. Methods

3.1. Day 1

1. Clean the bench space and instruments with RnaseZap (Ambion).
2. Place the sheet of the nylon filter between two filter papers, staple them together to hold them in position and mark on the filter paper the circular shape of the filter adding 2 mm beyond the diameter of the filter holder. Cut filters with scissor by hand.
3. Treat all the material used (filters, filter holders, and glass homogenizer) with DEPC* as follows: soak them in DEPC* dissolved freshly in water (for each 1 L of water add 2 mL of DEPC*, shake vigorously and pour in the container where you placed the tools). Let sit over night at room temperature.
4. Treat water with DEPC* to rinse the tools and to prepare the Agarose gel (about 2–3 L) (1 L of water plus 1 mL DEPC*, shake vigorously, and let sit over night at room temperature). From this point on, NO fresh DEPC is used.

3.2. Day 2

5. Autoclave DEPC treated water at least 30 min for each Liter. (We usually do 90 min for 2 L.).
6. Rinse with DEPC treated water all the tools soaked in DEPC solution.

7. Place the filters on the bottom part of the holder and let them dry under the hood.
8. Assemble the filter holders and place them in a sealing sterilization pouch (remember to mark the mesh pore size on the envelope or on the filter holder before placing them on the filter holder).
9. Autoclave everything for 20 min at 120°C (dry cycle).
10. Prepare HB and treat it with RNAsecure (Ambion).
    For 50 mL HB:
    25 mL 2× HB + 1 mL 0.5 M EDTA + 1.2 mL RNAsecure stock solution (25×) + DEPC Water (Ambion) to 30 mL. Incubate at 60°C for 30 min then place at 4°C till use.
11. Prepare PBS and treat it with RNAsecure (Ambion).
    For 10 mL use 1 mL 10× PBS + 0.2 mL 0.5 M EDTA + 60 μL RNAsecure + 240 μL DEPC water and incubate at 60°C for 30 min. Bring to 10 mL with DEPC water (Ambion).

3.3. Day 3

12. Before proceeding to the SynN extraction, add to the pretreated HB the proteinase and Rnase inhibitors (PRi): 500 μL stock solution PMSF and NEM, 50 μL stock solution of Leupeptin, pepstatin A, Aprotinin, 400 μL of SuperaseIN, and 200 μL of RNaseOUT.
13. Bring HB+PRi volume to 50 mL with DEPC water (Ambion). Keep on ice.
14. Assemble Syringes with filter holders; make sure the luer-lok is tight.
15. Place the assembled filter holders on top of a 50 mL polypropylene tube. The 50 mL tubes will collect the filtrate and therefore are kept on ice.
16. Dissect the forebrains in ice cold RNAse free PBS (prepared as described before).
17. Place the forebrains in 10 mL of RNAlater (Ambion) placed in a 50 mL polypropylene tube and keep on ice till ready to proceed.
18. Homogenize the 3 forebrains with the hand glass homogenizer with 7–10 strokes in 10 mL HB+PRi.
19. Record the final volume of the total homogenate.
20. Collect aliquots for RNA (400 μL), Protein (50 μL), Protein concentration measurement (50 μL) as follows:
    (a) *RNA*. Place 100 μL of sample to 1 mL of Trizol, follow manufacturer's instructions with the following modifications: add 1.5 μL of Glycoblue (or Glycogen 15 mg/mL) to the tube where you will place the aqueous phase and allow to precipitate over night at –20°C.

(b) *Protein*. Place 25 μL of sample in 1 mL MeOH and incubate over night at −20°C.

(c) *Protein concentration*. Place 50 μL in a tube and store on ice if performing protein concentration the same day, otherwise place the sample at −20°C.

21. Place the total homogenate in the first syringe connected to the first mesh (150 μm).
22. Push the homogenate through into the 50 mL collection tube placed on ice.
23. Add 2 mL of HB+PRi to the same syringe and push the HB+PRi through the filter into the same collection tube.
24. Place the collected filtrate into the next syringe connected to the 60 μm filter holder placed over a new collection tube and proceed as in steps 22 and 23.
25. Place the collected filtrate into the next syringe connected to the 30 μm filter holder placed over a new collection tube and proceed as in steps 22 and 23.
26. Place the collected filtrate into the next syringe connected to the 10 μm filter holder placed over a new collection tube and proceed as in steps 22 and 23.
27. Spin down the final filtrate at 1,500 × *g* for 1 min at 4°C, collect supernatant, and discard pellet.
28. Spin down supernatant at 20,000 × *g* for 4 min, discard supernatant, and collect pellet.
29. Rinse pellet once with HB+PRi, spin down again, and re-suspended in HB+PRi (550 μL).

Collect aliquots for RNA, Protein, and Protein concentration as mentioned above (RNA 4 × 100 μL, Protein 2 × 25 μL, Protein concentration 50 μL).

3.4. Day 4

30. Spin down the RNA samples from steps 20a and 29 at 20,000 × *g* for 25 min at 4°C. Discard supernatant carefully so as not to lose the pellet.
31. Add 250 μL 80% EtOH per tube and carefully put together all the pellets from the same group in a single tube and bring the volume to 1.5 mL with 80% EtOH.
32. Spin the RNA down again for 10 min at 20,000 × *g* for 10 min at 4°C, decant supernatant, and let the pellet dry for 5–10 min.
33. Re-suspend RNA with 40 μL RNAsecure water (40 μL RNAsecure solution + 960 μL DEPC water (Ambion)). Incubate at 60°C for 15 min, then place on ice.

34. To eliminate possible DNA contamination still present in the tube, perform DnaseI (Ambion) treatment (4 μL 10× buffer + 1 μL DnaseI enzyme for 25 min at 37°C. Stop reaction with the "Stop reaction buffer" and collect the RNA).
35. Check the RNA OD with Spectrophotometer. The RNA concentration should be in the 1 mg/mL range. The 260/280 ratio should be in the range of 1.9–2.0, which confirms the purity of the RNA. Also, it is advisable to run an aliquot of the RNA in a 1% Agarose gel and stain with ethidium bromide to confirm RNA quality—you should clearly see the 28S and 18S ribosomal RNA bands (their intensity ratio should be 2:1).
36. Spin down the protein sample from steps 20b and 29 at 20,000 × *g* for 20 min at 4°C.
37. Vacuum supernatant off, and let the pellet dry overnight under the hood.
38. Check protein concentration of samples from steps 20c and 29 using the Lowry or Bradford method.
39. Re-suspend to 2 mg/mL with 4× SDS-Laemmli buffer and 1% DTT (final).

Heat at 100°C for 5 min and load a series of aliquots at 20, 10, and 5 μg per lane in the appropriate polyacrylamide gel (7.5% would be fine for assessing distribution of marker proteins in the two fractions).

4. Notes

1. A variety of protein and RNA synaptic markers can be used to ensure the purity and enrichment of synaptic fractions (5, 6). For example, we and others have used semi-quantitative Western blotting of PSD95 (6), dystroglycan (8), and spinophilin (9) as postsynaptic markers. Synapsin I is a convenient presynaptic marker of synaptic vesicles (6). The nuclear protein PCNA can be used as a negative control (6). Among RNAs that should be enriched in synaptic fractions are mRNAs (e.g., CamKII-alpha (6), SynGAP1, MAP2 (10)) as well as the small RNAs BC1 (in rodent) or BC200 (in man) (6). Nuclear RNAs such as U40 (11) or MBII-52 (12) should be depleted in synaptoneurosomes.
2. The protocol shown in this paper used the divalent cation chelator EDTA to lower the free Mg and Ca concentrations; this was chosen to minimize activation of proteases and RNAses during sample preparation. However, Mg helps maintain RNA stability and is necessary for the maintenance of

some RNA-protein complexes; thus depending on the purposes of the experiment, one may want to carry out subcellular fractionation in the presence of Mg and/or Ca ions. Experiments studying phosphorylation or signaling-dependent events should include phosphatase inhibitors in the homogenization buffer.

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Chapter 15

Isolation of Total RNA and Detection Procedures for miRNA Present in Bovine-Cultured Adipocytes and Adipose Tissues

Scott L. Pratt, T. Ashley Burns, Marcy D. Owens, and Susan K. Duckett

Abstract

Micro-ribonucleic acids (miRNA) regulate gene expression posttranscriptionally by altering translation of protein(s) encoded by specific messenger RNA. Therefore the ability to detect and quantify the expression levels of specific miRNA present within a cell or tissue is necessary to thoroughly examine cellular physiology and gene expression. Here we describe procedures that allow for the isolation and quantification of miRNA in bovine adipocytes and adipose tissue.

Key words: Adipose tissue, Adipogenesis, Micro-ribonucleic acids, RT-PCR, Differentiation

1. Introduction

Micro-ribonucleic acids (miRNA) are small noncoding inhibitory RNA that are present and expressed in cells of all plants and animals examined to date (Fig. 1). miRNA are transcribed as large precursors by RNA polymerase II and processed into the small active miRNA by two RNAse-III enzymes called Drosha and Dicer (1). Cleavage by Drosha occurs in the nucleus leaving the pre-miRNA to be transported to the cytoplasm where cleavage by Dicer results in the mature ~22 bp miRNA containing a 2 nt 3′ overhang, and after cleavage, the miRNA associate with the RNA-induced silencing complex, which unwinds the miRNA to base pair with target mRNA sequence. miRNA are thought to regulate the translation of messenger RNA by repression, RNA interference and/or translational stimulation (Fig. 1).

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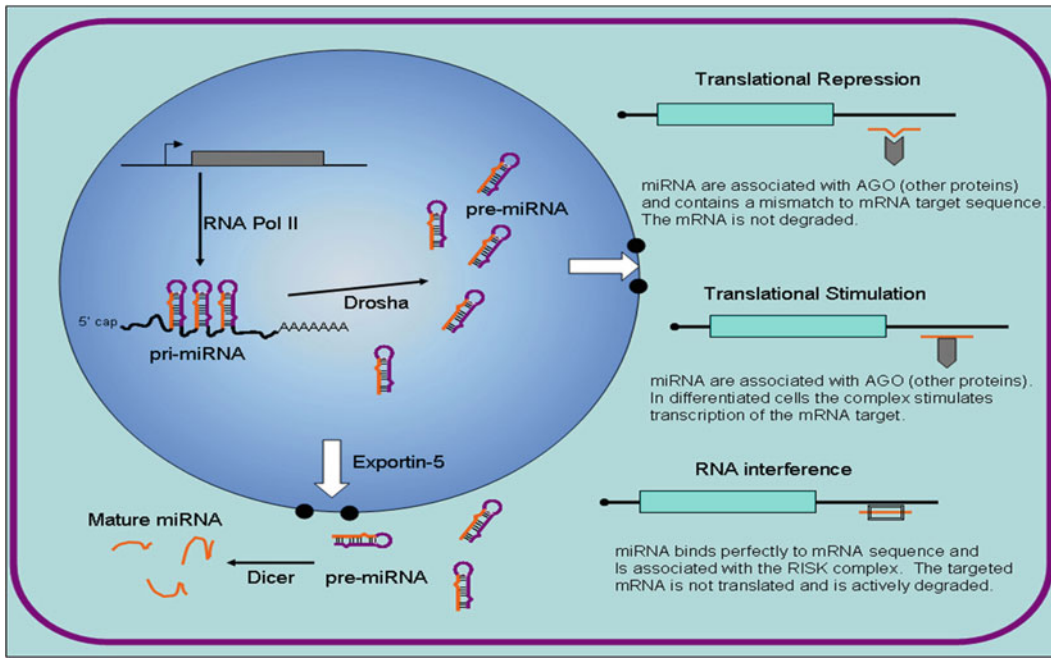

Fig. 1. Schematic of the synthesis, transport, and function of miRNA. miRNA are synthesized in the nuclease, transported to the cytoplasm where the processing into mature miRNA is completed. Shown are three possible mechanisms of action.

Multiple procedures have been developed to identify and quantitate miRNA expression in cells and tissues including cloning and sequencing (2–5), microarrays (3, 6–8), RT-PCR (9–11), and northern blotting (12, 13). More recently high throughput sequencing procedures have been adapted to generate the entire miRNA-ome of cells and/or tissues (14, 15). All procedures require the isolation of quality RNA for expression analysis. Tissues that our laboratory routinely analyze, such as oocytes, blastocysts, sperm cells (16), and adipose tissue (9, 17), present numerous difficulties in the isolation of quality RNA. The difficulty in isolating RNA from the aforementioned tissues include lipid content, amount of total RNA present within the cell, and/or total cell number. In addition, miRNA constitutes ~0.1% of the total RNA within a cell. Currently, the most common procedure to verify and quantify specific gene expression is quantitative reverse transcription polymerase reaction (qRT-PCR). qRT-PCR has been adapted for the detection and quantification of miRNA (18) including variations that allow for multiplexing of reactions (19). In fact multiple commercial products are available to facilitate research efforts utilizing human and rodent biological samples, but few reagents are readily available for other species. Fortunately, miRNA are typically highly conserved (16).

2. Materials

2.1. Assessment of RNA Isolation Procedures and Isolation of Total RNA from Bovine Adipose Tissue

1. Tissue Homogenizer.
2. DEPC-treated high speed centrifuge tubes (~30 mL vol). Add DEPC to a final concentration of 0.2% in 1 L of H_2O. Mix vigorously then dispense the DEPC-H_2O into the tubes (~20 mL per tube), seal and shake vigorously, let set for ~30 min, pour off the DEPC-H_2O and autoclave.
3. Sterile, RNase-free 50 mL conical tubes.
4. Sterile, RNase-free 1.5 mL microfuge tubes.
5. *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX).
6. TRIzol Reagent (Invitrogen, Carlsbad, CA), or other RNA isolation reagent/procedure of choice.
7. 10× MOPS ((41.85 g 4-morpholinopropanesulfonic acid (MOPS) and 6.80 g NaOAc—$3H_2O$ to 800 mL sterile nuclease-free H_2O and stir until completely dissolved (see Note 1)). Add 20 mL of a 0.5 M Na_2EDTA made in nuclease free H_2O and adjust pH to 7.0. Bring vol to 1 L with sterile nuclease free H_2O.
8. 37% Formaldehyde.
9. Formamide.
10. RNA sample buffer (0.5 mL 10× MOPS, 1.75 mL 37% formaldehyde, 5.0 mL formamide) stored as 1 mL aliquots in RNase-free tubes at –20°C.
11. RNA loading buffer (50% glycerol final concentration, 1 mM EDTA, and 0.25% bromophenol blue; bring to volume with nuclease free H_2O).
12. 40% acrylamide (19:1) (multiple suppliers are available), with 1.0 mm spacers and 10 well comb.
13. 1.2% denaturing formaldehyde gel (for a 50 mL gel: add 0.6 g of agarose to 36 mL of nuclease free H_2O in a container large enough not to boil over when microwaving to heat solution; microwave on high for ~1 min. Evaluate and repeat process until all agarose is in solution. Let the vessel cool briefly then add 5 mL of 10× MOPS and 9 mL of 37% formaldehyde and pour into prepared gel tray).
14. 15% denaturing acrylamide gel: 3.75 mL of 40% acrylamide (19:1), 0.5 mL 10× TBE (108 g Tris-base, 55 g Boric Acid, 9.3 g EDTA in 1 L of H_2O), 4.2 g urea, 2 mL H_2O. Allow urea to fully dissolve into solution, then add 80 μL 10% ammonium persulfate and 8 μL TEMED. Pour gel into preassembled BioRad mini-Protean gel plates to within ~1–2 cm of the top

of the glass. Insert comb gently and do not introduce air bubbles. Let the gel polymerize at room temperature (~30 min).

15. Prepared slab gel tray and buffer tank: clean with RNase AWAY or like product prior to use (see Note 2).
16. Electrophoresis power pack.
17. EtBr solution (10 mg/mL in DEPC-H_2O).
18. RNA ladder (multiple commercial products exist).
19. *N*,*N*,*N*,*N*′-tetramethyl-ethylenediamine (TEMED).
20. Mini-Protean glass plates (multiple versions exist).
21. 5 mL disposable syringes (order number: 5CCVLL; Vet-Syringe, Mettawa, IL).
22. Vac-Man manifold (Promega, Madison, WI).
23. 100% EtOH.
24. Nanodrop 1000 (Thermo Scientific).
25. Agilent Bioanalyzer 2100 (Agilent Technologies).
26. RNA 6000 Nano Kit (Agilent Technologies; see Note 3).

2.2. RT-PCR

1. Electrophoresis power pack.
2. Gel tray and gel tank.
3. EtBr solution (10 mg/mL in DEPC-H_2O).
4. 10× TBE (108 g Tris-base, 55 g Boric Acid, 9.3 g EDTA in 1 L of H_2O) or TAE (242 g Tris-base, 57.1 mL of glacial acetic acid, and 18.6 g of EDTA) buffer.
5. 3% Agarose non-denaturing slab gel. Weigh out 1.5 g of agarose and add to 0.5× TBE in a beaker large enough not to boil over upon microwaving (at least a 200 mL beaker). Microwave on high for 1 min and swirl the beaker contents to determine if the agarose is completely in solution. Repeat procedure until all of the agarose is in solution, and add 5 μL of EtBr from the 10 mg/mL stock. Swirl and pour into the gel tray. This mixture is very thick so if air bubbles are observed gently remove them using the well comb. Place the comb and let gel set for ~30 min prior to loading samples and electrophoresis.
6. 6× DNA loading dye (30% glycerol containing 0.25% bromophenol blue and xylene cyanol FF each).
7. 20 bp DNA ladder.
8. Ampicillin (Amp) stock (80 mg/mL; weigh out the Amp and suspend in sterile H_2O. Sterile filter and dispense into sterile 1.5 mL tubes and store at −20°C until needed).
9. Lauria Broth.
10. LB/Agar plates.

11. Taq polymerase (we used the manufacturer recommended SuperTaq™ from Ambion, Austin, TX).
12. Commercial miRNA primers (Ambion, Austin, TX).
13. *mir*Vana™ qRT-PCR miRNA Detection Kit (Ambion, Austin, TX).
14. Thermocycler.
15. Decontaminated pipettes. Decontaminate by treating with RNase AWAY and by UV crosslinking for a minimum of 20 min.

3. Methods

For all procedures, use appropriate safety protection such as protective eye wear, lab coats, and gloves. Dispose of spent reagents according to your laboratory and federal regulations.

3.1. Methods to Evaluate Total RNA Isolation for the Detection of miRNA (see Note 4)

Before the isolation of RNA from more difficult cell and/or tissue samples, assess total RNA isolation procedures that will meet your experimental goals. Choose the RNA isolation procedures you wish to evaluate and obtain samples that total RNA yield is not an issue. We usually use either bovine liver or ovary. Both are easily obtained at local abattoirs.

1. The *mir*Vana™ miRNA Isolation Kit isolates total RNA and is reported to maximally bind small RNA. TRIzol Reagent is widely used and yields high quality total RNA. We have utilized both reagents and we further evaluated the products for use in isolation of total RNA containing small RNA (Fig. 2).
2. For each isolation procedure, follow the manufacturer's detailed protocols.
3. Estimate mass and purity using the Nanodrop 1000 (Thermo Scientific, Wilmington, DE; see Note 5).
4. To quickly assess quality of total RNA isolation without the expense of chip technologies, run a 1.2% formaldehyde denaturing gel (Fig. 2).
   i. Place the gel in the gel tank and fill the chambers and cover the gel with 1× MOPS (10× MOPS diluted in DEPC-H_2O).
   ii. To separate RNase-free 1.5 mL tubes add the RNA sample(s) and RNA ladder in a total vol of 10 μL then add 10 μL of RNA sample buffer, 2 μL of RNA loading buffer, and 1 μL of EtBr. Spin all tubes and heat the samples at 80°C for 5 min. Spin samples again.

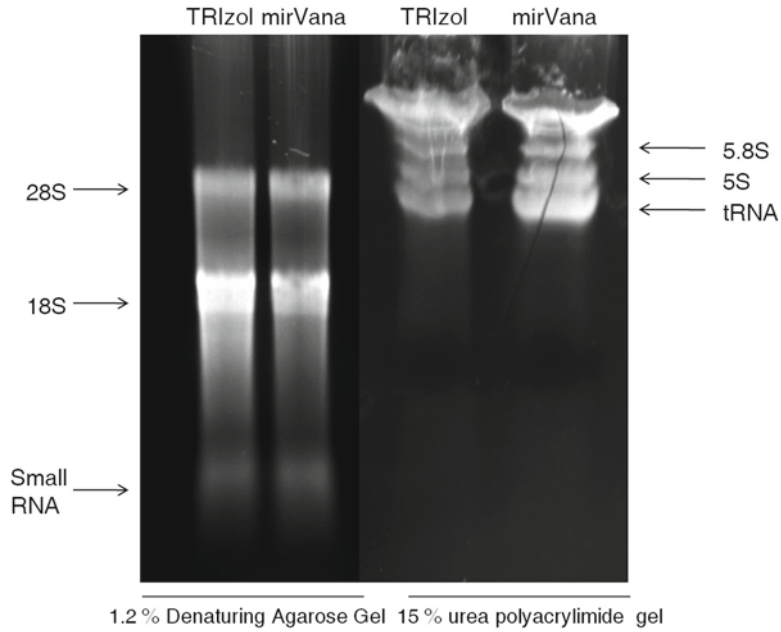

Fig. 2. Comparison of two RNA isolation methods to obtain total RNA samples possessing small RNA. Total RNA was isolated from porcine ovary using either the TRIzol procedure or the *mir*Vana™ miRNA Isolation Kit (lanes indicated at the *top* of the photo). Equal mass of each sample (5 μg) was subjected to electrophoresis under denaturing conditions either using 1.2% denaturing agarose gel or a 15% urea PAGE gel (gel type shown at the *bottom* of the figure). Both RNA isolation procedures produced acceptable RNA quality as represented by the presence of 18S and 28S ribosomal RNA (indicated on the *left*). In addition, both procedures were effective in isolating small RNA as indicated by the presence of 5.8S, 5S, and tRNA (indicated on the *right*); however, the *mir*Vana™ miRNA Isolation Kit was deemed slightly superior and a subsequent RNA precipitation step was not necessary where precious sample can be lost.

iii. Remove the well comb from the gel and carefully load the samples.

iv. Subject the gel to electrophoresis at 85 V for approximately 1 h. Check the gel by exposure to UV light and if it requires greater resolution, return the gel to the tank and continue electrophoresis.

v. Photograph the gel for your records.

5. To quickly assess the presence of small RNA in your sample of interest and for a qualitative comparison of RNA isolation methods based on the presence of small RNA, run a 15% denaturing polyacrylamide gel (Fig. 2).

i. After pouring the 15% denaturing gel (1.0 mm thick), assemble the BioRad mini-Protean cassette and place it in the tank buffer reservoir. Add 0.5× TBE to the upper buffer chamber and let stand for 10 min. Assess lower chamber after 10 min to verify no leaking from the upper gel chamber. Fill the lower buffer chamber (a pre-run prior to loading sample can be performed but I have found it not to be necessary for this procedure).

ii. Prepare your RNA samples as in Subheading 3.1, step 4.ii.

iii. Take a 1 mL syringe with an 18-gauge needle, draw up buffer and blow out each well. This is necessary as debris is problematic in the wells with urea gels.

iv. Load the samples and finish assembling the mini-Protean apparatus for the electrophoresis run (see Note 6).

v. Perform electrophoresis at 100 V and monitor the bromophenol blue dye front migration. Make a note as to where the dye front is and length of time when electrophoresis is stopped for repeatability.

vi. Disassemble the plates and place the gel on either an overhead transparency or plastic wrap and photograph for your records.

6. Once you have satisfactory procedures to repeatedly isolate quality RNA you can progress to RNA isolation of your samples and/or gene expression studies.

7. If sophisticated procedures such as microarray and/or deep sequencing are to be performed, we recommend further evaluation of your sample(s) by using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA; see Note 7).

8. Store all RNA samples at –80°C (see Note 8).

3.2. Total RNA Isolation from Cultured Adipocytes and/or Adipose Tissue

Isolation of total RNA from cultured adipocytes can be accomplished as outlined by the manufacturer. Isolation from adipose tissue is problematic due to the large amount of lipid and relatively small number of cells per gram of tissue compared to other tissues, such as liver, testis, or ovary. Therefore, to isolate quality total RNA to assess gene expression whether it be miRNA or mRNA quantification, procedures needed to be developed/altered.

1. The day prior to RNA isolation from tissue, thoroughly clean the homogenizer probe and soak in activated DEPC-H_2O (0.2%) overnight. The next morning, rinse in sterile DEPC-H_2O and autoclave the probe. In addition, if the high speed centrifuge tubes are not RNase-free, DEPC-treat the centrifuge tubes and autoclave. Alternatively, we have had success in cleaning all materials with RNase AWAY prior to RNA isolation. Duckett et al. (17) had described a total RNA isolation procedure that was sufficient for the detection and quantification of both mRNA and miRNA; however, the second step of that published procedure is not conducive to retaining high quantities of miRNA. Therefore we evaluated scaling up the *mir*Vana™ miRNA Isolation Kit for adipose tissue.

2. Prior to initiation of RNA isolation, evaluate the RNA isolation reagents. If the kit is new, add EtOH as directed to the

wash buffers. If the kit has been previously used make sure enough reagents, filter cartridges, and RNase-free 1.5 mL tubes are available to proceed. Always check for the presence of debris in the liquid components of the kit.

3. Assemble the tissue homogenizer per manufacturer's directions taking care to keep the probe RNase-free (use gloves at all times for RNA isolation).
4. Dispense 10 mL of the *mir*Vana™ miRNA Isolation Kit Lysis/Binding buffer to RNase-free tubes and place on ice.
5. With gloved hands, remove the desired samples from the −80°C and place on ice for transport to scales.
6. If tissue was not processed and frozen in small pieces, use RNase AWAY treated scissors or other implement to obtain small pieces to weigh. Weigh ~1 g of tissue into a clean weigh boat then place the tissue directly into tubes containing Lysis/Binding buffer and place on ice. Return remaining sample to its assigned space in the −80°C.
7. Immediately prior to homogenization of the sample, wash the homogenization probe by running it in sterile 50 mL tubes containing ~25 mL of DEPC-H_2O, followed by a MeOH and second run in a separate tube of DEPC-H_2O.
8. Homogenize with short bursts. If multiple samples are to be processed, repeat the sequence of wash procedures outlined in Subheading 3.2, step 6.
9. Add 1/10 vol of the *mir*Vana™ miRNA Isolation Kit Homogenate Additive as described by the manufacturer, mix and incubate on ice for 10 min.
10. Per manufacturer's directions, add an equal vol of the Acid-Phenol:Chloroform (equal vol to the original vol of the Lysis:Binding buffer) and vortex for a minimum of 30 s.
11. Centrifuge for 5 min at 10,000 × *g* (see Note 9).
12. Place 150 μL of the Elution buffer per sample in an RNase-free 1.5 mL centrifuge tube and place in a heat block set at 95°C. Hold the Elution buffer at 95°C until needed.
13. Harvest the upper aqueous phase from the centrifuge tube and place in a new RNase-free tube and keep the sample on ice.
14. Assemble the vacuum manifold device. Place a 5 mL syringe into the vacuum manifold (Promega Vac-Man® Laboratory Vacuum Manifold, Madison, WI; or similar model), then insert the filter cartridge into the top of the 5 mL syringe (see Note 10).
15. Turn on the vacuum and pass the sample over the filter cartridge then perform the washes as described by the manufacturer.

16. Move the filter cartridge possessing bound RNA into a manufacturer supplied 1.5 mL tube and spin at maximum RPM for 1 min to remove residual wash buffer.
17. Move the filter cartridge into a fresh manufacturer supplied 1.5 mL microcentrifuge tube, pipet 100 μL of 95°C Elution buffer into the center of the cartridge, and incubate for 1 min. Centrifuge at maximum speed for 30 s.
18. Dispose of the cartridge and either store the RNA sample or progress with RNA quantification and quality check (see Note 11).
19. Quantify RNA as described for Subheading 3.1, step 1.ii
20. Assess RNA quality using the Agilent Bioanalyser 2100 and the supporting RNA 6000 Nano Kit. This assay has become the industry standard and is usually performed prior to array analysis and sequencing (Fig. 3).
21. Store the total RNA sample at −80°C (see Note 12).

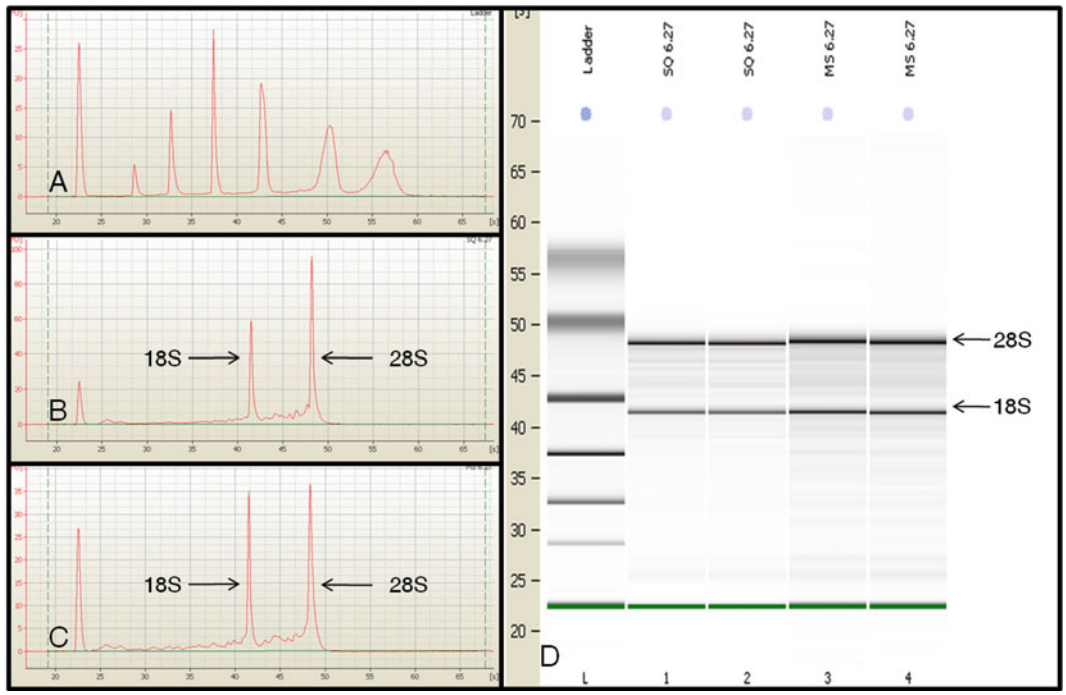

Fig. 3. Isolation of total RNA from subcutaneous and mesenteric bovine adipose tissue using the *mir*Vana™ miRNA Isolation Kit. (**a**–**c**) Are the electropherogram of the RNA ladder, bovine subcutaneous adipose tissue, and mesenteric adipose tissue, respectively. (**d**) Shows the gel image of the RNA ladder and two bovine adipose samples (in duplicate shown in (**b**, **c**)). Lane identity is given at the *top* of the gel image. The 28S and 18S ribosomal bands are indicated in (**b**–**d**) by *arrows*. The RNA integrity number was 8.9 and 8.4, and the estimated concentration was 204 and 116 ng/μL, respectively for subcutaneous and mesenteric adipose tissue. Estimates of RNA concentration and purity of the same samples using the Nanodrop 1000 was 280 ng/μL with a A° 260/280 ratio of 2.1 and 155 ng/μL with a A° 260/280 ratio of 2.1 for subcutaneous and mesenteric adipose, respectively.

3.3. RT-PCR to Validate Primers and Detect miRNA

Before expending energy and resources on qRT-PCR to determine expression levels for specific miRNA of interest, determine if available primers amplify the miRNA of interest and that the miRNA of interest is expressed in your samples (see Note 13). We conduct end-point PCR and sequencing to verify presence and identity of both miRNA and mRNA in samples of interest. Our work focuses on cattle and pigs and few if any specific reagents are available for these species; however, miRNA are fairly conserved across species allowing for the use of rodent and/or human designed reagents, but this makes the verification of the amplified product even more important. The following procedures utilized the *mir*Vana™ qRT-PCR miRNA Detection Kit (see Note 14); however other commercial reagents are available.

1. Prior to each round of RT and/or PCR reactions, decontaminate all pipettes using RNase AWAY as directed. In addition, prior to PCR expose pipettes to UV irradiation using a UV-cross linker for a minimum of 20 min.
2. Upon receiving miRNA primers, suspend and/or dilute primers and store at −20°C in RNase-free tubes. This is done to minimize contamination between RT-PCR runs.
3. For each primer set, conduct end-point RT-PCR to confirm detection of a product exhibiting the expected size in your sample of interest. In general use 5–10 ng of total RNA and perform the RT and PCR as described by the manufacturer, making sure to run a H_2O blank control.
4. To evaluate PCR product, after the reaction is complete, place the 3.0% agarose gel in the gel tank, fill both chambers and cover the gel with 0.5× TBE. Load a 20 bp DNA ladder (already in DNA loading buffer) and the end-point RT-PCR reaction for each primer set and its H_2O blank control (10 μL of each reaction and 2 μL of 6× DNA loading buffer) and subject to slab gel electrophoresis at 85 V for 30–40 min (a vertical 10% acrylamide gel can be utilized here as well). Keep the remaining reaction products on ice.
5. Verify the products are of the expected size by visualization using UV exposure and capture an image for your records (Fig. 4).
6. Once the bands have been visualized and verified the product is of the expected molecular weight, perform a subcloning reaction using the Qiagen PCR Cloning Kit (Valencia, CA). Perform ligation reactions using a maximum of 4 μL of the PCR amplification reaction. Incubate the ligation reaction(s) overnight at 4°C.

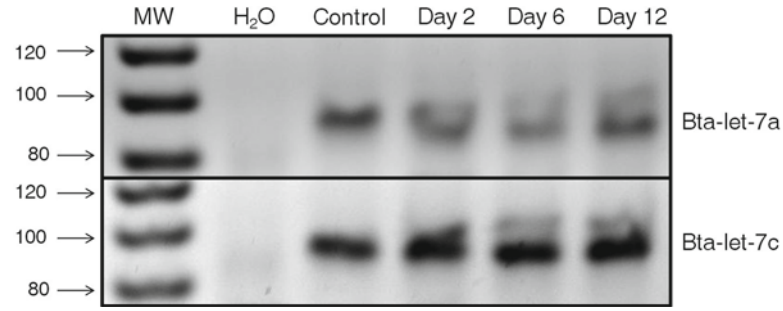

Fig. 4. End-point RT-PCR analysis for the miRNA let-7a and c in cultured bovine adipocytes. The respective primer set evaluated is shown to the *right* of the figure and the sizes of the DNA standards are shown to the *left*. Lanes are given at the *top* of the image. miRNA are usually 19–24 bp in length but primers used in these experiments contained flanking sequences for stabilization and the resulting products ranged in size from 86 to 90 bp. Control samples were preadipocytes that were held at confluence for 2 days. Day 2, 6, and 12 are adipocytes differentiated as described by (9, 17).

7. Transform and plate E. coli with the ligation reaction(s) as per the manufacturer's protocol (see Note 15). Place the competent E. coli cells obtained with the Qiagen kit immediately on ice after removing from the −80°C and label tubes with the ligation reaction that will be used to transform the E. coli.
8. Incubate the transformation reactions for a minimum of 30 min on ice.
9. Heat shock the transformation reactions by placing the tubes in a heating block or water bath at 37°C for 5 min then place on ice for 2 min (see Note 16).
10. Add 1 mL of room temperature LB containing 80 μg/mL Amp final concentration and incubate at 37°C for ~1 h with shaking at 250 rpm.
11. Plate 100 μL on an LB agar plate containing 80 μg/mL Amp and incubate inverted overnight at 37°C. You can use X-Gal and IPTG to perform blue/white screening (see Note 17).
12. The next day, pick single colonies, make a master plate (LB agar with antibiotic resistance) and inoculate LB media containing Amp then grow the clone of interest to saturation overnight at 37°C with shaking at 250 rpm.
13. Isolate plasmid DNA using a mini-prep procedure of choice and then sequence plasmid to verify (1) it contains an insert and (2) that the insert is the miRNA cDNA of interest. Once the primers are verified to work properly, proceed to performing real time qRT-PCR if expression levels are required for your experiment (see Note 18).

4. Notes

1. For large quantities of sterile, nuclease-free H_2O we use 0.2% DEPC-treated H_2O. Add 0.2% final vol of DEPC to H_2O, shake well and let stand for at least 30 min at room temperature and then deactivate and sterilize by autoclaving.
2. Alternatively to commercial RNase treatments, incubate equipment in 3% H_2O_2 diluted in DEPC-H_2O for a minimum of 30 min, then rinse in MeOH followed by DEPC-H_2O.
3. In case of limited and dilute samples use the RNA 6000 Pico Kit from Agilent. Also keep in mind that the kits have a relatively short shelf life so coordinate RNA isolation and analysis closely. Do not go through freeze thaw with the RNA ladder as they readily degrade.
4. For all procedures use sterile, nuclease-free aerosol barrier pipette tips.
5. Any spectrophotometer that can read at the 260 and 280 A° wave lengths will suffice. However, the Nanodrop 1000 spectrophotometer uses minimal sample for the estimation of concentration and is particularly handy for hard to obtain RNA samples of limited quantity.
6. A 1.0 mm thick PAGE with a 10 well comb will allow for loading a sample with a total vol of ~25 mL. If you need larger vol go to a thicker gel and/or use a comb with wider wells for preparing the gel.
7. Currently, Agilent supplies two RNA analysis chips, the Nano and the Pico. Use the Pico Kit when assessing RNA quality on samples with very low concentration such as oocytes, sperm and/or from tissues where RNA was isolated using laser-capture techniques.
8. For long term storage of your RNA samples it is best to store them precipitated at −80°C. The precipitation procedure we routinely utilize is adding 3 vol of ice-cold EtOH (at least 95%) to your sample with a final concentration of 0.1 M NaCl.
9. For these volumes of isolation described here using bovine adipose tissue, my laboratory uses the Sorval SS34 rotor at an RPM of 9500 giving ~ RCM of 10,000 × *g*. You will see three phases. The aqueous phase is what contains nucleic acids. You will observe a clear organic phase separated from the aqueous phase by a layer containing protein, DNA, and lipid. Do not carry any of this phase over to the next step when harvesting the aqueous phase as it will impact the purification of RNA and RNA quality.

10. I recommend using a vacuum manifold for this step. Centrifugation with the supplied tubes requires approximately ten individual spins in a microcentrifuge per sample to bind the volume used in this procedure. Directions for the Vac-man assembly can be viewed at http://www.promega.com/resources/multimedia/dna-and-rna-purification/setting-up-a-vacuum-manifold-video/.
11. Our yields range between 15 and 30 μg of RNA per gram of starting tissue for adipose.
12. While the *mir*Vana miRNA Isolation Kit recommends storage at −20°C, I recommend storage at −20°C for only a short time. It is best to store at RNA −80°C. If the quantity and/or concentration of RNA is low in your sample you may want to precipitate in the presence of a carrier such as glycogen prior to use or storage.
13. Commercial primers generated for bovine miRNA do not exist. However if there is a specific miRNA you wish to evaluate in bovine samples search www.mirbase.org (20, 21). A limited number of bovine miRNA are listed. If the miRNA you are interested in has not been identified in cattle you can compare across species to determine the degree of conservation. We have had success in detecting miRNA in both porcine and bovine samples using primers designed for human miRNA (9, 16).
14. The *mir*Vana™ qRT-PCR miRNA Kit has been discontinued after Ambion was purchased by Applied Biosystems. However, all steps outlined for RT-PCR and sequencing described here should work in conjunction with most other commercial kits using Taq polymerase. Subcloning and sequencing using the Applied Biosystems TaqMan™ miRNA Assay Detection kit has not worked for us, most likely due to the presence of the TaqMan probe.
15. A shorter ligation period can be performed; however, our most consistent results were generated from ligation reactions conducted overnight.
16. The heat shock as described by the manufacturer is conducted at 42°C for 30 s. Over time I have had more consistent results using 37°C for 5 min.
17. Store the remaining transformation reaction at 4°C. If little or no growth is observed plating 100 μL of the reaction you can increase the amount plated and repeat. Also, you can use an enriched media for E. coli called SOC for incubating the transformation reactions. However, LB will suffice for standard subcloning.
18. Many miRNA are highly similar, such as the multiple forms of let-7 miRNA, therefore it is advisable to verify that the primers being used are amplifying the expected product and not another miRNA or a random RNA product.

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Chapter 16

miRNA and shRNA Expression Vectors Based on mRNA and miRNA Processing

Ping Wu, Melissa A. Wilmarth, Feng Zhang, and Guangwei Du

Abstract

RNA interference (RNAi) has emerged as a powerful tool in basic research and therapeutics by silencing the expression of specific target genes. RNAi is triggered by double-stranded small interfering RNAs, which can be processed from naturally or artificially expressed microRNAs (miRNAs) or small hairpin RNAs (shRNAs). The development of reliable RNAi vectors encoding artificial and natural miRNAs would be useful tools for basic research and therapeutic applications. We reported a new type of new RNAi vectors, designated pSM155, and pSM30 that take into consideration of miRNA processing and RNA splicing by placing the miRNA-based artificial miRNA expression cassettes inside of synthetic introns. These vectors significantly improved the expression of an enhanced green fluorescent protein marker from the same mRNA transcript and also provide a simplified cloning method. We have described in this chapter the protocols for cloning artificial and natural miRNAs (or shRNAs), evaluating their efficiency in downregulating gene expression, and also discuss the potential applications of these vectors.

Key words: RNA interference, microRNA, Small-hairpin RNA, Intron, pSM155, pSM30

1. Introduction

RNA interference (RNAi) occurs naturally within cells to regulate gene expression at the post-transcriptional level. Scientists are able to mimic and manipulate this process in mammalian cells by introducing small interfering RNAs (siRNA) or by transfecting DNA-based vectors encoding short hairpin RNAs (shRNAs) (1, 2) or microRNAs (miRNAs) (3–5). Inhibition of target protein expression based on RNAi has become a widely used method for the functional studies of genes and is also attracting increasing attention as a therapeutic strategy for many human diseases (6).

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Most original shRNA expression vectors are driven by a RNA polymerase III promoter. Some newer vectors mimic the structure of miRNAs and are driven by a RNA polymerase II promoter. These miRNA-based expression vectors can be used to express both miRNAs and shRNAs, and offers some advantages over the RNA polymerase III promoter-based vectors, such as better control of inducible expression, simultaneous expression of several miRNAs or shRNAs from a single polycistronic transcript, and regulated or tissue-specific expression. We reported a new type of miRNA/shRNA expression vectors, pSM155 and pSM30, which were designed based on the knowledge of mRNA splicing and miRNA processing (7, 8). In addition to the strengths of the classic miRNA-based expression vectors, our design also offers some additional advantages including allowing an excellent expression of the marker protein, simple cloning strategy for shRNA/miRNA, and multiple miRNA and shRNA cassettes. In the past 4 years, pSM155 and pSM30 have been used in both in vitro and transgenic animal studies in many laboratories. In this chapter, we will describe the basic procedures of using this miRNA/shRNA expression system and discuss its potential applications.

2. Materials

2.1. DNA Cloning and Plasmid Maps

1. Restriction enzymes, *BsmB* I, *Msc* I, *Sal* I, *EcoR* V, *Mlu* I, *Xho* I, and *Nhe* I (New England Biolabs (NEB), Ipswich, MA).
2. QIAEX II gel extraction kit (Qiagen, Valencia, CA).
3. Rapid ligation kit (Roche Diagnostics, Indianapolis, IN).
4. VectorNTI program (Invitrogen, Carlsbad, CA, USA).

2.2. Cell Culture and Sample Preparation

1. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% calf serum (CS, HyClone, Ogden, UT).
2. Opti-MEM-I and LipofectAMINE LTX (Invitrogen).
3. Phosphate buffered saline (PBS): Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4 (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by dilution of one part of the stock solution with nine parts of water.
4. Complete protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN): Dissolve in PBS to make 25× stock solution. Keep the stock at −20°C.

5. Radio ImmunoPrecipitation Assay (RIPA) buffer: 20 mM Tris·Cl, pH 7.4, 137 mM NaCl, 10% (v/v) glycerol, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate, 1% (v/v) Triton X-100, 1.0 mM EDTA, 1× protease inhibitor cocktail. Keep the buffer at –4°C. Add the protease inhibitor cocktail from 25× stock before use.

2.3. Western Blotting

1. Ponceau S Staining Solution: 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid. The staining solution can be stored at room temperature and re-used many times.
2. Semi-dry transfer buffer: 48 mM Tris–HCl, pH 8.3, 39 mM glycine, 0.037% SDS, 20% methanol. Store the buffer at –4°C.
3. Tris-buffered saline (TBS): The 10× stock contains 1.37 M NaCl, 27 mM KCl, 250 mM Tris–HCl, pH 7.4, 1% Tween-20. Dilute 100 mL of the stock with 900 mL of water to make the 1× working solution.
4. Tris-buffered saline with Tween (TBS-T): 10× stock contains 1.37 M NaCl, 27 mM KCl, 250 mM Tris–HCl, pH 7.4, 1% Tween-20. Dilute 100 mL of the stock with 900 mL of water to make the working solution.
5. Blocking buffer: 1% (w/v) casein in TBS. Make sure the pH is around 7.4. Casein does not dissolve in acidic pH.
6. Antibody dilution buffer: 1% (w/v) casein in TBS-T.
7. Monoclonal anti-α-tubulin (Sigma-Aldrich, St Louis, MO).
8. Infrared IRDye-labeled secondary antibodies. Goat anti-mouse and anti-rabbit IgG conjugated to Alexa 680 (Invitrogen). Goat anti-mouse and anti-human IgG conjugated to IRDye 800 (Rockland Immunochemicals, Gilbertsville, PA).
9. Semi-dry blotting unit (Fisher Scientific, Pittsburg, PA).
10. Odyssey infrared imaging system (LI-COR Biosciences—Biotechnology, Lincoln, NE).

3. Methods

3.1. Rationale of the Intron-Expressed miRNA Expression System

Some original artificial miRNA expression vectors were directly coupled to the coding sequence of a fluorescent protein to provide a way to identify transfected cells genuinely expressing the artificial miRNA (5, 9). An example of this design is shown in Fig. 1a. The pri-miRNAs are processed in the nucleus by Drosha to generate pre-miRNAs. Pre-miRNAs are then transported to the cytoplasm, where they are further processed to siRNA. However, this design is not expected to allow simultaneous expression of both mature

miRNA and the fluorescent protein. In eukaryotes, after transcribed by RNA polymerase II in the nucleus, capped at the 5′ end and polyadenylated at the 3′ end, primary miRNAs (pri-miRNA) are processed by a nuclear microprocessor complex containing the RNase III enzyme Drosha (10, 11). The resultant 70–90 nt hairpin is recognized and transported out of the nucleus by Eportin-5. In the cytoplasm, the pre-miRNA is then cleaved by Dicer, another miRNA RNase III enzyme, to produce a ~22 nt double-stranded RNA duplex, which inhibits protein expression. In these original designs, generation of the mature miRNA initiated from Drosha activity would block the translation of the enhanced green fluorescent protein (EGFP) marker, because the resulting mRNA fragment lacks a 5′ CAP structure and is rapidly degraded. EGFP can be translated if the pri-miRNAs are exported to the cytoplasm before Drosha cleavage; but siRNAs are then not produced from these unprocessed pri-miRNAs (7). Indeed, our results have confirmed that the expression of the EGFP is very poor with this method (7). To achieve the expression of both miRNA and EGFP from the same vector, we inserted the artificial miRNA-expressing cassettes into a chimeric intron derived from pCI-neo (Promega) (Fig. 1b) (7). This design mimics the structure and processing of some natural miRNAs that locate in the introns of protein-coding sequences (10, 11). Separation of the miRNA component by mRNA splicing from the 5′ CAP-exon-EGFP-3′ poly(A) component should facilitate Drosha processing of the pri-miRNA rather than cytoplasmic export, and should also favor the cytoplasmic export and translation of the EGFP component rather than intranuclear degradation. Both pSM155 (Spliced miR155, Fig. 2) and pSM30 (Spliced miR30, Fig. 1b) that we designed significantly improved the expression of the EGFP marker without affecting the downregulation of target protein expression (see Note 1).

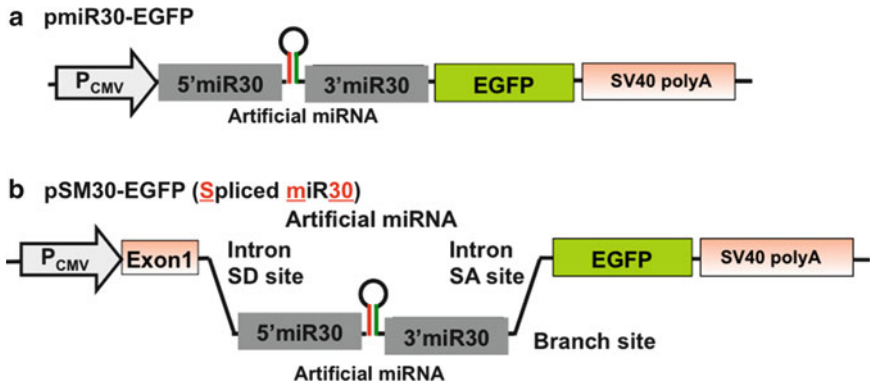

Fig. 1. Two strategies used to express both miRNA and EGFP. (**a**) An EGFP open reading frame is directly linked to the miRNA expression cassette in the traditional strategy. (**b**) The miRNA is located inside an intron in the new strategy (pSM30 is used as an example).

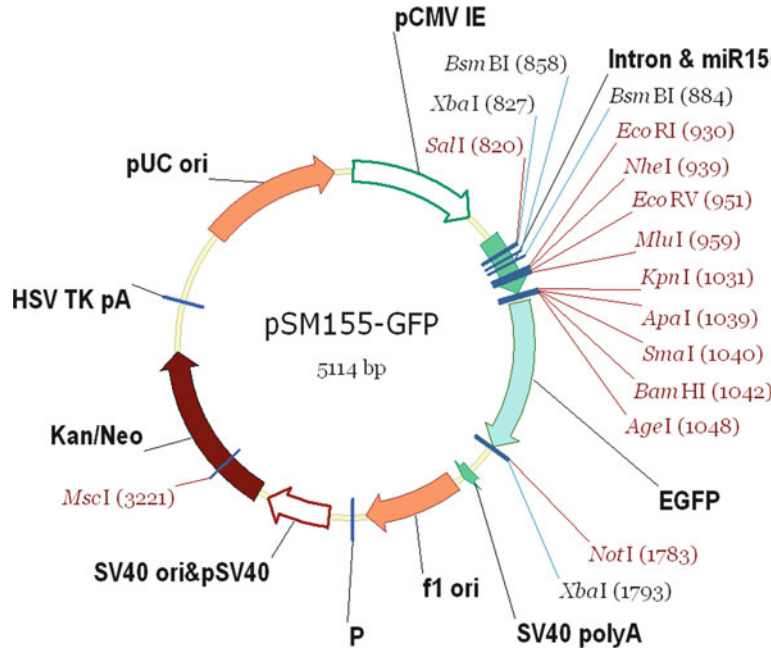

Fig. 2. Plasmid maps for the intron-based miRNA expression vectors (only pSM155 is shown). The vectors are used to express artificial miRNAs for RNAi experiments by incorporating the sequence of the human miR-155 miRNA precursor into a synthetic intron. The maps were generated using VectorNTI (full sequences and digital files of pSM155 and pSM30 are available upon request).

3.2. Design and Synthesis of Oligos

Many previous studies have generated the guidelines for choosing successful sequences. To select effective target sequences for artificial miRNA expression, we generally follow the guidelines for generating siRNA (12, 13). In summary, an effective artificial shRNA or miRNA must fit most but not necessarily all of the following rules (12): low to medium G/C content (30–50%), low internal stability at the 5′ anti-sense strand, high internal stability at the 5′ sense strand, absence of internal repeats or palindromes, A-form helix between miRNA and target mRNA, presence of an A at position 3 and 19 of sense strand, absence of a G or C at position 19 of sense strand, presence of a U at position 10 of sense strand, and absence of a G at position 13 of sense strand, etc. To enhance specificity, a miRNA also needs to have minimal homology with non-target RNAs and avoid low-stringency sequences such as a stretch of A or T. To clone any specific targets into the vectors, a pair of oligos includes cohesive ends, sense, and anti-sense specific sequences matching the target mRNA and a loop (64 nucleotides for the pSM155-based system and 67 nucleotides for the pSM30-based system). For each mRNA targets, we usually test four to five constructs and choose at least two for functional studies. The selection of candidate shRNAs can be assisted by many online websites for

siRNA and shRNA design, or more specifically, the algorithm for pSM155 and pSM30 on the Invitrogen website http://www.invitrogen.com/rnai and Cold Spring Harbor Lab website http://codex.cshl.edu, respectively.

3.2.1. Design of the Oligos for the Cloning Using pSM155 (An Example is Shown in Fig. 3a) (see Note 2)

Generating the Top Oligo Sequence. To Generate the Top Oligo Sequence, Combine These Elements (From 5′ End to 3′ End)

1. Start with 5′ TGCTG.
2. Reverse complement of the 21-nt sense target sequence (this is the mature miRNA sequence).
3. Add the loop GTTTTGGCCACTGACTGAC.
4. Add nucleotides 1–8 (5′-3′) of the sense target sequence and nucleotides 11–21 (5′-3′) of the sense target sequence.

To Generate the Bottom Oligo Sequence, Perform the Following Steps

1. Delete 5′ TGCT from the top oligo sequence.
2. Generate the reverse complement sequence of Step 1.
3. Add CCTG to the 5′ end of the sequence.

3.2.2. Synthesis of the Oligos for the Cloning Using pSM30 (An Example is Shown in Fig. 3b)

To Generate the Top Oligo Sequence, Combine These Elements (From 5′ End to 3′ End)

1. Start with 5′ AGCG.
2. Add 22-nt sense target sequence.
3. Change the first nucleotide to one that does not anneal to the last nucleotide in anti-sense, e.g., C to A.
4. Add TAGTGAAGCCACAGATGTA (loop).
5. Add nucleotides of the anti-sense target sequence.

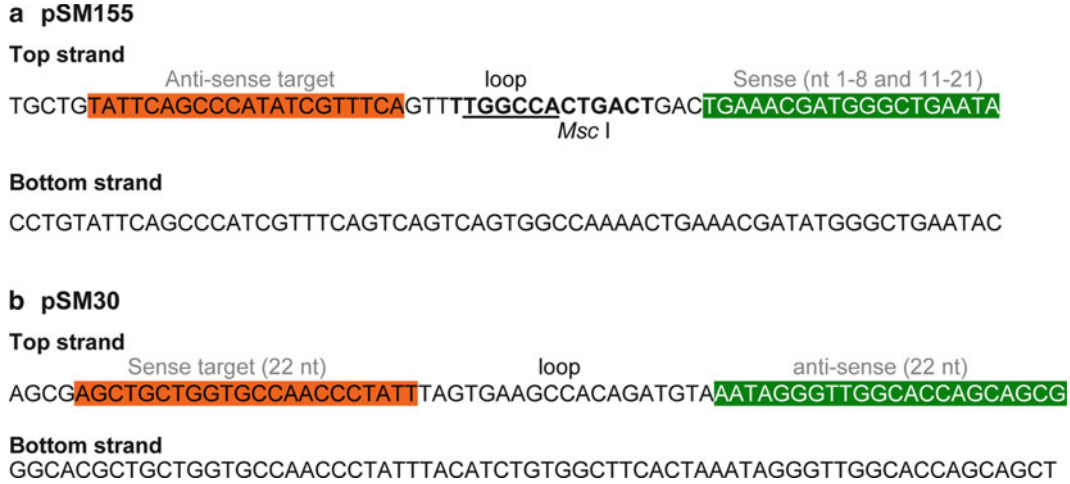

Fig. 3. Design of cloning oligos encoding artificial miRNAs into pSM155 (**a**) and pSM30 (**b**). A pair of oligos is required for cloning an artificial miRNA, which includes sequences for creating cohesive sites for the vector digested by *BsmB* I, sense strand, miRNA loop, and anti-sense strand. Both pairs of oligos shown are derived from firefly luciferase.

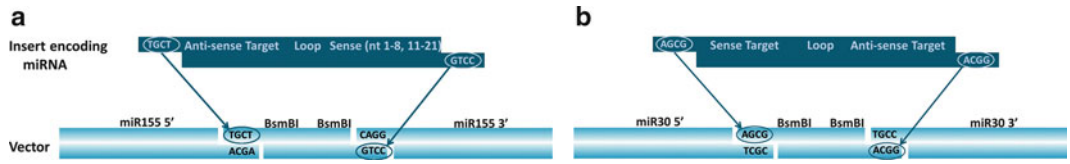

Fig. 4. Diagram of cloning strategy using pSM155 (**a**) and pSM30 (**b**). Cutting both vectors with *BsmB* I removes the two *BsmB* I recognition sites and generates cohesive ends for miRNA cloning.

To Generate the Bottom Oligo Sequence, Perform the Following Steps

1. Remove 5′ AGCG from the top oligo sequence.
2. Generate the reverse complement sequence of the one in B1 (this is the mature miRNA sequence).
3. Add GGCA to the 5′ end of the sequence from step 2.

3.3. Cloning of Artificial miRNAs

Two inverted *BsmB* I sites were placed internal to the arms of pSM155 and pSM30 (Fig. 4). *BsmB* I is a class IIS restriction enzyme, and has a cleavage site different but next to its recognition site. The unique properties of *BsmB* I make it possible to create a cohesive end of any required sequences. In this case, the cohesive ends are parts of the miRNA arms. A pair of oligos with appropriate four-nucleotide overhangs as described in Subheading 3.2 and Figs. 3 and 4, can be ligated to the cohesive sites of the vector generated by *BsmB* I digestion. Incorporation of the *BsmB* I sites greatly reduces the length of the oligos, thus lowering the cost in purchasing oligos and avoids the high error rate of long oligos.

1. Preparing the vectors. Cut 1–2 μg of pSM155 or pSM30 in 15 μL NEB restriction Buffer 3 at 55°C for 1 h. Run the digested vectors on 0.8% agarose gel and purify them using a gel purification kit following the manufacturer's instruction.
2. Annealing of oligos (25 μM final). Dissolve oligos with distilled water (100 μM stock concentration). Take 5 μL from each oligo (top and bottom) and add 8 μL water and 2 μL 10× NEB restriction buffer (We usually use Buffer 3). Boil the oligos for 4 min. Leave the denatured oligos at room temperature for 15 min and then move them to 4°C for 10–15 min. Dilute 2,500-fold to get 10 nM double-stranded oligos (250-fold dilution with water, quickly followed by another ten fold dilution with 1× NEB Buffer 3; or add 0.2 μL of annealed oligos to 500 μL 1× NEB Buffer 3) (see Note 3).
3. Ligation using rapid ligation kit. Mix 2 μL of the annealed oligos with 2 μL vector and H_2O (~5 ng or 1.5×10^3 pmol) (digested with *BsmB* I) (oligos: vector, ~15:1). Add 1 μL DNA dilution buffer, mix well, and then spin down briefly. Add 5 μL 2× ligation buffer and mix. Add 0.5 μL T4 DNA ligase, mix well, and spin down briefly. Incubate at RT for 5 min.

4. Transform bacteria. Any general *E. coli* strain used for cloning, e.g., DH5α, XL-Blue, are suitable. Add 3 μL of ligation mixture to 50 μL of competent cells, follow the regular transformation method (see Note 4).
5. Characterization of recombinant plasmids. The cloning efficiency is excellent with very low empty vector background. We often purify plasmids from two colonies for the characterization of correct ligation. For the cloning using the pSM155 vector, the plasmid DNA is digested with *Msc* I. The linearized parental vector is 5,114 bp, and recombinant construct generates a 2,784 and 2,368 bp fragments (see Note 5). For the pSM30 vector, the plasmid DNA is digested with *Xho* I/*Nhe* I (see Notes 5 and 6). The recombinant clone and the vector release a 239 and 200 bp insert fragments, respectively. Digestion of the empty pSM30 vector can be used as a negative control. Finally, the candidate recombinant clones need to be confirmed by DNA sequencing (see Note 7).

3.4. Cloning of Natural Endogenous miRNAs

Both pSM155 and pSM30 could also be used to express natural miRNAs, and thus label cells transfected by exogenous miRNAs. A full-length miRNA can be amplified by PCR using a top primer with a 5′ flanking *Sal* I restriction site and a bottom primer with a 5′ flanking *Nhe* I, *EcoR* V, or *Mlu* I restriction site. The PCR product digested with these restriction enzymes is then cloned into pSM155 or pSM30 cut with the same enzymes (Fig. 2).

3.5. Determination of Knockdown Efficiencies of pSM155 and pSM30 miRNA Constructs

The knockdown efficiency is often determined by Western blotting (see Note 8). An irrelevant artificial miRNA construct, such as that targets luciferase, can be used as a control. In some cases, immunofluorescent microscopy can also be used when the target cells are hard to be transfected. If an antibody for the gene of interest is unavailable, reverse transcription-PCR is then the preferred method. We describe Western blot analysis here using the Odyssey Infrared Imaging System from LI-COR Biosciences—Biotechnology.

1. Cell culture and transfection. HeLa cells are cultured in DMEM containing 10% CS using the standard method. One day before transfection, plate cells to a 6-well plate to get around 40–60% confluent at the time of transfection (see Note 9). On the day of transfection, transfect cells with 1–2 μg DNA using Lipofectamine LTX from Invitrogen (see Note 10).
2. Collect cell lysates for Western blotting. Remove culture medium from 6-well plates using vacuum. Add 1 mL ice-cold PBS containing 5 mM EDTA to cells. Leave cells on ice until they detach from the plate (see Note 11). After transferring to a 1.5 mL microtube, cells are collected by briefly spinning for 20–30 s at top speed. Remove supernatants and re-suspend pellets with 60 μL RIPA buffer containing 1× protease

inhibitor cocktail, keep them on ice for 5 min. Remove the nuclei by low speed spinning (about 2,000–3,000 ×*g*) at 4°C for 2–3 min. Transfer the supernatant to a new microtube containing SDS loading buffer (to make the final concentration to 1×).

3. After separated by standard SDS-PAGE, proteins are transferred to a nitrocellulose membrane by semi-dry transfer (see Note 12). A 9×6 mm mini-gel is transferred at 200 mA for 1 h. To check transfer efficiency, incubate the membrane for 1 min in Ponceau S staining solution with gentle agitation. Rinse the membrane in distilled water until the background is clean. The stain can be completely removed from the protein bands by continued washing.
4. Block the membrane in blocking buffer for 1 h (see Note 13). Be sure to use sufficient blocking buffer to cover the membrane.
5. Incubate blot in primary antibody for 60 min or longer at room temperature with gentle shaking. The dilution of the antibody depends on each individual antibody and should be determined empirically. An internal loading control such as α-tubulin or actin is recommended (see Note 14). To lower background, add 0.1–0.2% Tween-20 to the diluted antibody before incubation. Optimum incubation times vary for different primary antibodies. Use enough antibody solution to completely cover the membrane.
6. Wash membrane for 5 min at room temperature in TBS-T with gentle shaking. Repeat three times.
7. Incubate secondary antibody with membrane at room temperature for 60 min. Dilute the Alexa 680- or IRDye 800-labeled secondary antibody in blocking buffer with Tween-20 (see Note 15). Avoid prolonged exposure of antibody and membrane to light. Recommended starting dilution is 1:10,000 (dilution can be ranged from 1:5,000–1:25,000) (see Note 16). Add SDS if desired (see Note 17).
8. Wash membrane for 5 min at room temperature in TBS-T with gentle shaking. Repeat two more times. Rinse membrane with TBS or PBS for 5 min to remove residual Tween-20.
9. Scan the membrane in the appropriate channels. Alexa 680 and IRdye 800-labeled secondary antibodies are scanned at 700 and 800 channels, respectively (see Note 18).

3.6. Other Potential Applications and Improvements of the Current System

We described in the original papers that pSM155 and pSM30 vectors provide a reliable fluorescent marker for identifying shRNA-transfected cells. These vectors can be also used for other applications in basic research and therapeutics. Some of these applications have been demonstrated in the recent publications.

1. pSM155 and pSM30 can be used to express two or more miRNAs/shRNAs from a single vector, which can be designed against either the same target mRNA or two different target mRNAs. To clone two different miRNAs/shRNAs into pSM155, the first miRNA cassette purified by cutting with *Xba* I/*Mlu* I can be cloned into the second miRNA expression vector cut with *Nhe* I/*Mlu* I. The ability to effectively express two synthetic miRNAs from a single transcript has been proved in a similar design (3). A recent publication recommended that the number of concatenated miRNAs should be more than four (14). However, other studies expressed more than four miRNAs from the same mRNA transcript.
2. If other fluorescent proteins such as mCherry and dsRed are needed to label the transfected cells, the EGFP can be replaced by these open reading frames encoding these proteins in both vectors using *Not* I and *Kpn* I, *Apa* I, *Sma* I, *BamH* I, or *Age* I (see the map in Fig. 2). In addition, the EGFP marker can also be replaced by an antibiotic selection marker such as hygromycin in both pSM155 and pSM30 vectors. Expression of the antibiotic selection marker and miRNA from a single mRNA transcript allows to select "true clones" when stable cell lines expressing artificial miRNAs are generated.
3. RNAi and rescue experiments can be performed in the same vector. The cDNA containing wobble mutations at the sites where the artificial miRNA targets can be cloned into the vector in place of or in addition to the EGFP coding sequences.
4. The utilization of Pol II promoters in the new pSM155, pSM30, and other miRNA-based vectors provides more flexibility for the tissue-specific regulation or inducible expression. Targeting disease-specific tissues has been a challenge in RNAi-based therapy. The combination of a promoter that expresses specifically in the diseased tissues and miRNA-based expression cassettes will reduce the nonspecific targeting of normal tissues. A promoter inducibly controlled by small molecules can also be adapted to drive the expression of miRNAs to avoid chronic toxicity of RNAi.
5. One concern of using any miRNA-based retroviral vectors is that miRNA-based RNAi may not be as efficient as synthetic siRNAs and the traditional pol III-driven shRNAs in some cell types and tissues. The more complex structure of miRNAs requires additional processing steps, which may eventually lead to generation of less mature siRNAs. In fact, it has been reported that many miRNA primary transcripts are present at high levels but are not processed by the enzyme Drosha in early mouse development and human primary tumors (15). A solution for this problem may be to increase the activity of the proteins involved in miRNA processing by overexpression.

For example, overexpression of exportin 5, have been shown to enhance RNAi (16). Similar strategy may also be used to overexpress other enzymes such as Drosha and Dicer in the target cells.

4. Notes

1. The vectors and full sequences will be distributed upon request.
2. It is not necessary to use the very expensive phosphorylated oligos. If non-phosphorylated oligos are used for cloning, make sure that the vector is not dephosphorylated.
3. Keep the annealed oligos on ice; don't leave diluted oligos at temperatures higher than RT. Cooled samples can be stored at –20°C.
4. Avoid adding too much ligation product. High salts in the ligation buffer can reduce transformation efficiency.
5. The cloning efficiency is very high. We often directly sequence two candidate plasmids without the routine restriction enzyme characterization.
6. 1.5% agarose gel is used to separate the 239 bp fragment from the 200 bp fragment. The parental vector can be used as a negative control.
7. The mutation rate of the synthesized long oligos is usually high. Point and deletion mutations were often seen. It is important to confirm the desired sequences by DNA sequencing.
8. High transfection efficiency is required to get a consistent result. We only perform Western blotting when more than 80–90% cells are transfected. If transfection efficiency is a problem, co-transfection of the gene of interest targeted with an epi-tag such as flag or myc and the miRNA constructs would be an alternative method in the initial screening of the efficient miRNA constructs. In this case, a primary antibody against the epi-tag can be used. This method is very fast and reliable, especially when many constructs need to be tested and genes of interest express only in cells that are hard to be transfected.
9. Transfection efficiency is higher in healthy and fast proliferating cells.
10. The time required for effectively knocking down varies with different genes.
11. The time for detachment is dependent on cell types. It usually takes about for 3–5 min for most cell types.

12. Nitrocellulose membrane is preferred for the detection with fluorescently labeled secondary antibodies. If PVDF membrane has to be used, chose those with low fluorescence.
13. It is critical to avoid a blocking solution containing Tween-20. Tween-20 increases fluorescent background.
14. We often use primary antibodies generated from different species, i.e., rabbit and mouse, for the target protein and internal control, then choose Alexa 680-conjugated goat anti-mouse and IRDye 800-conjugated goat anti-rabbit antibodies. The target protein and internal control will be displayed as red or green colors, respectively.
15. Freshly diluted antibody gives the best sensitivity and performance.
16. For the detection of low abundant of proteins, try using more secondary antibody (1:3,000–1:5,000).
17. Adding 0.01–0.02% SDS to the diluted secondary antibody (in addition to Tween-20) will substantially reduce membrane background, particularly when using PVDF. However, DO NOT add SDS during blocking or to the diluted primary antibody.
18. Protect the membrane from light before scanning. Keep the membrane wet if re-blotting is planned. Once a membrane is dried, it cannot be stripped and re-used. Dry blots can be used for scanning. Signal strength may be enhanced on a dried membrane. The fluorescent signal on the membrane will remain stable for several months, or longer, if protected from light. Membranes may be stored dry or in PBS buffer at 4°C.

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Chapter 17

Gene Silencing In Vitro and In Vivo Using Intronic MicroRNAs

Shi-Lung Lin and Shao-Yao Ying

Abstract

MicroRNAs (miRNAs), small single-stranded regulatory RNAs capable of interfering with intracellular messenger RNAs (mRNAs) that contain either complete or partial complementarity, are useful for the design of new therapies against cancer polymorphism and viral mutation. Numerous miRNAs have been reported to induce RNA interference (RNAi), a posttranscriptional gene-silencing mechanism. Recent evidence also indicates that they are involved in the transcriptional regulation of genome activities. They were first discovered in *Caenorhabditis elegans* as native RNA fragments that modulate a wide range of genetic regulatory pathways during embryonic development and are now recognized as small gene silencers transcribed from the noncoding regions of a genome. In humans, nearly 97% of the genome is noncoding DNA, which varies from one individual to another, and changes in these sequences are frequently noted to manifest in clinical and circumstantial malfunction; for example, type 2 myotonic dystrophy and fragile X syndrome were found to be associated with miRNAs derived from introns. Intronic miRNA is a new class of miRNAs derived from the processing of nonprotein-coding regions of gene transcripts. The intronic miRNAs differ uniquely from previously described intergenic miRNAs in the requirement of RNA polymerase (Pol)-II and spliceosomal components for its biogenesis. Several kinds of intronic miRNAs have been identified in *C. elegans*, mouse, and human cells; however, their functions and applications have not been reported. Here, we show for the first time that intron-derived miRNA is not only able to induce RNAi in mammalian cells, but also in fish, chicken embryos, and adult mice cells, demonstrating the evolutionary preservation of this gene regulation system in vivo. These miRNA-mediated animal models provide artificial means to reproduce the mechanisms of miRNA-induced disease in vivo and will shed further light on miRNA-related therapies.

Key words: MicroRNA, RNA interference, RNA polymerase type II, RNA splicing, Intron, RNA-induced gene-silencing complex, Gene silencing in vivo

1. Introduction

Nearly 97% of the human genome is noncoding DNA, which varies from one species to another, and changes in these sequences are frequently noted to manifest in clinical and circumstantial malfunction. Numerous nonprotein-coding genes are recently found to encode MicroRNAs (miRNAs), which are responsible for

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RNA-mediated gene silencing through RNA interference (RNAi) like pathways (1–3). RNAi is a posttranscriptional gene-silencing mechanism that can be triggered by small regulatory RNA molecules, such as miRNA and small interfering RNA (siRNA). miRNAs, which are small single-stranded regulatory RNAs, can interfere with intracellular messenger RNAs (mRNAs) that contain either complete or partial complementarity; a trait useful for the design of new therapies against cancer polymorphism and viral mutation (4, 5). A much more rigid complementarity is required for double-stranded siRNA-induced RNAi gene silencing.

miRNAs were first discovered in *Caenorhabditis elegans* as native RNA fragments that modulate a wide range of genetic regulatory pathways during embryonic development (6). Currently, varieties of natural miRNAs have been found to be derived from hairpin-like RNA precursors in almost all eukaryotes, including yeast (*Schizosaccharomyces pombe*), plant (*Arabidopsis* spp.), nematodes (*C. elegans*), flies (*Drosophila melanogaster*), fishes, mice, and humans; they have been found to provide intracellular defense against viral infections and regulation of certain gene expressions during development (7–17). In contrast, natural siRNAs were abundantly discovered in plants and low-level animals (worms and flies), but rarely in mammals (18, 19). The intronic miRNA is a new class of miRNAs derived from the processing of gene introns. As shown in Fig. 1, the intronic miRNAs differ uniquely from previously described intergenic miRNAs in the requirement of Pol II and spliceosomal components for their biogenesis (20, 21). We have shown, for the first time, that these intron-derived miRNAs are able to induce RNA interference in not only human and mouse cells, but also in zebrafish, chicken embryos, and adult mice, demonstrating the evolutionary preservation of the intron-mediated gene regulation through miRNA-associated mechanisms in vertebrates in vitro and in vivo. These findings suggest the existence of an intracellular miRNA-mediated gene regulatory system for fine-tuning the degradation of protein-coding mRNAs (22).

The introns occupy the largest proportion of noncoding sequences in the protein-coding DNA of a genome. The transcription of the genomic protein-coding DNA generates precursor (pre)-mRNA with four major parts: a 5′-untranslated region (UTR), a protein-coding exon, a noncoding intron, and a 3′-UTR (23). In broad definition, both 5′- and 3′-UTRs can be seen as a kind of intron extension; however, their processing during mRNA translation is different from the intron located between two protein-coding exons, termed the in-frame intron. The in-frame intron can be up to several tens of kilobase nucleotides long and was thought to be a huge genetic waste in gene transcripts. Recently, this stereotypical misunderstanding was changed with the discovery of intronic miRNAs. Approximately 10–30% of some spliced introns are found in the cytoplasm, with moderate half-lives (24, 25).

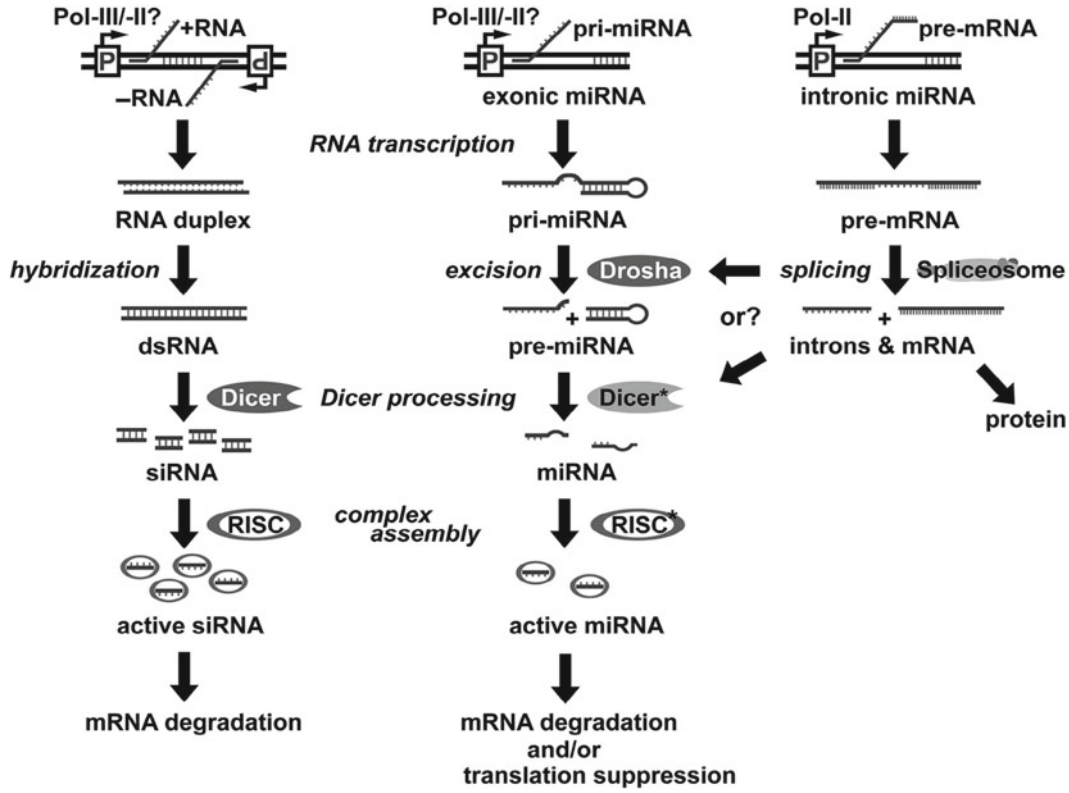

Fig. 1. Comparison of biogenesis and RNA interference (RNAi) mechanisms among small interfering RNA (siRNA), intergenic (exonic) microRNA (miRNA), and intronic miRNA. siRNA is likely formed by two perfectly complementary RNAs transcribed from two different promoters (remains to be determined) and further processing into 19–22-bp duplexes by the ribonuclease (RNase) III familial endonuclease, Dicer. The biogenesis of intergenic miRNAs, e.g., lin-4 and let-7, involves a long transcript primary precursor (pri-miRNA), which is probably generated by RNA polymerase (Pol) II or III RNA promoters, whereas intronic miRNAs are transcribed by the Pol II promoters of its encoded genes and coexpressed in the intronic regions of the gene transcripts (pre-messenger RNA (mRNA)). After RNA splicing and further processing, the spliced intron may function as a pri-miRNA for intronic miRNA generation. In the nucleus, the pri-miRNA is excised by Drosha RNase to form a hairpin-like pri-miRNA template and then exported to the cytoplasm for further processing by Dicer* to form mature miRNAs. siRNA and miRNA pathways are processed by different Dicers. All three small regulatory RNAs are finally incorporated into a RNA-induced silencing complex, which contains either a strand of siRNA or a single strand of miRNA. The effect of miRNA is considered more specific with fewer adverse consequences than that of siRNA because only one strand is involved. However, siRNAs primarily trigger mRNA degradation, whereas miRNAs can induce either mRNA degradation or suppression of protein synthesis depending on the sequence complementarity to the target gene transcripts.

The biogenic process of intronic miRNA presumably involves five steps (Fig. 1). First, miRNA is generated as a long primary precursor miRNA (pri-miRNA) encoded within a gene transcript (pri-mRNA) by Pol II (20, 26). Second, the pre-mRNA is excised by spliceosomal components and/or Drosha-like ribonuclease (RNase) III endonucleases to release hairpin-like intronic structures and form pri-miRNA (20, 27). Third, the pri-miRNA is exported out of the nucleus, probably by Ran–guanosine triphosphate and a receptor Exportin-5 (28, 29). In the cytoplasm,

Dicer-like nucleases cleave the pri-miRNA to form mature miRNA. Lastly, the mature miRNA is incorporated into a ribonuclear particle (RNP), which becomes the RNA-induced gene-silencing complex (RISC), capable of executing RNAi-associated gene-silencing effects (30, 31). Although the in vitro model of siRNA-associated RISC assembly has been studied, the link between the final miRNA maturation processes and RISC assembly remains to be determined.

The characteristics of Dicer and RISC have been reported to be distinct between the siRNA and miRNA mechanisms (32, 33). In zebrafish, we have recently observed that the stem-loop structure of pri-miRNAs is involved in the strand selection for mature miRNA during RISC assembly (21). These findings suggest that the duplex structure of siRNA may be not essential for the assembly of miRNA-associated RISC. Conceivably, a method to distinguish the individual properties and differences between miRNA and siRNA biogenesis would facilitate our understanding of the evolutional and functional relationship between these two RNA-mediated gene-silencing pathways. In addition, the differences may provide an explanation for the prevalence of native siRNAs in invertebrates and their rarity in mammals.

The intronic miRNA must fulfill the following conditions: first, they must share the same promoter with their encoded genes, and second, they are spliced out of the transcript of their encoded genes and further processed into mature miRNAs. Although some of the currently identified miRNAs are encoded in the genomic intron region of a gene but in the opposite orientation to the gene transcript, those miRNAs are not intronic miRNAs because they neither share the same promoter with the gene nor need to be released from the gene transcript by RNA splicing. The promoters of those miRNAs are located in the antisense direction to the gene, likely using the gene transcript as a potential target for the antisense miRNAs. For example, let-7c was found to be an intergenic miRNA located in the antisense region of a gene intron.

More than 90 intronic miRNAs have been identified using bioinformatics approaches (34, 35), but the functions of the vast majority of these intronic molecules remain undetermined. According to the strictly expressive correlation of intronic miRNAs to their encoded genes, one may speculate that the levels of condition-specific, time-specific, and individual-specific gene expressions are determined by interactions of different miRNAs on single or multiple genes. This interpretation accounts for a more accurate genetic expression of various traits, and any deregulation of the interactions, thus, will result in genetic diseases. For instance, monozygotic twins frequently demonstrate slightly, but definitely distinguishing, disease susceptibility and physiological behaviors. For instance, a long CCTG expansion in intron 1 of the zinc finger protein-9 gene has been correlated to type 2 myotonic dystrophy

in one twin with a higher susceptibility (36). Although the expansion motif confers high affinity to certain RNA-binding proteins, the interfering role of intron-derived expansion fragments remains to be elucidated.

Another more-established example, involving intronic expansion fragments in its pathogenesis, is fragile X syndrome, which represents approx 30% of human inherited mental retardation. An intronic CGG repeat (rCGG) expansion in the 5′-UTR of the FMR1 gene causes mutation in 99% of individuals with fragile X syndrome (37). FMR1 encodes an RNA-binding protein, fragile X mental retardation protein, which is associated with polyribosome assembly in an RNP-dependent manner, and is capable of suppressing translation through an RNAi-like pathway. Fragile X mental retardation protein also contains a nuclear localization signal and a nuclear export signal for shuttling certain mRNAs between the nucleus and cytoplasm (38). Jin et al. proposed an RNAi-mediated methylation model in the CpG region of the FMR1 rCGG expansion, which is targeted by a hairpin RNA derived from the 3′-UTR of the FMR1 expanded allele transcript (37). The Dicer-processed hairpin RNA triggers the formation of RNA-induced initiator of transcriptional gene silencing on the homologous rCGG sequences and leads to heterochromatin repression of the FMR1 locus. These examples suggest that natural evolution gives rise to more complexity and more variety of introns in higher animals and plants for coordinating their vast gene expression volumes and interactions; therefore, any deregulation of miRNAs derived from introns may lead to genetic diseases involving intronic expansion or deletion, such as myotonic dystrophy and fragile X mental retardation.

To understand diseases caused by the deregulation of intronic miRNAs, an artificial expression system is needed to recreate the function and mechanism of the miRNA in vitro and in vivo. The same approach may be used to design and develop therapies for various intronic miRNA-related diseases. Using artificial introns carrying hairpin-like pri-miRNA, we successfully generated mature miRNA molecules with a full capacity to trigger RNAi-like gene silencing in human prostate cancer (LNCaP), human cervical cancer (HeLa), and rat neuronal stem (HCN-A94-2) cells (20, 39).

The artificial intron of Fig. 2a was located in a mutated HcRed1 red fluorescent membrane protein (rGFP) gene to form a recombined splicing-competent RNA intron (SpRNAi)–rGFP gene, in which the functional fluorescent structure was disrupted by the SpRNAi insertion. Thus, we were able to determine the occurrence of intron splicing and rGFP–mRNA maturation through the appearance of red fluorescent emission on the membranes of transfected cells. There is no homology or complementarity between the SpRNAi–rGFP gene and its expression vectors. After transfection of SpRNAi–rGFP genes containing synthetic inserts

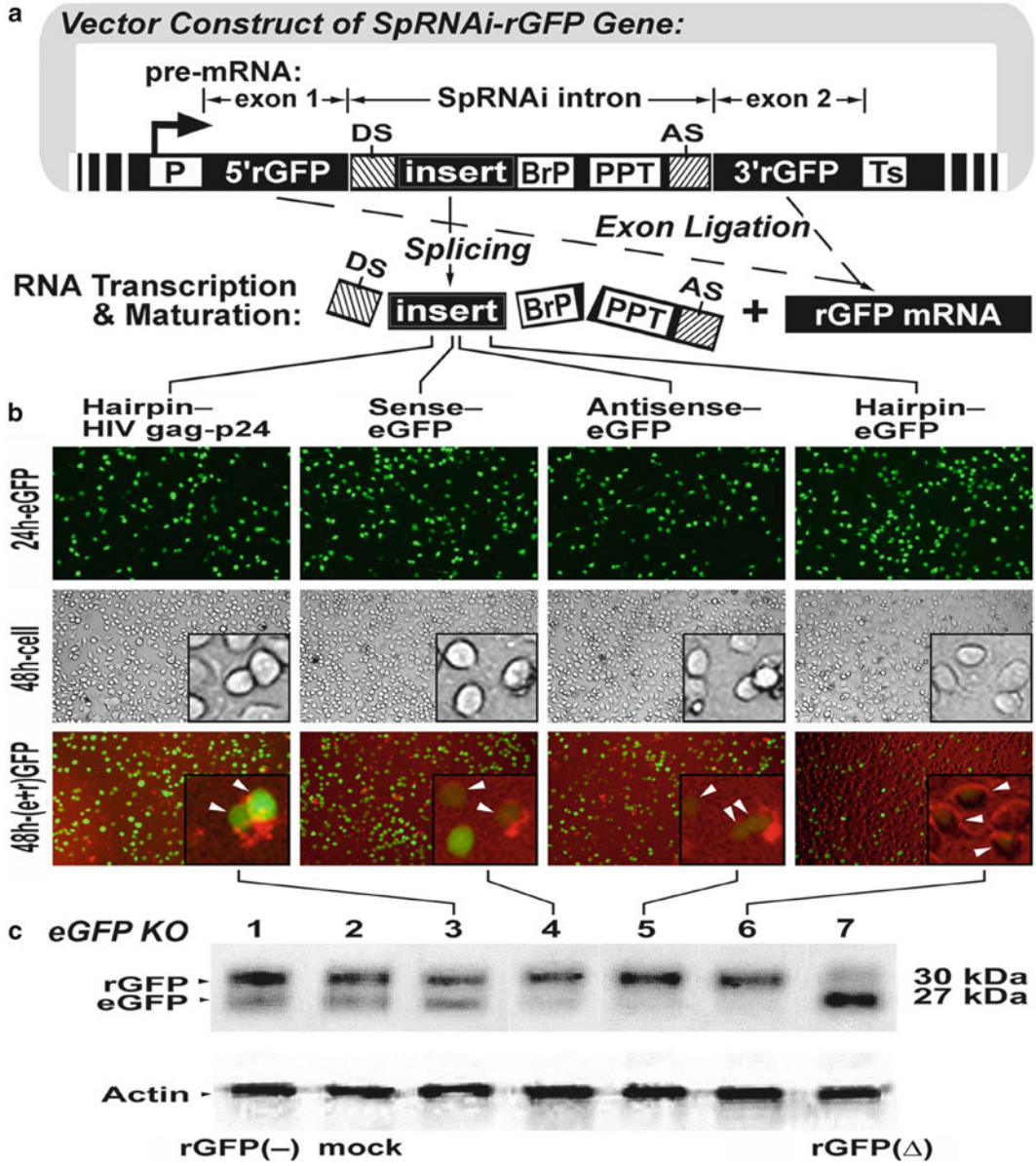

Fig. 2. Strategy for analysis of intronic miRNA mechanisms using artificial splicing-competent RNA intron (SpRNAi)–red fluorescent membrane protein (rGFP) gene vectors. (**a**) The *SpRNAi–rGFP* gene consists of a 5′-RNA promoter (P), an artificial intron (SpRNAi) flanked with two red fluorescent proteins (rGFP) exon fragments, and a 3′-proximity of transcription and translation termination codons (Ts). The construction of SpRNAi includes a 5′-splice donor site (DS), a 3′-splice acceptor site (AS), a poly-pyrimidine tract (PPT), a branch-point domain (BrP), and an inserted pri-miRNA oligonucleotide (insert) in the 5′-proximity of SpRNAi between the DS and BrP sites. During messenger RNA (mRNA) maturation, the SpRNAi is spliced out of the *SpRNAi–rGFP* pre-mRNA and further processed into miRNAs for gene silencing, whereas the mature rGFP mRNA is translated into rGFP for the target identification. (**b**) Simultaneous expression of rGFP and silencing of *Aequorea victoria* green fluorescent protein (eGFP) by various *SpRNAi–rGFP* transfections. At 24 h after transfection, approximate total cell numbers and eGFP-positive cell populations were observed with very few apoptotic or differentiated cells,

homologous to a targeted gene exon, we found that a hairpin insert comprising both sense and antisense exon strands resulted in maximal effects of gene silencing.

As shown in Fig. 2, the transfection of various SpRNAi–rGFP genes targeting the nucleotides 279–303 open-reading frame region of the enhanced Aequorea victoria green fluorescent protein (eGFP) was found to be highly significant ($n = 4$; $p < 0.01$) in silencing eGFP protein expression. The use of eGFP-positive HCN-A94-2 rat neuronal stem cells offered an excellent visual aid to observe the decreased green fluorescent emission of eGFP in the red fluorescent rGFP reporter gene-expressing cells. Silencing of eGFP was detected 42–48 h after transfection, indicating a potential requirement for precise timing of the production of sufficient small interfering intron inserts from the SpRNAi–rGFP gene.

Quantitative knock-down levels of eGFP protein were significantly altered (Fig. 2b), and there were modest reduction rates of 56 ± 6% for the transfection of inserts homologous to the sense strand of the eGFP target, of 50 ± 4% for the antisense strand of the GFP target, and a significant rate of 81 ± 2% for the hairpin inserts containing both strands of the eGFP target. No knock-down specificity to eGFP was detected by the transfection of intron-free rGFP gene, or for the SpRNAi–rGFP gene containing hairpin inserts homologous to either integrin-β1 exon 1 or to the human immunodeficiency virus (HIV)-1 gag-p24 gene. The Western blot results shown in Fig. 2c confirmed the knock-down regulation observed and demonstrated that such a gene-silencing effect is determined by the hairpin structures of the pri-miRNA inserts.

The intron-derived miRNA system is able to be activated in a specific cell type under the control of a Pol II-directed transcriptional machinery. Our research group was the first to discover the biogenesis of miRNA-like precursors from the 5′-proximal intron regions of gene transcripts (pre-mRNAs) produced by the mammalian Pol II. Depending on the promoter of the miRNA-encoded gene transcript, intronic miRNA is coexpressed with its encoding gene in the specific cell population, which activates the promoter

Fig. 2. (continued) whereas no detectable silencing of eGFP occurred. The RNAi effect was detected 42 h after transfection, showing that the gene knock-down potency of the *SpRNAi–rGFP* genes containing inserts homologous to hairpin-eGFP was much greater than the sense-eGFP, which was approximately equal to the antisense-eGFP, which was much greater than the hairpin-human immunodeficiency virus (HIV) p24 (negative controls). (**c**) Western blot analyses confirmed the knock-down potency of (B). The lanes from left to right indicate the *SpRNAi–rGFP* transfection with genes containing various inserts homologous to the open-reading frame of eGFP, namely: 1, rGFP(–) (blank controls); 2, hairpin-integrin-β1 exon 1 (negative controls); 3, hairpin-HIV gag-p24; 4, sense-eGFP; 5, antisense-eGFP; 6, hairpin-eGFP; and 7, rGFPΔ (DS-defective controls).

and expresses the gene. This type of miRNA generation relies on the coupled interaction of nascent Pol II-mediated pre-mRNA transcription and intron excision, occurring within certain nuclear regions proximal to genomic perichromatin fibrils (4, 20, 40, 41).

After Pol II RNA processing and splicing excision, some of the intron-derived miRNA fragments can form mature miRNAs and effectively silence the target genes through the RNAi mechanism, whereas the exons of pre-mRNA are ligated together to form a mature mRNA for protein synthesis (4, 20). Because miRNAs are single-stranded molecules insensitive to double-stranded RNA-dependent protein kinase R (PKR) and 2′,5′-oligoadenylate synthetase (2-5A)-induced interferon systems, the use of this Pol II-mediated miRNA generation can be safe in vitro and in vivo, preventing the cytotoxic effects of double-stranded RNAs (dsRNAs) and siRNAs. Interferon-induced protein kinase PKR can trigger cell apoptosis, whereas activation of the interferon-induced 2-5A system leads to extensive cleavage of single-stranded RNAs (i.e., mRNAs) (42, 43). Although both the PKR and the 2-5A systems contain dsRNA-binding motifs that are highly conserved for binding to dsRNAs, these motifs do not bind to either single-stranded RNAs or RNA–DNA hybrids. These findings indicate a new function for mammalian introns in intracellular miRNA generation and gene regulation, which can be used as a tool for analysis of gene functions, improvement of current RNAi technology, and development of gene-specific therapeutics against cancers and viral infections.

The components of the Pol II-mediated SpRNAi system include several consensual nucleotide elements: a 5′-splice site, a branch-point domain, a poly-pyrimidine tract (PPT), and a 3′-splice site (Fig. 2a). Additionally, a pri-miRNA insert-sequence is placed within the artificial intron between the 5σψ-splice site and the branch-point domain. This portion of the intron would normally form a lariat structure during RNA splicing and processing. We currently know that spliceosomal U2 and U6 small nuclear RNPs, both helicases, may be involved in the unwinding and excision of the lariat RNA fragment into pri-miRNA; however, the detailed processing remains to be elucidated. Further, the SpRNAi contains translation stop codon domains in its 3′-proximal region to facilitate the accuracy of RNA splicing, which, if present in a cytoplasmic mRNA, would signal the diversion of a splicing-defective pre-mRNA to the nonsense-mediated decay pathway and, thus, cause the elimination of any unspliced pre-mRNA in the cell.

For intracellular expression of the SpRNAi, we need to insert the SpRNAi construct into the DraII cleavage site of an rGFP gene from mutated chromoproteins of coral reef Heteractis crispa. The cleavage of rGFP at its 208th nucleotide site by the restriction enzyme, DraII, generates an AG–GN nucleotide break, with three recessing nucleotides in each end, which forms 5′ and 3′ splice

sites, respectively, after the SpRNAi insertion. Because this intronic insertion disrupts the expression of functional rGFP, it becomes possible to determine the occurrence of intron splicing and rGFP–mRNA maturation via the appearance of red fluorescent emission around the membrane surface of the transfected cells. The rGFP also provides multiple exonic splicing enhancers to increase RNA-splicing efficiency.

To test the requirement of an siRNA-like duplex construct in miRNA-associated RISC (miRISC) assembly, the pri-miRNAs were designed to contain perfectly matched stem arm domains. Although most of the native pri-miRNAs contain a mismatched area in their stem arms, it is not necessary for us to construct an imperfectly paired stem arm to trigger RNAi-related gene silencing. Previous studies have demonstrated that a mature miRNA can be generated by placing a perfectly matched siRNA duplex in the miR-30 pri-miRNA structure (27, 44). Further, there are many genes not subjected to the regulation of native miRNAs, in particular, eGFP, which can be otherwise silenced by intracellular transfection of a pri-miRNA containing a perfectly matched stem arm construct. Therefore, we define a mature miRNA based on its biogenetic function and mechanism, rather than the structural complementarity of its precursor. In this view, any small hairpin RNA can be a pri-miRNA, if a mature miRNA is successfully processed from the small hairpin RNA and further assembled into miRISC for target gene silencing.

This designed miRNA system has been tested in zebrafish, establishing the fact that intronic miRNAs can be used as an effective strategy to silence specific target genes in vivo. We first tried to resolve the structural design of pri-miRNA inserts for the best gene-silencing effect and found that a strong structural bias exists in the selection of a mature miRNA strand during assembly of the RNAi effector, RISC. RISC is a protein–RNA complex that directs either target gene transcript degradation or translational repression through the RNAi mechanism. Formation of siRNA duplexes has been reported to play a key role in assembly of the siRNA-associated RISC. The two strands of the siRNA duplex are functionally asymmetric, but assembly into the RISC complex is preferential for only one strand. Such preference is determined by the thermodynamic stability of each 5′-end base pairing in the strand.

Based on this siRNA model, the formation of miRNA and its complementary miRNA (miRNA*) duplexes was thought to be an essential step for the assembly of miRISC. If this were true, no functional bias would be observed in the stem-loop of a pri-miRNA. Nevertheless, we observed that the stem-loop of the intronic pri-miRNA was involved in the strand selection of a mature miRNA for RISC assembly in zebrafish. In these experiments, we constructed miRNA-expressing SpRNAi–rGFP vectors, as previously described (4, 20); and two symmetric

pri-miRNAs, miRNA–stem-loop–miRNA* (0) and miRNA* –stem-loop–miRNA (8), were synthesized and inserted into the vectors, respectively. Both pri-miRNAs contained the same double-stranded stem arm region, which was directed against the eGFP nucleotide 280–302 sequence. Because the intronic insert region of the SpRNAi–rGFP recombined gene is flanked with a PvuI and an MluI restriction site at the 5′- and 3′-ends, respectively, the primary insert can be easily removed and replaced by various gene-specific inserts (e.g., anti-eGFP) possessing cohesive ends. By changing the pri-miRNA inserts directed against different gene transcripts, this intronic miRNA generation system provides a valuable tool for genetic and miRNA-associated research in vivo.

To determine the structural preference of the designed pri-miRNAs, we isolated the zebrafish small RNAs by mirVana® miRNA isolation columns (Ambion, Austin, TX) and precipitated all potential miRNAs complementary to the target eGFP region by latex beads containing the target RNA sequence. One effective miRNA identity, miR-eGFP (280/302), was verified in the transfections of the 5′-miRNA–stem-loop–miRNA*-3′ construct, as shown in Fig. 3a (gray-shading sequences). Because the effective mature miRNA was detected only in the zebrafish when transfected by the 5′-miRNA–stem-loop–miRNA*-3′ construct, the miRISC seems to prefer interacting with the 8 construct rather than the 0 pri-miRNA. The eGFP expression was constitutively driven by the β-actin promoter located in almost all zebrafish cells, whereas Fig. 3b shows that transfection of the SpRNAi–rGFP vector into the transgenic (UAS:gfp) zebrafish coexpressed rGFP, serving as a positive indicator for the miRNA generation in the transfected cells. This approach has been successfully used in several mouse and human cell lines to demonstrate RNAi effects (20, 39).

We applied the liposome-capsulated vector (total 60 μg) to the fish and found that the vector easily penetrated almost all tissues of the 2-week-old zebrafish larvae within 24 h, achieving fully systemic delivery of the miRNA effect. The indicator rGFP was detected in all of the fishes transfected by either 5′-miRNA*–stem-loop–miRNA-3′ or 5′-miRNA–stem-loop–miRNA*-3′ premiRNA, whereas the silencing of target eGFP expression (green) was observed only in the fishes transfected by the 5′-miRNA–stem-loop–miRNA*-3′ pri-miRNA (Fig. 3b, c).

The suppression level of eGFP in the gastrointestinal tract was found to be less effective, probably because of the high RNase activity in this region. Switching the stem-loop position has changed the thermostability of the 5′-end of the siRNA-like stem arm, resulting in different miRNA maturation patterns; thus, we suggest that the stem-loop of a premiRNA may be involved in Dicer recognition and strand selection of a mature miRNA for

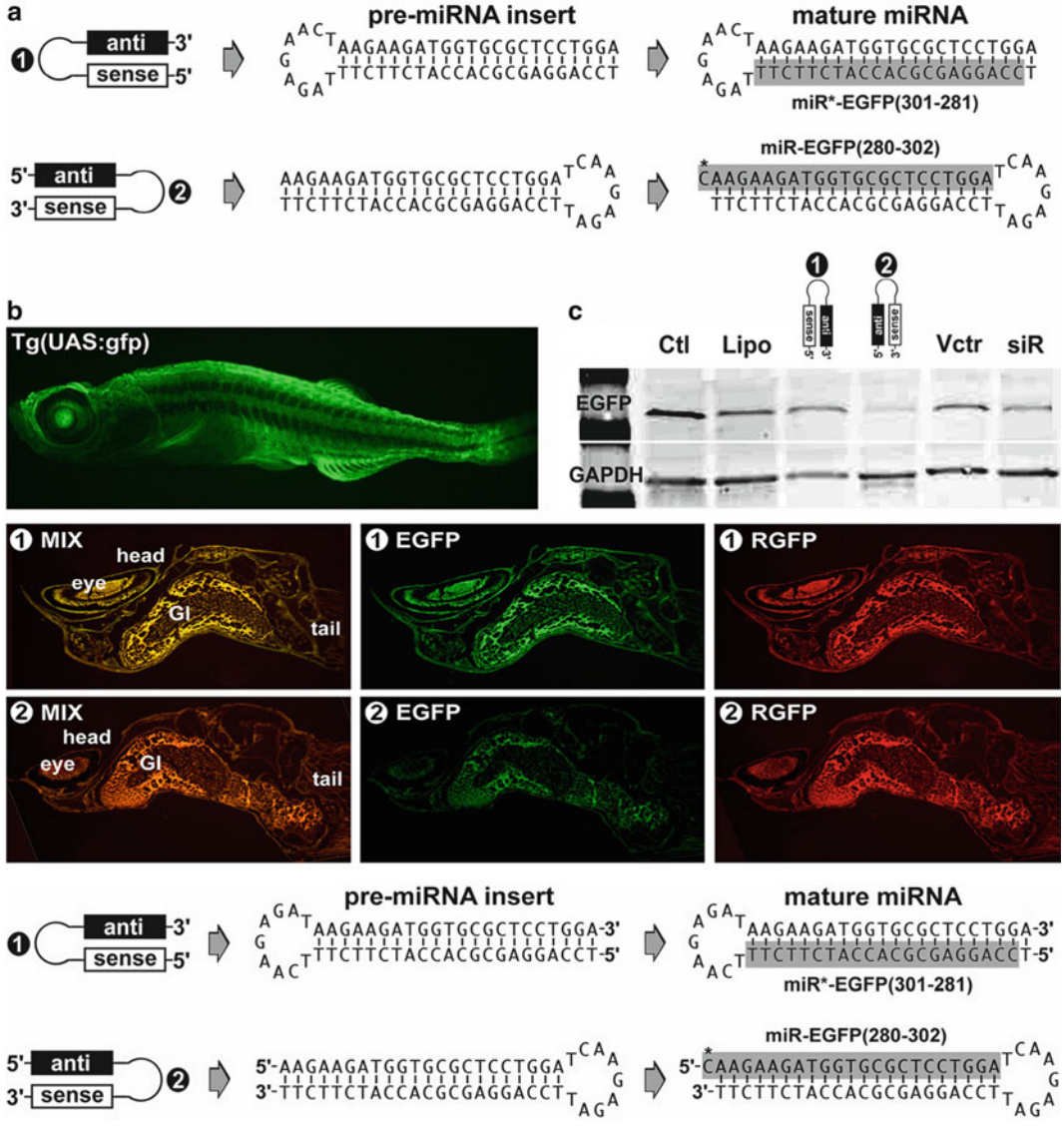

Fig. 3. Structural preference of miRNA–miRNA* asymmetry in miRNA-induced gene silencing complex (RISC) in vivo. Different preferences of RISC assembly were observed by transfection of 5′-miRNA*-stem-loop-miRNA-3′ (❶) and 5′-miRNA-stem-loop-miRNA*-3′ (❷) pri-miRNA constructs in zebrafish, respectively. (**a**) Based on the RISC assembly rule of siRNA, the processing of both ❶ and ❷ should result in the same siRNA duplex for RISC assembly; however, the experiments demonstrate that only the ❷ construct was used in RISC assembly for silencing target EGFR. Due to the fact that miRNA is predicted to be complementary to its target messenger RNA, the "antisense" (*black bar*) refers to the miRNA and the "sense" (*white bar*) refers to its complementarity, miRNA*. One mature miRNA, namely miR-eGFP-(280/302), was detected in the ❷-transfected zebrafishes, whereas the ❶ transfection produced different miRNA: miR*-EGFR(301–281), which was partially complementary to the miR-eGFP(280/320). (**b**) In vivo gene silencing efficacy was only observed in the transfection of the ❷ pri-miRNA construct, but not the ❶ construct. Because the color combination of EGFP and RGFP displayed more red than green (as shown in *deep orange*), the expression level of target EGFP (*green*) was significantly reduced in ❷, while miRNA indicator RGFP (*red*) was evenly present in all vector transfections. (**c**) Western blot analysis of the EGFP protein levels confirmed the specific silencing result of (**b**). No detectable gene silencing was observed in fishes without (Ctl) and with liposome only (Lipo) treatments. The transfection of either a U6-driven siRNA vector (siR) or an empty vector (Vctr) without the designed pri-miRNA insert resulted in no gene silencing significance.

effective RISC assembly and the resultant gene silencing. Given that the cleavage site of Dicer in the stem arm determines the strand selection of mature miRNA (27), the stem-loop may function as a determinant for the recognition of specific cleavage sites. Therefore, different from the dual open-ends of siRNA, a hairpin-like pri-miRNA has the advantage of using its stem-loop structure to control the asymmetry of miRNA maturation for more efficient RISC assembly.

Consistent evidence of miRNA-induced gene-silencing effects in mammalian cell lines and zebrafish demonstrates the preservation of an ancient intron-mediated gene regulation system in eukaryotes. In these in vitro and in vivo models, the intron-derived miRNAs determine the activation of RNAi-associated gene-silencing pathways. We herein provide the first evidence for the biogenesis and function of intronic miRNAs, both in vitro and in vivo. Evolution gives rise to more complexity and more variety of introns in higher animal and plant species for coordinating their vast gene expression volumes and interactions. Deregulation of these miRNAs due to intronic expansion or deletion could be the likely cause of various genetic diseases, such as myotonic dystrophy and fragile X mental retardation. Gene expression produces not only a gene transcript for its own protein synthesis but also for intronic miRNAs, capable of interfering with the expression of other genes. Thus, the expression of a particular gene may result in the gain of function of that particular gene but the consequent loss of function of other genes that are complementary to the mature intronic miRNAs. In volatile environments, an array of genes can swiftly and accurately coordinate their expression patterns with each other through the mediation of their intronic miRNAs, bypassing time-consuming translation processes.

Conceivably, intron-mediated gene regulation may be as important as the mechanisms by which transcription factors regulate the gene expression. It is likely that intronic miRNA is able to trigger cell transitions quickly in response to external stimuli without tedious protein synthesis. Undesired gene products are reduced by both transcriptional inhibition and/or translational suppression via miRNA regulation. This could enable a rapid switch to a new gene expression pattern without the need to produce various transcription factors. This regulatory property of miRNAs may serve as one of the most ancient gene modulation systems before the emergence of proteins. With the vast variety of miRNAs and the complexity of genomic introns, a thorough investigation of miRNA variants in the human genome will markedly improve the understanding of genetic diseases and improve the design of miRNA-based drugs. Learning how to exploit such a novel gene regulation system for future therapies will be a forthcoming challenge.

2. Materials

2.1. Synthetic Oligonucleotides Used for SpRNAi–rGFP Gene Construction

1. Sense SpRNAi sequence: 5′-dephosphorylated GTAAGTGGTC CGATCGTCGC GACG CGTCAT TACTAACACTAT CAT ACTTATC CTGTCCCTTT TTTTTCCACA GCTAG GAC CT TCGTGCA-3′ (100 pmol/μL in autoclaved ddH_2O).
2. Antisense SpRNAi sequence: 5′-phosphorylated TGCACGAA GG TCCTAGCTGT GGA AAAAAAA GGGACAGGAT AAG TATGATA GTTAGTAATG ACGCGTCGCG ACG ATCGG AC CACTTAC-3′ (100 pmol/μL in autoclaved ddH_2O).
3. 2× Hybridization buffer: 200 mM KOAc, 60 mM HEPES-KOH, 4 mM MgOAc, pH 7.4 at 25°C.
4. 0.5 μg/μL pHcRed1-N1/1 plasmid vector (BD Biosciences, Palo Alto, CA).
5. Incubation chambers at 94°C, 65°C, and 4°C.

2.2. Restriction Enzyme Digestion and Sequential Ligation With Cohesive Ends

1. 10× L buffer: 100 mM Tris–HCl, pH 7.5 at 37°C; 100 mM $MgCl_2$; and 10 mM dithiothreitol (DTT).
2. Restriction enzymes, including DraII, BfrI, NheI, and BsmI.
3. DraII digestion reaction mix: 14 μL autoclaved ddH_2O, 4 μL of 10× L buffer, and 2 μL DraII; prepare the reaction mix just before use.
4. DraII/BfrI digestion reaction mix: 2 μL autoclaved ddH_2O, 4 μL of 10× L buffer, 2 μL DraII, and 2 μL BfrI; prepare the reaction mix just before use.
5. 10× Ligation buffer: 660 mM Tris–HCl, pH 7.5 at 20°C; 50 mM $MgCl_2$; 50 mM DTT; and 10 mM adenosine triphosphate (ATP).
6. 5 U/μL T4 DNA ligase.
7. Ligation reaction mix: 4 μL autoclaved ddH_2O, 4 μL of 10× ligation buffer, and 2 μL T4 ligase; prepare the reaction mix just before use.
8. 10× M buffer: 100 mM Tris–HCl, pH 7.5 at 37°C; 500 mM NaCl; 100 mM $MgCl_2$; and 10 mM DTT.
9. NheI digestion reaxtion mix: 4 μL autoclaved ddH_2O, 4 μL of 10× M buffer, and 2 μL NheI; prepare the reaction mix just before use.
10. 10× H buffer: 500 mM Tris–HCl, pH 7.5 at 37°C; 1 M NaCl; 100 mM $MgCl_2$; and 10 mM DTT.
11. BsmI digestion reaction mix: 4 μL autoclaved ddH_2O, 4 μL of 10× H buffer and 2 μL BsmI; prepare the reaction mix just before use.

12. 10 U/μL T4 polynucleotide kinase.
13. Ligation/phosphorylation reaction mix: 2 μL autoclaved ddH_2O, 4 μL of 10× ligation buffer, 2 μL T4 ligase, and 2 μL T4 polynucleotide kinase; prepare the reaction mix just before use.
14. Incubation chambers at 65°C, 37°C, 16°C, and 4°C.
15. 1% Agarose gel electrophoresis.
16. Gel extraction kit (Qiagen, Valencia, CA).
17. Microcentrifuge: 17,900 × *g*.

2.3. Cloning of the SpRNAi–rGFP Gene Construct

1. 10× H buffer: 500 mM Tris–HCl, pH 7.5 at 37°C; 1 M NaCl; 100 mM $MgCl_2$; and 10 mM DTT.
2. Restriction enzymes, including XhoI and XbaI.
3. XhoI/XbaI digestion reaction mix: 2 μL autoclaved ddH_2O, 4 μL of 10× H buffer, 2 μL XhoI, and 2 μL XbaI; prepare the reaction mix just before use.
4. 10× Ligation buffer: 660 mM Tris–HCl, pH 7.5 at 20°C; 50 mM $MgCl_2$; 50 mM DTT; and 10 mM ATP.
5. 5 U/μL T4 DNA ligase.
6. Ligation reaction mix: 4 μL autoclaved ddH_2O, 4 μL of 10× ligation buffer, and 2 μL T4 ligase; prepare the reaction mix just before use.
7. Low salt Luria-Bertani culture broth.
8. Expand cloning kit (Roche Diagnostics, Indianapolis, IN).
9. DH5α transformation-competent *Escherichia coli* cells (Roche).
10. 10× $MgSO_4$ solution: 1 M $MgSO_4$.
11. 1× $CaCl_2$ solution: 0.1 M $CaCl_2$.
12. 10× Glucose solution: 1 M glucose.
13. Incubation shaker: 37°C; 285 rpm vortex.
14. Incubation chambers: 37°C, 16°C, and 4°C.
15. Luria-Bertani agar plate containing 50 mg/mL kanamycin.
16. Spin Miniprep kit (Qiagen).
17. Microcentrifuge: 17,900 × *g*.

2.4. Insertion of Pri-miRNA Into the SpRNAi–rGFP Gene Construct

1. Sense pri-miRNA sequence: 5′-GTCCGATCGT CAAGAA GATG GTGCGCTCCT GGA TCAAGAG ATTCCAGGAG CGCACCATCT TCTTCGACGC GTCAT-3′ (100 pmol/μL in autoclaved ddH_2O).
2. Antisense pri-miRNA sequence: 5′-ATGACGCGTC GAAGA AGATG GTGCGCTCCT GGAATCTCTT GATCCAGGAG CGCACCATCT TCTTGACGAT CGGAC-3′ (100 pmol/μL in autoclaved ddH_2O).

3. 2× Hybridization buffer: 200 mM KOAc, 60 mM HEPES-KOH, and 4 mM MgOAc, pH 7.4 at 25°C.
4. 10× H buffer: 500 mM Tris–HCl, pH 7.5 at 37°C; 1 M NaCl; 100 mM $MgCl_2$; and 10 mM DTT.
5. Restriction enzymes, including PuvI and MluI.
6. PuvI/MluI digestion reaction mix: 2 μL autoclaved ddH_2O, 4 μL of 10× H buffer, 2 μL PuvI, and 2 μL MluI; prepare the reaction mix just before use.
7. 10× Ligation buffer: 660 mM Tris–HCl, pH 7.5 at 20°C; 50 mM $MgCl_2$; 50 mM DTT; and 10 mM ATP.
8. 5 U/μL T4 DNA ligase.
9. Ligation reaction mix: 4 μL autoclaved ddH_2O, 4 μL of 10× ligation buffer, and 2 μL T4 ligase; prepare the reaction mix just before use.
10. Incubation chambers: 65°C, 37°C, and 16°C.
11. 1% Agarose gel electrophoresis.
12. Gel extraction kit (Qiagen).
13. Microcentrifuge: 17,900×*g*.

2.5. Liposomal Transfection of the SpRNAi–rGFP Gene Construct

1. RPMI-1640 cell culture medium, serum-free.
2. FuGENE transfection reagent (Roche).
3. Cell culture incubator.

3. Methods

3.1. Synthetic Oligonucleotides Used for SpRNAi–rGFP Gene Construction

The SpRNAi artificial intron is formed by hybridization of the sense and antisense SpRNAi sequences, which are synthesized to be perfectly complementary to each other. Both of the SpRNAi sequences must be purified by polyacrylamide gel electrophoresis (PAGE) before use and stored at –20°C.

1. Hybridization: mix the sense and antisense SpRNAi sequences (5 μL for each sequence) in 10 μL of 2× hybridization buffer, heat to 94°C for 3 min, and cool to 65°C for 10 min. Stop the reaction on ice.

3.2. Restriction Enzyme Digestion and Sequential Ligation with Cohesive Ends

Two rGFP exons are provided by DraII cleavage of the pHcRed1-N1/1 plasmid vector between the 881st and 882nd nucleotide site, forming an AG–GN nucleotide break with 5′-G(T/A)C protruding nucleotides in the cleaved ends. The 5′-GTC protruding nucleotides need to be removed from the end of the first exon for blunt-end ligation, whereas the 5′-GAC protruding end of the second exon is used to ligate with the 3′-DraII-restricted end of the

SpRNAi intron. After the ligation of the SpRNAi intron and the second rGFP exon, add the first rGFP exon to the 5′ end of the ligated sequence by blunt-end ligation, so as to form a complete SpRNAi–rGFP gene cassette (see Note 1).

1. DraII cleavage: add the DraII digestion reaction mix to the SpRNAi hybrid. Add the DraII/BfrI digestion reaction mix to 30 μL of the pHcRed1-N1/1 plasmid vector. Incubate both of the reactions at 37°C for 4 h and stop the reaction on ice.
2. Purification of the DraII- and DraII/BfrI-digested sequences: load and run the above reactions from step 1 in 1% agarose gel electrophoresis and cut out of the DraII-digested SpRNAi hybrid sequence and two other oligonucleotide fragments (one 1,760 bp and another 715 bp), which are derived from the DraII/BfrI-cleaved pHcRed1-N1/1 plasmid vector. Separately recover these three oligonucleotide sequences into different tubes in 30 μL autoclaved ddH2O, using the gel extraction columns and following the manufacturer's suggestions. Store the 1,760-bp pHcRed1-N1/1 fragment at 4°C for 2 week before use (see Note 2).
3. Ligation: mix 15 μL of the DraII-digested SpRNAi hybrid sequence with 15 μL of the 715 bp pHcRed1-N1/1 fragment and add the ligation reaction mix. Incubate the reaction at 16°C for 16 h and stop the reaction on ice.
4. Purification of the ligation product: load and run the ligation in 1% agarose gel electrophoresis and cut out the ligated sequence (~800 bp) using a clean surgical blade. Recover the sequence in one tube of 30 μL autoclaved ddH2O, using the gel extraction column and following the manufacturer's suggestions.
5. Cleavage by NheI and BsmI: add the BsmI digestion reaction mix to the ligation product. Add the NheI digestion reaction mix to the 1,760-bp pHcRed1-N1/1 fragment. Incubate both of the reactions at 37°C for 4 h and stop the reaction on ice.
6. Purification of the NheI and BsmI-digested sequences: load and run the reactions in 1% agarose gel electrophoresis and cut out the NheI- and BsmI-digested sequences, respectively, using a clean surgical blade. Recover the two oligonucleotide sequences in one tube of 30 μL autoclaved ddH2O, using the gel extraction columns and following manufacturer's suggestions.
7. Ligation: add the ligation/phosphorylation reaction mix to the extraction. Incubate the reaction at 16°C for 16 h and stop the reaction on ice.
8. Purification of the ligation product: load and run the ligation in 1% agarose gel electrophoresis and cut out the ligated

sequences using a clean surgical blade. Recover the sequence in one tube of 30 μL autoclaved ddH_2O, using the gel extraction column and following the manufacturer's suggestions. The final ligation product forms the SpRNAi–rGFP gene cassette (see Note 1).

3.3. Cloning of the SpRNAi–rGFP Gene Construct

To express the SpRNAi–rGFP gene in transfected cells, clone the SpRNAi–rGFP gene cassette into the pHcRed1-N1/1 plasmid vector, replacing the original HcRed protein sequence. Because the functional fluorescent structure of HcRed is disrupted by the SpRNAi intron insertion, one can determine the occurrence of intron splicing and miRNA maturation through the appearance of red fluorescent emission on the cell membranes. The red rGFP serves as a visual indicator for the generation of intronic miRNAs. This intron-derived miRNA system is activated under the control of cytomegalovirus-IE promoter.

1. Cleavage by XhoI and XbaI: add the XhoI/XbaI digestion reaction mix to the SpRNAi–rGFP gene cassette and the pHcRed1-N1/1 plasmid vector, respectively. Incubate both of the reactions at 37°C for 4 h and stop the reactions on ice.
2. Purification of the XhoI/XbaI-digested sequences: load and run the reactions in 1% agarose gel electrophoresis and cut out the XhoI/XbaI-digested SpRNAi–rGFP sequence and the 4,000-bp pHcRed1-N1/1 fragment, respectively, using a clean surgical blade. Recover the two oligonucleotide sequences in one tube of 30 μL autoclaved ddH2O, using the gel extraction column and following the manufacturer's suggestions.
3. Ligation: add the ligation reaction mix to the extraction. Incubate the reaction at 16°C for 16 h and stop the reaction on ice.
4. Plasmid amplification: transfect the ligation product into the DH5α transformation-competent *E. coli* cells using the expand cloning kit and following the manufacturer's suggestions.
5. Plasmid recovery: isolate and collect the amplified SpRNAi–rGFP plasmid in one tube of 30 μL autoclaved ddH2O, using a spin Miniprep filter and following the manufacturer's suggestions.

3.4. Insertion of Pri-miRNA Into the SpRNAi–rGFP Gene Construct

The SpRNAi–rGFP vector does not contain any intronic pri-miRNA structure. Because the intronic insert region of the SpRNAi–rGFP vector is flanked with a PvuI and an MluI restriction site at the 5′ and 3′ ends, respectively, the primary insert can be easily removed and replaced by various gene-specific inserts (e.g., anti-eGFP) possessing cohesive ends (see Note 3).

1. Hybridization: mix the sense and antisense pri-miRNA sequences (5 μL for each sequence) in 10 μL of 2× hybridization

buffer, heat to 94°C for 3 min, and cool to 65°C for 10 min. Stop the reaction on ice.

2. Cleavage by MluI and PvuI: add the MluI/PvuI digestion reaction mix to the SpRNAi–rGFP vector and the pri-miRNA hybrid construct, respectively. Incubate the reaction at 37°C for 4 h and stop the reaction on ice.
3. Purification of the MluI/PvuI-digested sequences: load and run the reactions in 1% agarose gel electrophoresis and cut out the MluI/PvuI-digested SpRNAi–rGFP sequence and the pri-miRNA fragment, respectively, using a clean surgical blade. Recover the two oligonucleotide sequences in one tube of 30 μL autoclaved ddH2O, using the gel extraction column and following the manufacturer's suggestions.
4. Ligation: add the ligation reaction mix to the extraction. Incubate the reaction at 16°C for 16 h and stop the reaction on ice.
5. Plasmid amplification: transfect the ligation product into the DH5α transformation-competent *E. coli* cells using the expand cloning kit and following the manufacturer's suggestions.
6. Plasmid recovery: isolate and collect the amplified SpRNAi–rGFP plasmid in one tube of 30 μL autoclaved ddH2O, using a spin Miniprep filter and following the manufacturer's suggestions (Note 4).

3.5. Liposomal Transfection of the SpRNAi–rGFP Gene Construct

To increase transfection efficiency, we use liposomal reagents to facilitate the delivery of the *SpRNAi–rGFP* vector into target cells.

1. Preparation of FuGENE: add 6 μL of the FuGENE reagent into 100 μL of RPMI-1640 medium in a clean tube and gently mix the solution, following the manufacturer's suggestions. Add 20 μg (in less than 50 μL) of the *SpRNAi–rGFP* vector into the liposomal dilution from Subheading 3.4, **step 6**, and gently mix the solution following the manufacturer's suggestions. Store the mixture at 4°C for 30 min.
2. Transfection: add the mixture into the center of the cell culture and gently mix the cell culture medium.
3. Cell culture: culture the treated cells in a cell culture incubator under the condition essential for the cell type.

4. Notes

1. Because of the low efficiency of blunt-end ligation and 5′-nucleotide hydrolysis of the first *rGFP* exon, the chance to obtain a correct *SpRNAi–rGFP* gene sequence is approx 1 in 50 (2%).

The final *SpRNAi–rGFP* gene sequence must be confirmed by DNA sequencing.

2. Because there is no enzymatic method to remove the 5′-protruding trinucleotide of the first *rGFP* exon, we need to use hydrolysis, which takes approx 2–3 week to remove three nucleotides from the end of an oligonucleotide sequence.
3. The synthetic pri-miRNA sequences that we present here are directed against the 279–303-nt region of enhanced eGFP. The principle for designing an intronic pri-miRNA insert is to synthesize two mutually complementary oligonucleotides, including one 5′-GTCCG.

   ATCGTC, 19–27-nt antisense target gene sequence—TCAAGAGAT (stem-loop)—19–27-nt sense target gene sequence, CGACGCGTCAT-3′; and another 5′-ATGACGGTCG, 19–27-nt antisense target gene sequence—ATCTCTTGA (stem-loop)—19–27-nt sense target gene sequence, GACGATCGGAC-3′. The hybridization of these two oligonucleotide sequences forms the intronic pri-miRNA insert, which contains a 5′-PuvI and a 3′Mlu1 restriction site for further ligation into the intron region of an *SpRNAi–rGFP* gene cassette. All synthetic oligonucleotides must be purified by PAGE to ensure their highest purity and integrity.
4. The sequence of the final *SpRNAi–rGFP* gene cassette and its pri-miRNA insert must be confirmed by DNA sequencing.

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Chapter 18

Vector-Free Methods for Manipulating miRNA Activity In Vitro and In Vivo

Angeliki Pantazi and Athanasios Zovoilis

Abstract

Vector-based methods for manipulating microRNA (miRNA) activity in vivo and in vitro face a number of disadvantages regarding biosafety, workload, and potential for therapeutic use in patients. Use of miRNA mimics and inhibitors provides an alternative for enhancing or inhibiting translational repression of target genes that could be easier applied to elucidate miRNA function. Here, we describe in detail application of miRNA mimics and inhibitors in vitro using adherent cell lines. In addition, we describe a method how they could be applied in vivo in a possible therapeutic context and provide a protocol for validation of this intervention using miRNA target protectors. We present these methods within a standard experimental design workflow that could be followed and we discuss the technical challenges that have to be taken into account.

Key words: miRNA mimics, miRNA inhibitors, miRNA target protectors

1. Introduction

In this chapter we describe a vector-free approach for enhancing or inhibiting microRNA (miRNA) activity both in vitro and in vivo. Since numerous high-throughput expression studies have associated expression of different miRNAs with a wide range of cellular processes and diseases (1–9), the need for methods for manipulating activity of candidate miRNAs has increased in order to reveal their exact regulatory, pathological, or even therapeutic potential in these contexts. Manipulating miRNA activity comprises either enhancing miRNA impact on their target mRNAs and their translation or inhibiting their ability to interfere to this process. In both cases, various strategies to modulate miRNA activity can be applied depending on how they are affecting biogenesis of target miRNA or its association with RISC complex (1, 10) (Fig. 1).

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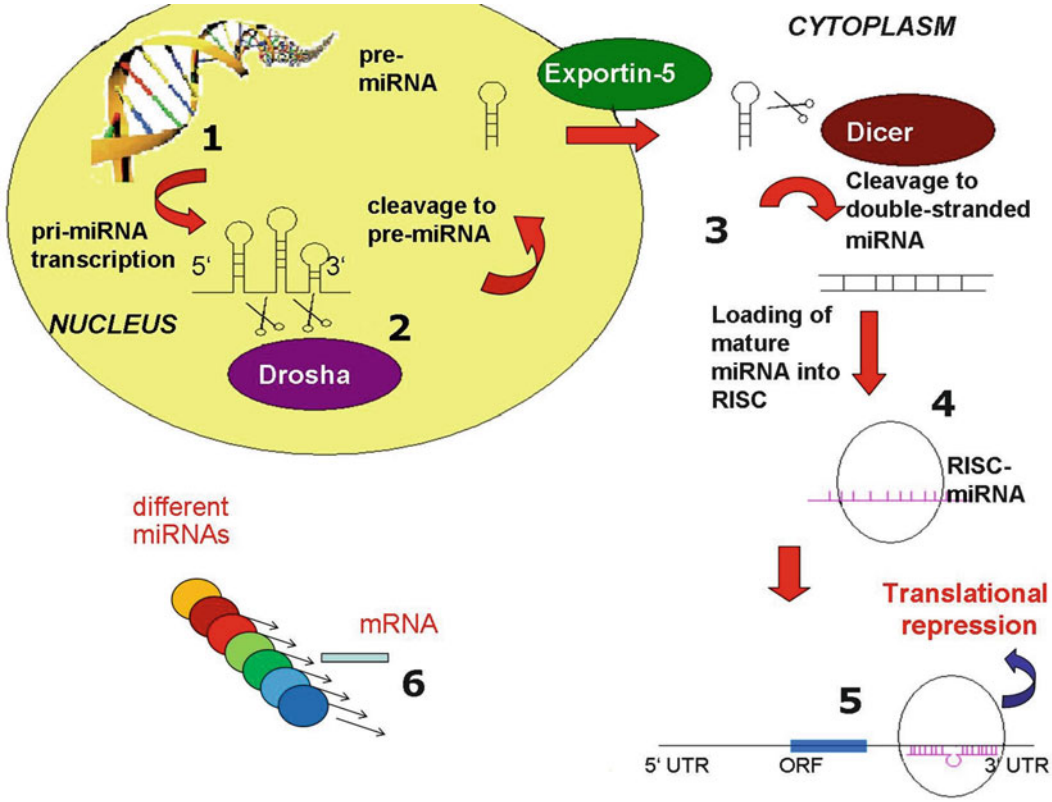

Fig. 1. MicroRNA (miRNA) biogenesis and strategies to manipulate miRNA activity. The figure depicts different stages of miRNA biogenesis and function. Numbers in *bold* represent steps in this process that can be targeted to modify miRNA processing rate and activity. (*1*) Transcription of the miRNA gene by Pol II produces a primary transcript (pri-miRNA). (*2*) Pri-miRNA is further processed to the precursor miRNA (pre-miRNA). Processing rate may be influenced by intra-cellular signaling. Vector-based transcription reproduces step 1 and 2, increasing the amount of starting product to be later processed by Dicer. (*3*) pre-miRNA hairpin is identified and processed from Drosha to a double strand RNA. miRNA mimics reproduce this step by increasing the amount of this RNA to be later loaded to RISC. (*4*) Mature miRNA is loaded to RISC. miRNA inhibitors bind irreversible to this miRNA and prevent subsequent translational repression. (*5*) RISC prevent translation of target mRNA by binding of the miRNA to the mRNA's 3′UTR. miRNA target protectors interfere to this process by selectively binding to a specific mRNA and inhibiting this recognition. In this way, miRNA target protectors inhibit miRNA activity only regarding a specific target of a miRNA and not all targets as in case of miRNA inhibitors. (*6*) One mRNA may be targeted in a specific site of its 3′UTR by more than one miRNAs. Production of artificial mRNAs that contain this site multiple times (miRNA sponges) absorb all these miRNAs and inhibit miRNA activity for this recognition site (miRNA seed).

Initial studies applied methods similar to those previously used to decipher function of protein coding genes using expression vectors and mainly transcription under RNA Pol II or III promoters (11, 12). In this way, miRNA precursor sequences (pri-miRNA and pre-miRNA) were transcribed under constitutive promoters in order to increase the initial substrate of Drosha and/or Dicer and thus the mature miRNA end product. Subsequently, this would increase miRNA activity and enable identification of possible mRNA targets of this miRNA by determination of their mRNA and protein levels. However, this

approach had the disadvantage that it interfered very early in miRNA biogenesis process. This process has been shown to be under strict regulation control affecting the processing rate by Drosha or Dicer or export from the nucleus (13). As a result, normal cell escape mechanisms could reduce significantly the end product. This concern has been mainly addressed by constructing vectors of "artificial" miRNA precursors (14, 15). These precursors combined the desired oligo sequence in the center of a miRNA cassette with flanking sequences. These miRNA precursor flanking sequences belong to a miRNA expected to be constitutively expressed. This approach, initially introduced for production of siRNAs, was later used for miRNA production of selected miRNAs. However, the efficiency of miRNA processing needs to be assessed in every different cell type and tissue to be used and is connected with the efficiency of processing of the constitutive miRNA in these cells. Moreover, this approach inserts size limitations on the miRNAs that could fit into the cassette without disturbing the precursor conformation and Dicer processing and demands laborious cloning when transcribing miRNA clusters. Most importantly, as noted below this process is connected with all disadvantages that methods that use vectors may have in a variety of cases. An alternative approach, which is described here, would be to insert directly into the cell the end product of miRNA biogenesis such as a miRNA or miRNA precursor analog. This RNA oligonucleotide can mimic the potential of the original miRNA to repress translation (miRNA mimic).

The second type of miRNA activity manipulation, miRNA activity inhibition, poses even more challenges. Classical genetic approaches, like generation of knock-out organisms, have been applied (16), but these approaches have the obvious limitations set by the need to generate these organisms. A more straightforward approach employed transcription under a vector promoter of a transcript with multiple target sites for a specific miRNA seed. As a result, miRNAs would be "absorbed" in these transcripts (17). This method, which was named miRNA sponges, presented remarkable results but lacked specificity regarding manipulation of miRNAs that shared the same targets. Moreover, as every vector-based method, miRNA sponges are also facing the limitations that vector technology has. An alternative would be again to insert into the cell an RNA oligonucleotide with the ability to specifically recognize by base pairing the mature miRNA and affect its incorporation into RISC complex and thus its activity (miRNA inhibitor).

These two vector-free approaches described here present a number of advantages compared to more classical vector approaches. Vector-based technologies are frequently combined with use of viral transfection systems, which demand high lab

security standards and a great amount of time consuming cloning and transfection efforts. Use of miRNA mimics and inhibitors does not require any cloning and has been shown to be able to transfect cells in vivo without use of any viral transfection system. However, the most serious disadvantage of vector-based technologies is the barriers they pose for therapeutic use in human patients. Even, when only minimal integration to the host genome is achieved, the effect of transfection can be long lasting, reducing control over the applied therapy. Vector approaches comprise a major change of the genetic material of the host cell (insertion of DNA) and therefore affect multiple cell functions (transcription, replication and DNA repair, splicing and mRNA processing, and translation). Despite these disadvantages, in case of expression of protein coding genes, transcription and translation of the selected gene cannot be easily by-passed in order to get the end product that determines organisms' phenotype, the protein. Insertion of whole mRNA molecules or modified proteins faces great challenges in in vivo transfection systems (18) and transcription of a vector is usually the only available choice. However, in case of miRNAs, the target product (protein) is not directly connected with the miRNA gene, since miRNA genes produce no proteins, but indirectly, as miRNAs regulate protein levels of target genes. Since small RNA molecules can be easily transfected into cells (19), transcription of miRNAs within the cell can be easier by-passed. This finding has opened a new avenue in the way we can regulate protein levels without the need to transcribe the original miRNA gene. In order to increase protein levels, it is sufficient to decrease activity of a miRNA that targets the respective mRNA; reversely, enhancing the activity of the same miRNA lowers target protein levels. Transfection with miRNA analogs and inhibitors that will be described here targets only a later step of gene expression flow, protein translation, and does not alter genetic content of the organism. Although various technical challenges remain, the methods described here could have a great impact in the way we manipulate protein expression levels for basic research or therapeutic uses.

The presented miRNA vector-free methods are divided into two parts, one regarding applications in vitro and another one describing one of the first attempts to apply them in vivo in a possible therapeutic context. In the first case, we describe use of these methods in a widely used cell culture system, mouse embryonic stem cells, in which we successfully over-expressed and inhibited members of the miR-290 miRNA cluster, in order to identify their potential to control differentiation (20). A similar approach has also been applied to multipotent germline stem cells (21). In the second case we present the application of miR-34c over-expression and inhibition in mouse hippocampus for possible

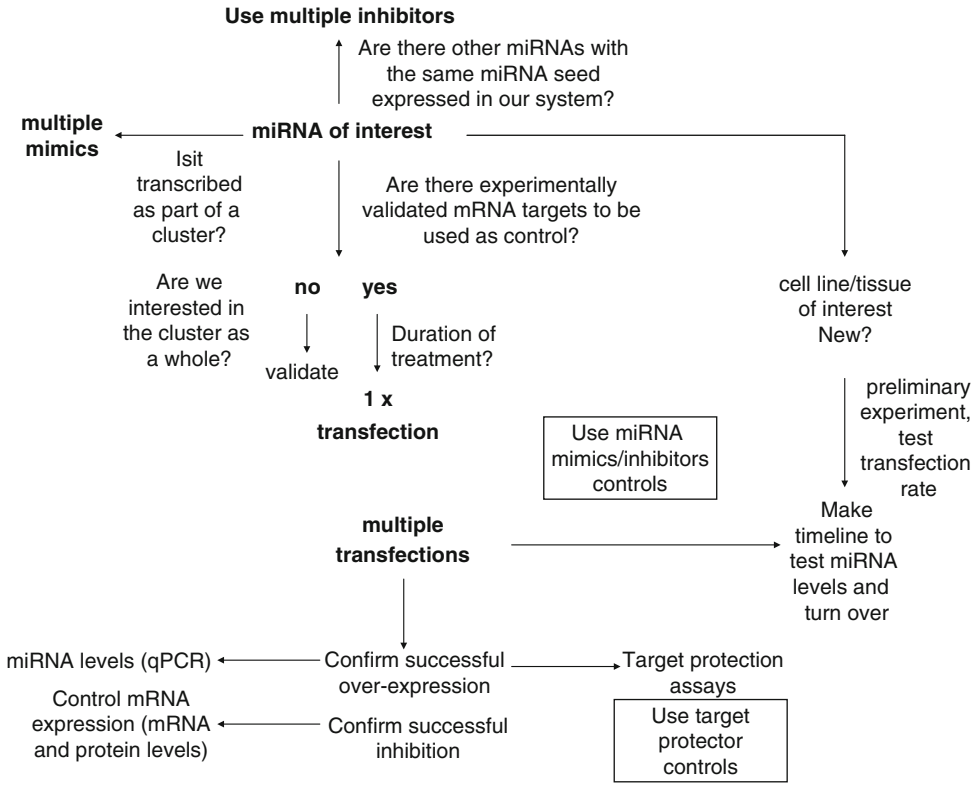

Fig. 2. Outline of experimental design.

treatment of cognitive impairment in mouse models (22). In addition, we present these methods within a standard experimental workflow that can be followed in any study analyzing impact of over-expression and inhibition of a specific miRNA in a model system (Fig. 2). Finally, at our end remarks (see Note 18), we present the technical challenges that this approach still faces.

2. Materials

2.1 Cell Culture

1. A mouse embryonic stem cell line or another adherent cell line of preference (see Note 1).
2. Culture media: diluted to DMEM to the following final concentration: 0.1 mM non-essential amino-acids, 1 mM Sodium pyruvate, 1 μM β-Mercaptoethanol, 2 mM L-Glutamine, 1 mM Penicillin/Streptomycin, 20 % Fetal Calf Serum, 1,000 U/mL Leukemia Inhibition Factor (LIF).
3. Inactivated mouse embryonic fibroblasts to form a feeder layer on a culture plate coated with gelatine.

4. PBS, trypsin (e.g., TrypLE Express from Invitrogen, Carlsbach).
5. Centrifuge, water bath, culture hood, cell culture incubator, Neubauer cell chamber.
6. Ethanol.

2.2. In Vivo miRNA Injections (see Note 2)

1. Canulated mice for intrahippocampal injections. For example, using microcannulae (1.5 mm-gauge needle that extends 0.5 mm beyond the tip of the guide cannula) stereotactically implanted to the hippocampus (1.0 mm posterior to the bregma; 1.0 mm lateral from midline; and 1.5 mm ventral).
2. Appropriate injection devise (to keep flow at a rate of 0.3 μL/min) and appropriate anesthetic.

2.3. miRNA Over-Expression and Inhibition

1. miRNA mimics or inhibitors for the miRNA(s) of interest (see Notes 3 and 4).
2. Appropriate miRNA mimic and inhibitor controls (see Notes 5 and 6).
3. PBS.
4. Sterile RNase free water.
5. Cell culture hood.
6. HiPerfect transfection reagent, Qiagen.
7. Basal Culture Medium, for example DMEM (only for the in vitro application).

2.4. miRNA Target Protection

1. miRNA Target protector (see Note 7).
2. miRNA mimic of the miRNA of interest.
3. PBS.
4. Sterile RNase free water.
5. HiPerfect transfection reagent, Qiagen.

3. Methods

3.1. Experimental Design (see Note 8)

The experimental workflow described here refers to the case where function of a known miRNA in a specific cell type and biological context is investigated (see Fig. 2). A critical point is the use of at least one already known target of this miRNA to assess successful enhancement or inhibition of miRNA activity. Here it is important to note that we refer to already experimentally validated mRNA targets in the literature and not to predicted miRNA targets, as derived by a variety of bioinformatics tools and platforms like Targetscan, Pictar, etc. The latter are an invaluable tool for identifying pairs of miRNA target mRNA candidates, but are characterized

by high false positive rates. Thus, they have first to be experimentally validated. Moreover, for many miRNAs such validated targets have not yet been identified or they are expressed only in specific cell types. Therefore, the users should first apply a reporter assay described in this book or elsewhere (20, 22) in a standard cell culture system in order to validate a potential miRNA target. Such a reporter assay involves vectors containing luciferase or GFP ORFs (open reading frames) combined downstream with parts or the full length of the 3′UTR of the predicted target mRNA. These vectors are co-transfected into cells with the miRNA of interest. Overexpression of the desired miRNA is accomplished either by the transfection method with mimics described here or by a vector-based method. However, for these preliminary experiments use of both systems is recommended, since co-transfection of mimics with vectors has not yet been tested in all systems regarding efficiency. This validated target can later be used as a read-out of the activity of the miRNA in the tested system, and combined with the approach mentioned in Subheading 3.5, consists a sufficient guarantee that the observed effects in the tested system are due to changes in miRNA activity.

Another critical point that has to be determined before the beginning of the experiments is whether the miRNA to be tested is transcribed in a cluster or not. Since the mimics used here are specific for each miRNA, if the effect of transcription of the whole miRNA cluster is to be tested, multiple mimics have to be used, each for every different cluster member.

The last important point to be taken into account during experiment design is the duration of the desired miRNA activity manipulation. The presented methods are transient transfections and, although the duration of their impact may vary per cell type, experiments demanding prolongation of this effect for more than 48 h should definitely employ multiple transfections (see Note 8). Use of miRNA mimics for as long as 10 days in vitro and in vivo with the current described protocols did not show any signs of significant cell toxicity in cultured embryonic stem cells, affecting their viability or proliferation rate, and hippocampal cells in vivo, altering animal behavior. However, in cases of prolonged exposure to mimics and inhibitors, users are recommended to perform a series of preliminary transfections (timeline) with the miRNA mimic or inhibitor so that expression of the target gene as well as possible toxic events in the cell type of interest can be assessed.

3.2. Determination of miRNAs Sharing the Same Seed

It is possible that a specific miRNA recognition motif (seed) on the UTR of a specific mRNA is shared by more than one miRNAs. When designing an inhibition experiment, it is critical to take into account this fact, since it can heavily affect evaluation of the impact that inhibition can have on cell. For example, other miRNAs sharing the same seed may compensate for loss of activity through

inhibition of a specific miRNA, resulting in moderate or no increase of the protein markers. This information itself might be of great importance regarding how indispensable a miRNA might be. This phenomenon could hinder the users' efforts to identify the function of miRNA of interest, since practically no inhibition of the miRNA activity takes place. A possible solution that can be applied in many cases is to inhibit at the same time all miRNAs that share this seed and are expressed in this cell system. This will enable to decipher in which pathways this miRNA participates through translational repression of its targets. Although not specific, combined with the reverse procedure, miRNA over-expression, which is more specific, can deliver this information.

3.3. In Vitro Application of miRNA Mimics and Inhibitors

1. Preparation of stock solutions.

   For miRNA mimics: miRNA mimics get shipped lyophilized and must be kept in −80°C before and after their resuspension. Before resuspension spin down for 10 s the vial. Under the cell culture hood, working aseptically add sterile RNase free water to a total concentration of 50 μM to produce a stock solution. For example in a vial containing 5 nmol mimic add 100 μL water and mix at RT (room temperature) by pipetting up and down for at least ten times. To prepare the "working" solution to be used in transfections, dilute further the stock solution to a final concentration of 2 μM, for example by adding 240 μL RNase free water to 10 μL of stock solution (since mimic concentrations to be used in different cell types in possible future experiments may vary, you are advised not to dilute all the stock solution into the working solution but keep the half in −80°C in case higher concentrations of working solution may be needed in the future). Aliquot the working solution in order to avoid more that two freezing-defreezing cycles during its use for transfections. Keep working solution also in −80°C.

   For miRNA inhibitors: Use the same procedure as described above for the mimics but towards a working solution of 8 μM.

2. If cells to be transfected are not currently in culture, defreeze them at least 1 week before the planned transfection of the cells (see Note 9). Transfection experiments described here are performed on 6-well cell culture plates and 5×10^5 cells/well will be needed. For lower or higher cell amounts scale culture media, cell number, and transfection, mix volumes described below accordingly.
3. Subculture cells 2 days before so that the day of transfection is not fully confluent. Change culture medium 1 day before planned transfection.
4. The day of transfection.

miRNA mimics: Shortly before transfection, wash cells with PBS, trypsinize, and subculture them to a new 6-well plate at 5×10^5 cells/well in 2.3 mL of the appropriate culture medium. Put them in the incubator. Per well: under the hood, without delay dilute 6 μL of working solution of the miRNA mimic in DMEM to a final volume of 100 μL (see Note 10). Add 12 μL of HiPerFect to the mix and mix thoroughly by pipetting for 30 s. Leave the mix at room temperature for 5–10 min and then add it dropwise on the recently subcultured cells in the well. Using a 5 mL culture pipet, mix gently up and down the content of the well. The final concentration of the mimic in the culture medium is 5 nM per miRNA mimic.

miRNA inhibitors: Proceed as with miRNA mimics but add 18 μL of HiPerFect instead of 12 (see Note 11). Due to the different concentration of the working solution, final concentration of the inhibitor in the culture medium is 20 nM per miRNA inhibitor.

5. Cells can be incubated under normal growth conditions for 24 h. Do not change cell culture medium before. After that cells can be collected and tested for successful over-expression or inhibition (see Note 12).
6. If a long-term exposure is desired, cells should be passaged and retransfected as described above 48 h after each transfection (see Note 13).

3.4. In Vivo Application of miRNA Mimics and Inhibitors (see Note 14)

1. All handling must be done under hood and aseptic conditions.
2. Preparation of stock solutions (see Note 15).

miRNA mimics: Add sterile cold PBS (water can be also used) to the lyophylized mimic as described in step 1 of Subheading 3.3 to a final concentration of 1 mM. Mix intensively and leave it for 3 min at room temperature, then store at −80°C. For the working solution dilute further to a final volume of 100 μM and aliquot. Aliquotes should not be frozen more than once.

miRNA inhibitors: Due to the higher concentrations needed in in vivo applications of inhibitors, stock solutions are at the same time working solutions to be used directly for transfections. At the moment, for many companies 20 nmol is the maximum amount per vial and multiple vials may be needed per experiment. Add sterile cold PBS (water can be also used) to the lyophylized inhibitor as described in step 1 of Subheading 3.3 to a final concentration of 1 mM. For example, for a 20 nmol vial add 20 μL PBS direct at the bottom of the vial and mix carefully up and down. Due to the very small amount of the PBS, try to dispense the drop around to ensure that you do not let a part of the lyophylized "powder" undiluted. Mix intensively and leave at room temperature for 3 min (see Note 16).

3. Calculate the volume of transfection mix that will be needed for the injections (V μL). For example, for intahippocampal injections 0.5–1 μL of the mix per hemisphere are needed. miRNA mimics: Dilute the working solution to a total volume of V μL and a final concentration of 100 μM. For example, if 100 μL of injection volume is necessary, add 90 μL PBS to 10 μL of the working solution and mix. Transfer into 0.2 mL tubes and add 1.35 μL of the HiPerfect reagent per 10 μL of the V μL volume. miRNA inhibitors: Transfer V μL of the working/stock solution (it is the same) containing the inhibitor into 0.2 mL tubes and add 1.35 μL of the HiPerfect reagent per 10 μL of the V μL volume.
4. Mix thoroughly up and down for at least 30 s and leave at room temperature for 5 min. Then warm it in your hand before aspirating it and injecting it in vivo.
5. Do not freeze or use again what it remains.

3.5. In Vivo Application of miRNA Target Protectors

Final target protector concentration to be used is the same with that of miRNA mimics. Apply the previous protocol for miRNA mimics with the following modification:

Step 3

Dilute the miRNA mimic working solution to a total volume of V/2 μL and a final concentration of 200 μM. Do the same for the target protector working solution. For example, if 100 μL of injection volume is necessary, add 40 μL PBS to 10 μL of each working solution and mix. Then combine the two solutions. Transfer the total volume into 0.2 mL tubes and add 1.35 μL of the HiPerfect reagent per 10 μL of the V μL volume (see Note 17).

4. Notes

1. The methods described in this chapter have been optimized for use within embryonic stem cells and other types of pluripotent stem cells like multipotent germline stem cells. Stem cells are characterized by a high proliferation rate which may influence transfection efficiency and turnover of applied mimics and inhibitors. Thus, for other adherent cell types, users are recommended to perform preliminary experiments testing these parameters, especially if they intend to perform long-term transfections. Many companies provide control miRNA mimics conjugated with fluorophores that could be used in initial transfection experiments, and followed by FACS sorting, could define transfection efficiency. For example, for embryonic stem cells, the transfection protocol mentioned here could provide

more than 70 % transfection rate as defined by FACS sorting of a Cy-5 conjugated control miRNA 24 h post-transfection. For mouse embryonic fibroblasts, this percentage was around 50 %. If the long-term transfection protocol is needed, perform also an initial time line experiment in your cell line testing miRNA levels throughout time during the multiple transfections with the miRNA mimic.

2. The protocol refers to intra-hippocampal injections. For direct application in vivo to other tissues, adapt injection protocol accordingly. For systematic application, possible metabolism through hepatocytes must be taken into account.
3. miRNA mimics and inhibitors are available from many companies. Although exact chemical structure and modifications are usually not disclosed, they mainly consist of a single or double strand synthetic RNA which resembles mature or precursor miRNA (mimic) or can stably bind to it (inhibitor). A different approach which has also depicted remarkable results is the use of LNA technology that significantly stabilizes the RNAs. The protocols described in this chapter have been optimized for use with mimics and inhibitors from two different companies: miScript miRNA mimics and inhibitors, Qiagen/Ambion Pre-miR miRNA Precursors and Anti-miR miRNA inhibitors, Ambion, Invitrogen. The first were used during in vivo studies, the second during in vitro, but to our knowledge, at the moment, there is no indication that either perform better in one application rather in the other one. Selection of products from each company for a specific application (in vivo or in vitro) was not based on any comparison between these two products for the specific application and authors encourage the users to test themselves which of these or other products fits to their research and financial requirements.
4. Since in vivo applications demand much higher concentrations, especially regarding inhibitors, it is likely that more than one vials of 20 nmol may be needed for each experiment. For example, for intrahippocampal injections of 20 mice, more than 40 μL of transfection solution will be needed (see Subheading 3), thus more than one vials of the miRNA inhibitor.
5. Use of appropriate controls is crucial for evaluation of non-specific effects of mimics and inhibitors to be used. These are usually provided also by the companies delivering the inhibitors and are scrambled oligonucleotides with the same chemical modifications that have been tested and have no effect on gene expression. Do not use control inhibitors as controls with miRNA mimics experiments and the reverse, since they have different biochemical characteristics (single vs. double strand, different length and chemical modifications). For the same reason, do not mix controls with mimics or inhibitors of different

companies. Some companies provide their controls also conjugated with fluorophores which can be used for evaluating transfection efficiency at preliminary experiments (see Note 1). However, do not use them as controls in the subsequent miRNA mimic/inhibitor experiments. Use the unconjugated ones.

6. If multiple mimics and inhibitors are to be tested (see Subheading 3), then the amount of the control to be ordered should be multiplied to the number of mimics or inhibitors to be tested.
7. Here we describe the use of miScript Target Protectors provided from the company Qiagen. These are modified RNAs that specifically interfere with the interaction of a miRNA with a single target, while leaving the regulation of other targets of the same miRNA unaffected (Qiagen, http://www.qiagen.com). The design of the protectors has to be done using the specific software provided by the company at http://www.qiagen.com/products/miscripttargetprotectors.aspx (http://www.qiagen.com). For each miRNA/target mRNA pair you want to test, you have to design as many protectors as the number of bindings sites this miRNA has to mRNA's 3′UTR. If a possible target site remains unprotected, there is a risk that target protection will fail due to compensatory translational repression by the miRNA to the unprotected site. For example, in case of Sirt-1 and miR-34c, there are two predicted binding sites on Sirt-1 3′UTR and thus two different protectors have to be designed and used for the binding sites starting either at position 780 nt or at 1,270 nt of the Sirt-1 mRNA 3′UTR. The respective control, the Negative Control miScript Target Protector, has also to be ordered and used at quantities multiplied to the number of protectors to be used (in case of Sirt-1/miR-34c, two times).
8. Although the cell type where a selected miRNA is to be tested is usually standard, selection of the right biological context to test function of this miRNA can sometimes be of a specific importance. Particularly, miRNAs have been shown to perform fine tuning of gene expression acting like "cellular buffers." To this end, when aims of the study allow it, it is worth testing miRNA function in dynamic cell systems rather than static. Challenging the system would enable to capture more easily a possible miRNA function that is normally hidden under this buffering capacity of miRNAs.
9. Do not use recently resuscitated frozen cells, it reduces transfection efficiency.
10. If multiple miRNA mimics have to be transfected (e.g., in case of miRNA clusters), modify this step as follows: Pool 6 μL of

working solution of each miRNA mimic and then dilute in DMEM to a final volume of 100 μL. For example, for six members of the miR-290 cluster (6 × 6 μL), 36 μL of pooled miRNA mimics will be added to 64 μL DMEM. For use of more than two miRNA mimics, add 18 μL instead of 12 μL of HiPerFect to the mix. It is important to note that volume of HiPerfect to be used is not scaled linearly according to the concentration of miRNA mimics. Too much transfection reagent can be toxic for the cells. The final concentration of each miRNA mimic in the medium remains the same; however, the control mimic concentration to be used should be multiplied with the number of mimics that are used. For example, in the case of the 6 miRNA mimics mentioned above, for the respective control mix, 36 μL of control mimic and not 6 μL should be diluted with 64 μL DMEM. Use again 18 μL HiPerfect.

11. For multiple inhibitors use 36 μL HiPerfect, do not scale it linearly. However, as in miRNA mimics (see Note 10), final inhibitor control concentration has to be scaled linearly based on the number of inhibitors.

12. For over-expression experiments, you can evaluate expression levels of the miRNA. miRNA mimic contributes to expression levels of mature miRNA, thus expression assays determining levels of the mature miRNA (not the precursor) should be able to assess them. In contrast, in the inhibition experiments, miRNA levels are not informative of successful inhibition, since very often binding of the inhibitor does not lead to direct degradation of the miRNA. The inhibitory effect is on the level of mRNA translation. In both over-expression and inhibition, assessment of protein levels of the validated target consists of a better read-out method of successful manipulation of miRNA activity. In contrast, evaluation of mRNA levels may not be informative, since many miRNAs do not lead to degradation of their mRNA targets, but only repress their translation. In in vitro studies confirmation can be also combined with co-transfection into the cells of the reporter GFP or luciferase vector containing the target gene's 3′UTR as mentioned in Subheading 3.1 and subsequent assessment of reported gene levels/fluorescence, etc. However, transfection efficiency of the vector might not be the same using conditions applied for miRNA mimics and inhibitors, and different transfection reagents might be needed. Moreover, in case of in vivo transfections, use of a viral vector might be needed. Alternatively, miRNA target protection assays described in Subheading 3.5 are able to evaluate in vivo whether observed repression of the control gene should be attributed to our manipulations and transfection with the miRNA mimic, being thus no indirect effect.

13. This applies to embryonic stem cells that need frequent subculture. For cell lines that proliferate in low rates, the protocol could be modified as follows: For every second transfection, do not trypsinize the cells but just change culture medium of the cells and add the transfection mixture dropwise in the wells (retaining the final volume the same, e.g., for 6-well plates to 2.4 mL). Make a time line to assess efficient repression or over-expression of the target gene to evaluate efficiency of your transfection scheme.
14. For in vivo studies, we recommend also the use of an already validated siRNA against a known mRNA target (known to be expressed in these cells). Apply the same transfection conditions for the miRNA mimics before use of the mimics. Successful silencing of target gene would provide a sufficient hint that in our in vivo model system use of HiPerfect is sufficient for delivering of small RNAs into the cells, which subsequently can be successfully incorporated into RISC.
15. For in vivo studies, we recommend direct use of the mimic or inhibitor after resuspension.
16. For use of multiple inhibitors, we recommend to use the resuspended solution of one inhibitor to resuspend all the others. This is necessary as many companies deliver a maximum amount of 20 nmol/vial, which means that for multiple inhibitors less than 20 μL/vial would be needed to retain final concentration, but with such amount it is difficult to sufficiently resuspend the lyophilized product. Do not increase the amount of HiPerfect to be used, if using until three inhibitors. For more, this amount might be modified based on case-specific preliminary transfection experiments and assessment of transfection efficiency.
17. Transfections should be combined with the miRNA mimic transfection protocol retaining the same final miRNA mimic concentrations, but not increasing the final HiPerfect volume to be used. Use controls for both the miRNA mimics and protectors.
18. The major challenge that methods described in this chapter face is the inability to selectively apply miRNA mimics and inhibitors to specific cell types in vivo. With vector-based methods, use of issue-specific promoters can achieve this, but for miRNA mimics and inhibitors no such specificity is possible at the moment. Future works should focus on the development of small RNA delivery methods that could specifically target different cell populations in vivo.

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Chapter 19

MicroRNA Expression Profiling of Human-Induced Pluripotent and Embryonic Stem Cells

Amit Sharma and Joseph C. Wu

Abstract

Clinical implications of induced pluripotent stem (iPS) cell technology are enormous for personalized medicine. However, extensive use of viral approach for ectopic expression of reprogramming factors is a major hurdle in realization of its true potential. Non-viral methods for making iPS cells, although plausible, are impractical because of high cost. MicroRNAs are important cellular modulators that have been shown to rival transcription factors and are important players in embryonic development. We have generated distinct "microRNA-omes" signature of iPS cells that remain in a near embryonic stem (ES) cell state and distinct from differentiated cells. Recent advances in the microRNA field and experimentally validated microRNAs warrant a review in experimental protocols for microRNA expression profile.

Key words: ES cells, iPS cells, MicroRNA, Microarray

1. Introduction

One of the most clinically relevant findings of the past decade is the ability to reprogram a terminally differentiated cell to an ES cell-like state. Takahashi and Yamanaka utilized ectopic expression of transcription factors OCT4, SOX2, KLF4, and cMYC in terminally differentiated somatic cells to achieve pluripotency (1). Later, Yu, Thompson and colleagues published NANOG and LIN28 along with OCT4 and SOX2 to make iPS cell lines (2). Despite rapid advancements in the field, two major hurdles in clinical application of iPS cells remaining are; the inability to avoid stable genomic integration of viral vectors used to overexpress "pluripotency factors" and the residual epigenetic signature from the cells used to derive iPS cells (3) (Fig. 1). Efforts have been made to address these issues with limited success, and all of these approaches have their shortcomings. For instance, one promising approach is to use

Shao-Yao Ying (ed.), *MicroRNA Protocols*, Methods in Molecular Biology, vol. 936,
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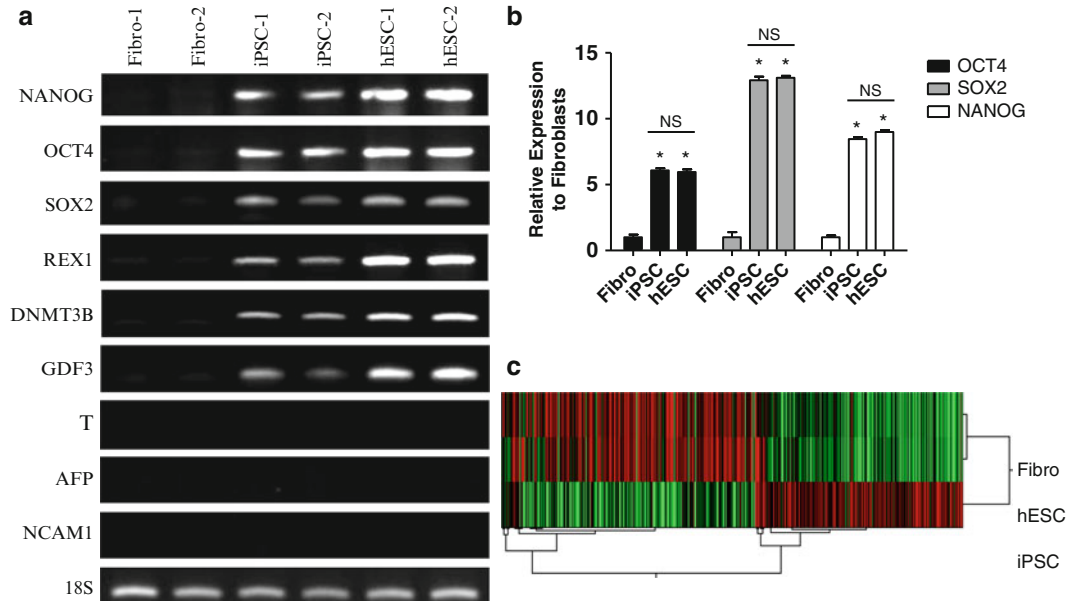

Fig. 1. RT-PCR and quantitative RT-PCR analysis of selected genes. (**a**) RT-PCR demonstrated robust expression of the embryonic genes (OCT4, NANOG, SOX2, REX1, DNMT3B, GDF3) in human iPS cells and ES cells, but not in fetal fibroblasts. Undifferentiated state of ES and iPS cells was also confirmed by performing RT-PCR for markers of mesoderm (Brachyury,T), endoderm (AFP), and neuroectoderm (NCAM1), which were all negative. Equal loading of RNA for each sample was confirmed by Human 18S. (**b**) Quantitative RT-PCR analysis of the pluripotency genes OCT4, SOX2, and NANOG confirms similar expression levels of these genes in human iPS cells and ES cells. Error bars represent one standard deviation from the mean (**P* value < 0.05 vs. fibroblasts; NS Not Significant). (**c**) Microarray data comparing global gene expression profiles of Fibroblasts, hESC and iPSC, heat map and hierarchical clustering analysis by Person correlation showing iPSC cells have similar expression profile to hES cells.

full-length mRNAs of "pluripotency factors" (4). However, this approach is still too expensive at present. Another approach is to use piggyback vectors in reprograming, but this also has its limitations in being cumbersome (5). Efforts are therefore underway to device a new technique to generate iPS cells without confounding factors to realize their true clinical potential. However, more effective strategies of cellular reprograming will require a better understanding of underlying changes that take place during the process of reprograming (6).

Along with transcription factors, microRNAs have emerged as a novel class of regulators of gene expression (7). These small RNAs are increasingly being recognized as important regulators of various biological pathways that control gene expression by either mediating target mRNA degradation or stalling protein synthesis (8, 9). Interestingly, the involvement of various microRNA families has long been known in various developmental processes (10). Therefore, it is not surprising that microRNAs play a key role in the generation and maintenance of iPS cell-like state. We have previously shown a distinct microRNA expression

profile in ES cells compared to terminally differentiated somatic cells and the similarity of "microRNA-omes" of iPS cells to ES cells (11). We have also assessed dynamic microRNA expression programs during cardiac differentiation of human ES cells (12). Others have analyzed common changes in the expression of microRNAs (miRNAs) and mRNAs in different human ES cell lines during early commitment and examined the expression of key ES cell-enriched miRNAs in earlier developmental states (12). Several microRNAs clusters such as miR-17-92, miR-106a-363, miR-302-367, and miR-200 showed a similar expression signature in ES and iPS cell lines. Recent advances in techniques for microRNA expression profiling and the ever growing list of validated microRNAs make it would be worthwhile to establish the protocols for genome-wide "microRNA-omes" expression profiling, which will help us understand molecular events that culminate in cellular dedifferentiation.

2. Materials

2.1. Cell Culture Reagents

1. Dulbecco's modified eagle medium contains 4.5 g/L glucose (DMEM, Gibico).
2. Phosphate-buffered saline (PBS) without calcium and magnesium.
3. Penicillin/streptomycin (Invitrogen).
4. Triple E solution (Invitrogen).
5. DMEM/F-12 Medium (Invitrogen).
6. mTeSR1 media and 5× supplement (STEMCELL Technologies, Canada).
7. BD Matrigel hESC-qualified (BD biosciences, USA).
8. Accutase solution (Sigma-Aldrich, USA).
9. miRNeasy Mini Kit (Qiagen Inc., Valencia, CA).

2.2. Fibroblast Medium Composition

DMEM containing 10% FBS and 50 U/mL penicillin/50 mg/mL streptomycin. To prepare 450 mL of DMEM medium, mix 50 mL of FBS and 2.5 mL of penicillin/streptomycin, and then fill up to 500 mL with DMEM. Store at 4°C up to a week.

2.3. MteSR1 Media and 5× Supplement

1. Thaw 5× supplement at 4°C overnight and mix with mTeSR1 media. Store at 4°C.

2.4. miRNA isolation and microarray

1. miRNA isolation and hybridization.
2. miRNeasy Mini Kit (Qiagen, USA).
3. Microcon centrifugal filter (Millipore, Billerica, MA).
4. μ Paraflo microfluidics chip (Atactic Technologies, Houston, TX).

5. 6× SSPE buffer (0.90 M NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 6.8).
6. Slide scanner (GenePix 4000B, Molecular Devices, Sunnyvale, CA).
7. Array-Pro image analysis software (Media Cybernetics, Bethesda, MD).

3. Methods

3.1. RNA Preparation

The miRNeasy Mini Kit combines phenol/guanidine-based lysis of samples and silica membrane-based purification of total RNA. The upper, aqueous phase is extracted, and ethanol is added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. A specialized protocol is provided for enrichment of miRNAs and other small RNAs (less than ~200 nt) in a separate fraction. The manufacturer's instructions should be followed to efficiently isolate the small RNA from fibroblasts, ES cells and iPS cells.

3.2. MicroRNA Microarray Construction, Hybridization, and Scanning

Microarray assay can be performed by outsourcing to service provider, or by using following protocol.

1. Enrich small RNA fraction from 4 to 8 μg total RNA sample, by size-fractionation using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA).
2. Extend the 3′-end of the small RNAs (<300 nt) isolated with a poly(A) tail using poly(A) polymerase.
3. Ligate an oligonucleotide tag to the poly(A) tail for later fluorescent dye staining.
4. Perform hybridization overnight on a μ Paraflo microfluidic chip using a microcirculation pump (Atactic Technologies, Houston, TX). The microfluidic chip has detection probes which are chemically modified nucleotides, complimentary to the target micrRNA (from miRBase, http://microrna.sanger.ac.uk/sequences/) or other control RNA, and a spacer segment of polyethylene glycol is used to extend the coding segment away from the substrate. The detection probes were made by in situ synthesis using photo-generated reagent chemistry.
5. Hybridization buffer is composed of 6× SSPE buffer (0.90 M NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C.
6. After RNA hybridization, circulate tag-conjugating Cy3 or Cy5 dyes through the microfluidic chip for dye staining.

7. Fluorescence images can then be collected using a laser scanner (GenePix 4000B, Molecular Devices, Sunnyvale, CA) and can be digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD).

3.3. MicroRNA Microarray Normalization and Data Analysis

In order to determine functionally relevant differences in microRNA expression, normalization needs to be performed to reduce system-related variations, including variations such as difference in sample amount, efficiency of labeling dyes, and signal gain that is subject to the scanner used to acquire the image.

1. The first step in data analysis is to subtract the background noise and then to normalize the signals using a LOWESS filter (locally weighted regression).
2. Background can be determined using a regression-based background mapping method.
3. The regression can be performed on 5–25% of the lowest intensity data points, excluding blank spots. The background matrix can then be subtracted from the raw data matrix.
4. The data filtering removes miRNAs with normalized intensity values below a threshold value of 32 or a predetermined baseline value across all samples.
5. Gene centering and normalization may be used to transform the Log2 values using the mean and the standard deviation of individual genes across all samples. The *t* values are calculated for each miRNA between groups, and *P* values are computed from the theoretical *t*-distribution. miRNAs with *P* values below a critical *P*-value (typically 0.01) are selected for cluster analysis.
6. The clustering can be done using various methods. However, hierarchical clustering is the favorable method, with average linkage and Euclidean distance metrics.
7. We used TIGR MeV (Multiple Experimental Viewer) software from The Institute for Genomic Research to generate the clustering plot.
8. We performed principal component analysis using the *R* statistical package (cran.r-project.org) (Figs. 2 and 3).

4. Notes

4.1. RNA Preparation

1. Determine the integrity of the input RNA for labeling and hybridization prior to use to increase the likelihood of a successful experiment
2. To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.

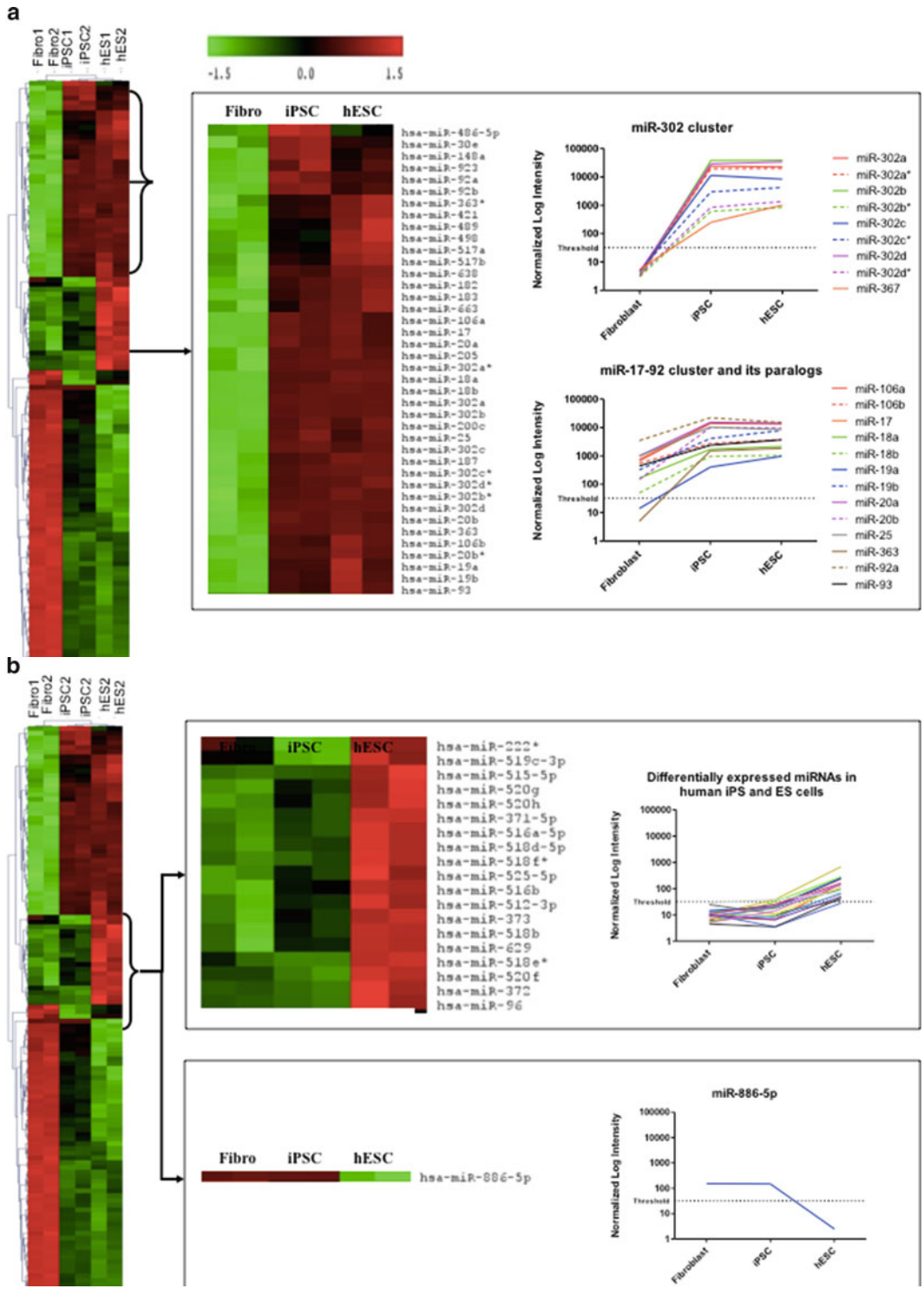

Fig. 2. Heat maps and signal intensity plots of microRNA expression across fibroblasts, iPS cells, and ES cells. ANOVA analysis demonstrates statistically significant differential microRNA regulation across the three samples. microRNAs with *P* values below 0.01 were selected for cluster analysis. (**a**) microRNAs upregulated in both iPS cells and ES cells compared to fibroblasts. Highlighted are the normalized signal intensity plots for the miR-302 cluster and miR-17–92 cluster, including

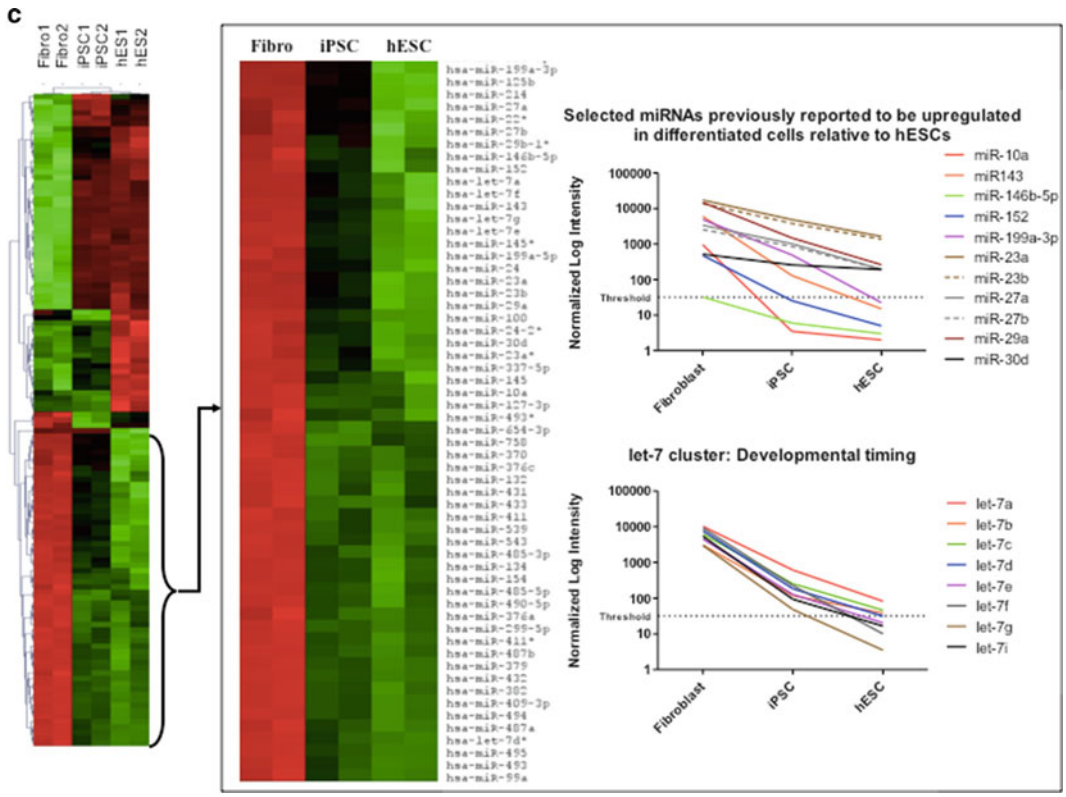

Fig. 2. (continued) its paralogs miR-106a-92 and miR-106b-25. (**b**) MicroRNAs exhibit opposite expression in iPS cells and ES cells. Note that the normalized signal intensities of the microRNAs are not dramatic, but still statistically significant. In contrast to the group of microRNAs upregulated in ES cells but not in iPS cells (*top panel*), we observed only one microRNA (miR-886–5p) that was upregulated in iPS cells but not in ES cells (*bottom panel*). Fig.2 (**c**) MicroRNAs are upregulated in fibroblasts but down-regulated in iPS cells and ES cells. Highlighted in the *top* and *bottom panels* are selected microRNAs known to be important in ES cell biology and/or organismal development, such as the let-7 cluster that has a well-established role in late development timing. The clustering was done using hierarchical methods with average linkage and Euclidean distance metrices. The "threshold" value denotes signal intensities <32, a cutoff used by the microarray service provider, below which quantitation of signal may be inaccurate.

3. Maintain a clean work area.
4. When preparing frozen reagent stock solutions for use:
   (a) Thaw the aliquot as rapidly as possible without heating above room temperature.
   (b) Mix briefly on a vortex mixer, then centrifuge for 5–10 s to drive the contents off of walls and lid.
   (c) Store on ice or in a cold block until use.
5. Total RNA extraction methods differ in numerous ways and may impact the yield. Some kits that are recommended for use are:
   (a) Qiagen miRNeasy Mini Kit—catalog number 217004.

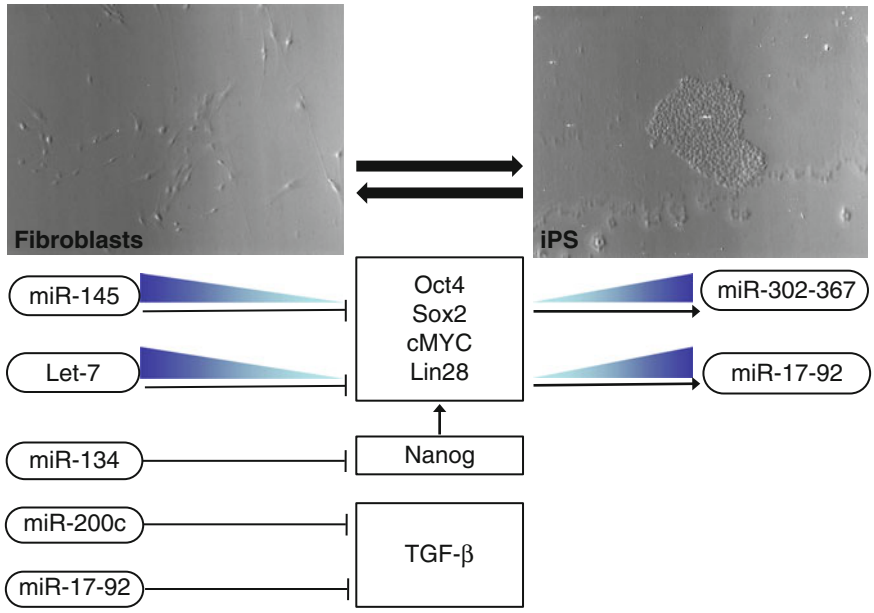

Fig. 3. Schematic representation depicting microRNAs as regulators of cellular pluripotency. Various transcription factors and signaling pathways are known to induce and maintain pluripotent state. However, various microRNAs are shown to regulate these factors. The microRNA miR-145 has been shown to regulate OCT4, SOX2, and KLF4 posttranscriptionally; this microRNA is high in differentiated cells while its impression is reduced in ES and iPSC cells. Also, miR-302-367 cluster is transcriptionally modulated by OCT4 and hence is much higher in undifferentiated cells. Similarly, cMYC is known to upregulate miR-17-92 family. This microNRA family also affects TGF-β signaling members, that are known to modulate cellular differentiation. Thus, interplay between microRNA and transcription factors may contribute to maintain a pluripotent state.

(b) Applied Biosystem mirVana RNA Isolation Kit—catalog number AM1560.

(c) Invitrogen TRIZOL Reagent (100 mL)—catalog number 15596-026.

We recommend these or any other commercial kits for the extraction of the total RNA including small RNA fraction. Do not use the size-fractionation or small RNA enrichment protocol. You must use the same total RNA extraction methods to obtain consistent results for comparative experiments. Different total RNA extraction methods may result in slightly different miRNA profiles. Extraction methods that use organic solvents such as TRIZOL may result in inaccurate quantification because organic solvent contamination from carry-over during the RNA extraction may compress the OD 260/230 ratio. The affected 260 measurements may result in inaccurate quantification of the total RNA.

4.2. Preparation of Samples for Hybridization

1. Do not leave samples in the ice water bath for more than 15 min. Longer incubations may adversely affect the hybridization results.

2. Do not allow the pipette tip or the hybridization solution to touch the gasket walls. Allowing liquid to touch the gasket wall greatly increases the likelihood of gasket leakage.
3. When you assemble the array slide to the gasket slide, keep the array slide parallel to the gasket slide at all times and do not drop the array slide onto the gasket slide. Doing so increases the chances of samples mixing between gasket wells.
4. Be sure that the arrays are hybridized for at least 20 h. Hybridization can occur for longer than 20 h, but the actual hybridization time should be consistent if the results are to be compared. Failure to maintain consistent hybridization times may adversely affect your data.
5. If you are not loading all the available positions on the hybridization rotator rack, be sure to balance the loaded hybridization chambers on the rack so that there are an equal number of empty positions on each of the four rows on the hybridization rack.
6. During washing following hybridization, care must be taken as some detergents may leave fluorescent residue on the dishes. Do not use any detergent in the washing of the staining dishes. If detergent is used, all traces must be removed by copiously rinsing with Milli-Q water.
7. As the dyes cy3 and cy5 are sensitive to ozone levels, all assays must be performed in environment with ozone levels of 50 ppb or less.
8. The scanning settings used to acquire images must be noted and maintained for all slides.

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Chapter 20

MicroRNA Expression During Neuronal Differentiation of Human Teratocarcinoma NTera2D1 and Mouse Embryonic Carcinoma P19 Cells

Hirohiko Hohjoh

Abstract

MicroRNAs (miRNAs) are 21–23-nucleotide-long small noncoding RNAs, function as mediators in gene silencing, and play essential roles in gene regulation in development, differentiation, and proliferation. Hundreds of miRNAs have been found, and tissue-specific or organ-specific expression of miRNAs has been detected. Here, I describe procedures for detection of miRNAs in the course of neuronal differentiation of human teratocarcinoma NTera2D1 and mouse embryonic carcinoma P19 cells.

Key words: Neuronal differentiation, Retinoic acid, miR-302, miR-124a, miR-125, let-7, RT-qPCR, DNA chip

1. Introduction

MicroRNAs (miRNAs) are small noncoding RNAs, typically 21–23 nt in length, which are processed from primary miRNA transcripts forming stem-loop structure by digestion with Drosha in the nucleus and Dicer in the cytoplasm (1–3). After processing by Dicer, miRNA duplexes undergo strand selection, and single-strand miRNA elements are incorporated into the RNA-induced silencing complex (RISC) and function as mediators in gene silencing (4). miRNAs play important roles in gene regulation by inhibiting translation of messenger RNAs (mRNAs), which are partially complementary to the incorporated miRNAs, and/or by digestion of mRNAs which are nearly complementary to the miRNAs, such as RNA interference (RNAi), in the process of development, differentiation, and proliferation (5–9).

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DOI 10.1007/978-1-62703-083-0_20,

Hundreds of miRNA genes have been identified in plants and animals (1, 5, 10), and most of them appear to be expressed by RNA polymerase II (11). In addition, tissue- or organ-specific and stage-specific expression of miRNAs has been detected. In mammals, a major small RNA class transition from retrotransposon-derived small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) to zygotically expressed miRNAs occurs in the course of early development of pre-implantation embryos (12), and tissue- or organ-specific expression patterns of miRNAs are generated thereafter.

Human teratocarcinoma NTera2D1 cells and mouse embryonic carcinoma P19 cells can be induced to differentiate into neurons or neuron-like cells which exhibit differentiated morphologies with long neuritic processes (13). Since miRNAs play essential roles in cell differentiation and proliferation, it is likely that they can participate in the differentiation of NTera2D1 and P19 cells and also maintenance of stemness in an undifferentiated state of the cells. We have investigated the expression profiles of miRNAs and also mRNAs over the course of neuronal differentiation of both NTera2D1 and P19 cells (13, 14). I herein describe procedures allowing for the detection of miRNAs and mRNAs expressed in the course of the differentiation of NTera2D1 and P19 cells.

2. Materials

2.1. Cell Culture

1. Dulbecco's Modified Eagle's medium (DMEM) with 4,500 mg/L glucose (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (Sigma-Aldrich Corporation, St. Louis, MO, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich).
2. Alpha-Minimum Essential medium (α-MEM) (Wako) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich).
3. Neurobasal® medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with B-27® supplement (Invitrogen).
4. Dulbecco's Phosphate Buffered Saline [D-PBS(−)] (Sigma-Aldrich).
5. BD BioCoat™ Poly-D-Lysine plates (BD Biosciences, Franklin Lakes, NJ, USA).
6. 6-well plates with Ultra-Low Attachment surface (Corning Incorporated Life Sciences, Lowell, MA, USA).
7. All-*trans*-retinoic acid (RA) (Sigma-Aldrich) (see Note 1).
8. Dimethyl sulfoxide (DMSO) Hybri-Max™ (Sigma-Aldrich) (see Note 1).

9. Cytosine β-D-arabinofuranoside (Ara-C) (Sigma-Aldrich) (see Note 2).
10. Trypsin-EDTA solution (Sigma-Aldrich).

2.2. Isolation of Total RNAs and Preparation of Small-Sized RNAs

1. TRIzol® reagent (Invitrogen).
2. RNasay® MinElute® Cleanup kit (Qiagen, Venlo, The Netherlands).
3. UltraPure™ DNase/RNase-free distilled water (Invitrogen).

2.3. Detection of MicroRNAs and Messenger RNAs

1. Platinum*Bright* 647 Infrared nucleic acid labeling kit (Kreatech Diagnostics, Amsterdam, The Netherlands).
2. *Genopal*®-MICM07 and -MICH07 DNA chips for mouse and human miRNAs, respectively (Mitsubishi Rayon Co., Lid., Tokyo, Japan) (see Note 3).
3. *Genopal*®- DNA chip reader (Yokogawa Electric Corporation, Tokyo, Japan).
4. TaqMan® Universal PCR Master Mix (Applied Biosystems, Faster City, CA, USA).
5. TaqMan® MicroRNA Assays (Applied Biosystems).
6. NanoDrop-3300 fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA).
7. AB 7300 Real Time PCR System (Applied Biosystems).
8. AB GeneAmp® PCR System 9700 (Applied Biosystems).
9. TURBO DNA-free™ (Applied Biosystems).
10. Oligo$(dT)_{15}$ primer (Promega, Madison, WI, USA).
11. SuperScript® III Reverse Transcriptase (Invitrogen).
12. *mir*Vana™ qRT-PCR miRNA detection kit and primer sets (Applied Biosystems (Ambion)).
13. Synthetic DNA oligonucleotides for PCR primers (Sigma-Aldrich).
14. Perfect Real Time Primers (TAKARA BIO INC, Shiga, Japan).
15. TaKaRa LA Taq® DNA polymerase (TAKARA BIO).
16. NuSieve™ 3:1 agarose (TAKARA BIO).
17. Tris-Acetate-EDTA Buffer (50×) (Nacalai Tesque, Inc., Kyoto, Japan).
18. 40% Acrylamide/Bis solution, 19:1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).
19. Tris-Borate-EDTA (TBE) Buffer (10×) (Nacalai Tesque).
20. 20× SSC buffer (Bio-Rad).
21. Sodium Dodecyl Sulfate (Wako).

3. Methods

3.1. Culture of Human Teratocarcinoma NTera2D1 Cells in Normal Medium

NTera2D1 cells are grown in DMEM (Wako) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) at 37°C in 5% CO_2-humidified chamber.

3.2. Induction of Neuronal Differentiation of NTera2D1 Cells (Fig. 1)

1. The day before induction, cultured cells are trypsinized, diluted with fresh medium, seeded onto T25 (25 cm^2) tissue culture flasks (approximately 2×10^4 cells/cm^2), and incubated at 37°C in 5% CO_2-humidified chamber.
2. Induction of neuronal differentiation is initiated by changing the medium to fresh medium containing 1×10^{-5} M all-*trans*-retinoic acid (RA) (Sigma-Aldrich).
3. Culture medium is changed to fresh medium containing RA twice a week.
4. After 3~4 weeks of RA treatment, RA-treated NTera2D1 cells are trypsinized, diluted with fresh normal medium without

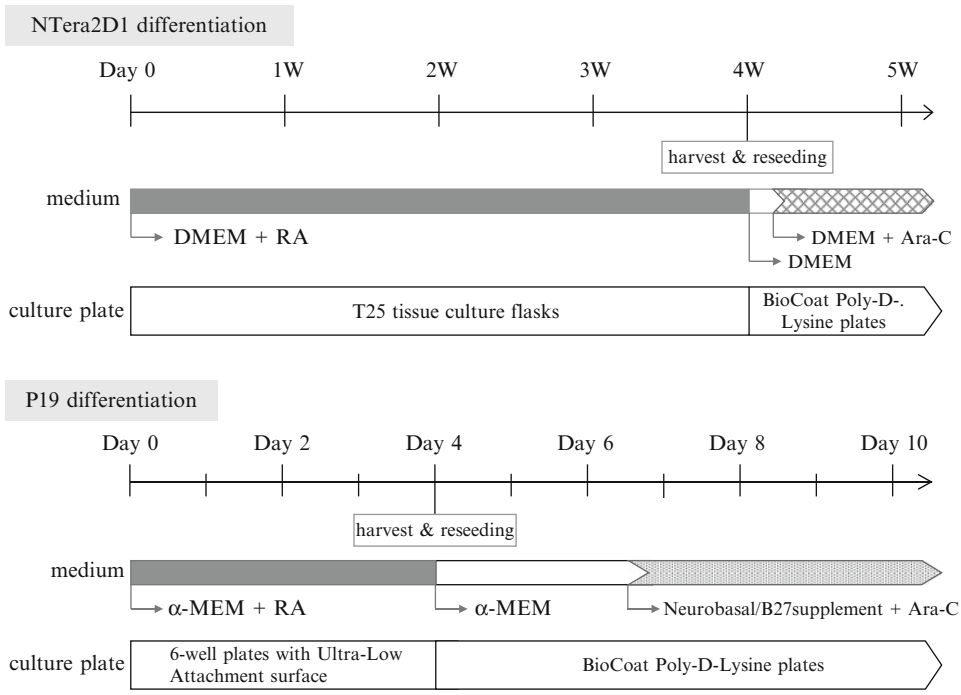

Fig. 1. Time schedule of neuronal differentiation of human NTera2D1 and mouse P19 cells. The start of the neuronal differentiation of the cells by retinoic acid (RA) is indicated by day 0. The schedules of the NTera2D1 and P19 differentiation are shown on the week (w) and day time scales, respectively. Culture medium conditions and culture plates used are indicated.

RA, seeded onto 12-well BD BioCoat™ Poly-D-Lysine plates (BD Biosciences) (approximately 1×10^5 cells/cm^2), and incubated at 37°C in 5% CO_2-humidified chamber.

5. After 2-day incubation, medium is changed to fresh medium containing 10 μM cytosine β-D-arabinofuranoside (Ara-C) (Sigma-Aldrich) (see Note 4), and further incubation is carried out.
6. Culture medium is changed to fresh medium containing Ara-C twice a week. Neurons or neuron-like cells with long neuritic processes can be observed during the incubation (Fig. 2).

3.3. Culture of Mouse Embryonic Carcinoma P19 Cells in Normal Medium

P19 cells are grown in α-MEM (Wako) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) at 37°C in 5% CO_2-humidified chamber.

3.4. Induction of Neuronal Differentiation of P19 Cells (Fig. 1)

1. Cultured P19 cells are trypsinized, diluted with fresh medium containing 5×10^{-7} M RA (Sigma-Aldrich) (approximately 1×10^5 cells/mL), seeded into 6-well plates with Ultra-Low Attachment surface (Corning) (2 mL/well) (see Note 5), and then incubated at 37°C in 5% CO_2-humidified chamber.

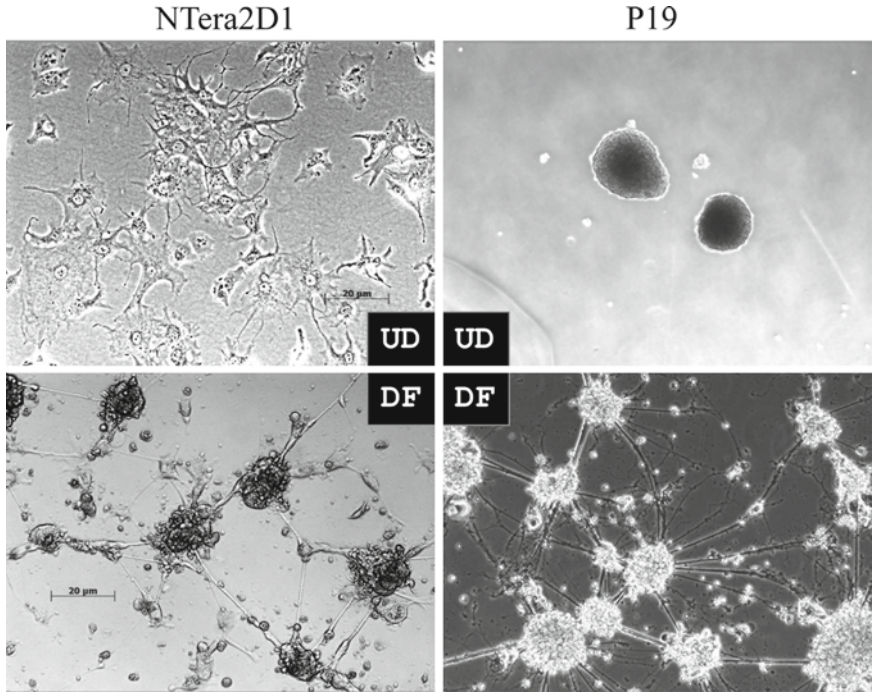

Fig. 2. Morphological differentiation of NTera2D1 and P19 cells. Neuronal differentiation of NTera2D1 and P19 cells was carried out. Undifferentiated (UD) and completely differentiated (DF) cells are shown. Undifferentiated P19 cells indicate clumps of the cells which are grown in culture plates with Ultra-Low Attachment surface. The photo of undifferentiated NTera2D1 cells was reprinted from (13).

2. After 2-day incubation, culture medium is changed to fresh medium containing RA.
   (a) Collect culture medium, where clumps of P19 cells are present (see Note 6), by using a 5 mL measuring pipette and transfer the medium into a 15 mL centrifuge tube.
   (b) Stand the tube straight up for 5 ~ 10 min to precipitate the cells spontaneously.
   (c) Remove the supernatant by a pipette (see Note 7).
   (d) Add fresh medium containing 5×10^{-7} M RA and pipette twice carefully such that the clumps of P19 cells are not collapsed.
   (e) Transfer the medium containing the clumps of the cells into 6-well plates with Ultra-Low Attachment surface (Corning) (2 mL/well).
3. Incubate cells at 37°C in 5% CO_2-humidified chamber for two more days.
4. After 4-day induction with RA, clumps of the cells are collected by spontaneous precipitation as described above, and rinsed twice with D-PBS(−) followed by centrifugation.
5. Precipitated cells are subjected to trypsinization as follows:
   (a) Add approximately 1/10 packed cell volume of Trypsin-EDTA solution (Sigma-Aldrich) to the precipitated cells, and mix them well by pipetting several times.
   (b) Add fresh normal medium (~ 5 mL) for quenching trypsin activity.
   (c) Collect the cells by centrifugation and re-suspend them with fresh normal medium.
6. Trypsinized cells are seeded onto 12-well BD BioCoat Poly-D-Lysine plates (BD Biosciences) (approximately 1×10^5 cells/cm^2), and incubated at 37°C in 5% CO_2-humidified chamber.
7. After 3-day incubation, medium is changed to Neurobasal medium (Invitrogen) containing B-27 supplement (Invitrogen) and 10 μM Ara-C (Sigma-Aldrich) (see Note 4), and cells are further incubated.
8. Medium is changed to fresh Neurobasal medium containing B-27 supplement and 10 μM Ara-C every 2 or 3 days. Neurons or neuron-like cells with long neuritic processes can be observed during the incubation (Fig. 2).

3.5. Extraction of Total RNAs

1. Total RNAs are extracted from undifferentiated and differentiated cells at various time points in the course of neuronal differentiation by TRIzol reagent (Invitrogen) according to the instructions of the manufacturer.

2. Extracted RNAs are dissolved in DNase/RNase-free distilled water (Invitrogen).
3. Store at –20°C.

3.6. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

1. Total RNAs are treated with TURBO DNA-free™ (Applied Biosystems) according to the instructions of the manufacturer.
2. Complementary DNA (cDNA) synthesis is carried out with the treated RNA (1 μg/test) as a template using oligo(dT) primer (Promega) and SuperScript® III reverse transcriptase (Invitrogen), according to the instructions of the manufacturer.
3. The resultant cDNA is subjected to PCR analysis using the AB GeneAmp® PCR System 9700 (Applied Biosystems) with TaKaRa LA Taq® DNA polymerase (TAKARA BIO) and following synthetic primers:

   Mouse *Pou5f1*-F; 5′-AAGCTGCTGAAGCAGAAGAGGATC-3′.

   Mouse *Pou5f1*-R; 5′-ACCTCACACGGTTCTCAATGCTAG-3′.

   Mouse *Map2*-F; 5′-TTAAACAGGCGAAGGATAAAGTCAC-3′.

   Mouse *Map2*-R; 5′-TGATTGCAGTTGATCCAGGGGTAG-3′.

   Perfect Real Time Primers (TAKARA Bio) against the human *POU5F1*, *MAP2*, *NEFL*, and *GAPDH* genes, and against the mouse *Nefl* and *Gapdh* genes.
4. Thermal cycling profile:
   (a) Heat denaturation at 95°C at 2 min.
   (b) 25~30 cycles of amplification including denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s.
   (c) Final extension at 72°C for 5 min.
5. PCR products are examined by agarose gel electrophoresis with 3% NuSieve™ 3:1 agarose gels (TAKARA BIO) (see Note 8) and ethidium bromide staining (Fig. 3).

3.7. Expression Profile Analysis of miRNAs Using DNA Chips

1. Small-sized RNAs containing miRNAs are prepared from total RNAs by an RNeasy® MinElute® Cleanup kit (Qiagen) according to the instructions of the manufacturer.
2. The small-sized RNAs (~1 μg) are subjected to direct labeling with a fluorescent dye using a Platinum*Bright* 647 Infrared nucleic acid labeling kit (KREATECH) according to the instructions of the manufacturer.

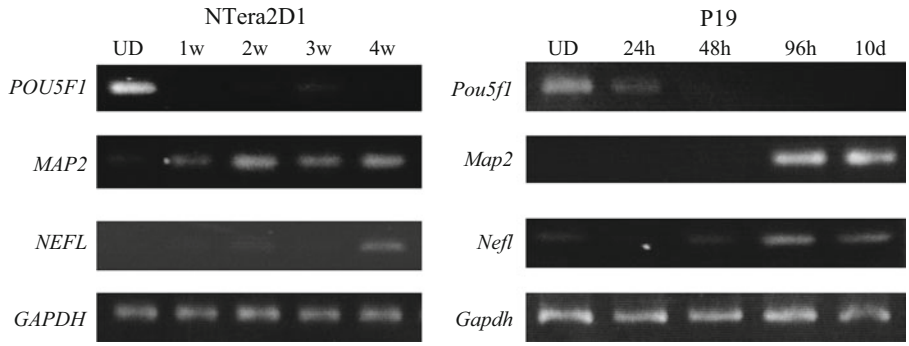

Fig. 3. Expression profiles of protein-coding genes related to neuronal differentiation. Total RNAs were prepared from undifferentiated (UD) NTera2D1 and P19 cells and from the cells at various periods (indicated) after induction of differentiation: 1–4 weeks (1w–4w) in NTera2D1 cells and 24 h to 10 days (24 h–10 d) in P19 cells. The RNAs were subjected to RT-PCR analysis to examine the expression of genes indicated. The examined genes were as follows: *POU Domain*, *Class 5*, *Transcription Factor 1* (*POU5F1*); *Microtubule-Associated Protein 2* (*MAP2*); *Neurofilament Protein*, *Light Polypeptide* (*NEFL*); and *Glyceraldehyde-3-Phosphate Dehydrogenase* (*GAPDH*) as a control. The results indicate that: (i) the *POU5F1* expression was detected in both the undifferentiated NTera2D1 and P19 cells, but rapidly reduced by retinoic acid (RA) treatment, and (ii) the expression of *MAP2* and *NEFL*, which are specific to the neuron, increased after RA treatment and their expression was also detected in the differentiated neuronal cells. The data were reprinted from (13).

3. After fluorescent labeling, the labeled RNAs are purified from free fluorescent substrates by the KREA*pure* columns (KREATECH).
4. The fluorescent intensity of the labeled RNAs is measured by the NanoDrop-3300 fluorospectrometer (Thermo Fisher Scientific).
5. Approximately 10^5 relative fluorescence units (rfu) of each labeled RNA sample is used in hybridization.
6. Hybridization is carried out with the *Genopal*® DNA chips (Mitsubishi Rayon) in 100 μL of hybridization buffer (6× SSC, 0.2% SDS and heat-denatured labeled RNAs (~10^5 rfu)) at 42°C overnight using a hybridization chamber specific for the *Genopal*®-DNA chip (Mitsubishi Rayon) (see Note 3).
7. After hybridization, the DNA chips are washed twice in 2× SSC containing 0.2% SDS at 50°C for 20 min followed by washing in 2× SSC at 50°C for 10 min.
8. Hybridization signals are examined by a DNA chip reader adopting multi-beam excitation technology according to the instructions of the manufacturer (Yokogawa Electric Corporation, Tokyo, Japan) (see Note 9) (Figs. 4 and 5).

3.8. RT-quantitative PCR (RT-qPCR) for miRNAs

1. An aliquot of total RNA isolated is diluted in DNase/RNase-free water, and the concentration of the total RNA is adjusted to 2 μg/mL.
2. 10 ng of total RNA per test is used (see Note 10) for synthesizing cDNAs of miRNAs.

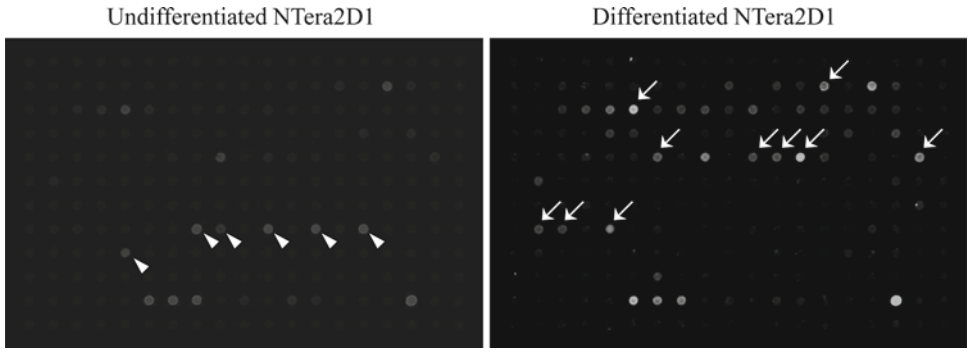

Fig. 4. Expression profiles of miRNAs between undifferentiated and differentiated NTera2D1 cells. Small-sized RNAs prepared from undifferentiated and differentiated NTera2D1 cells were examined by *Genopal*®-MICH07 DNA chips. MicroRNAs markedly expressed in undifferentiated and differentiated NTera2D1 cells are indicated by *arrowheads* and *arrows*, respectively. The data were reprinted from (13).

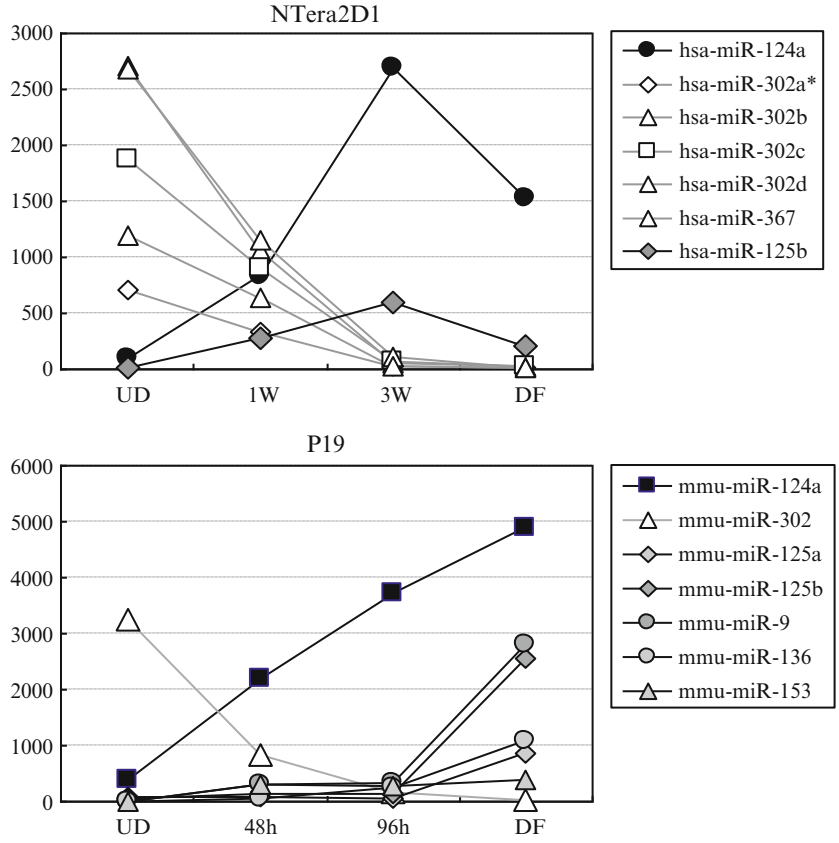

Fig. 5. Expression profiles of miRNAs during neuronal differentiation of NTera2D1 and P19 cells. Small-sized RNA samples were extracted from the cells at the indicated time points after retinoic acid (RA) treatment (1 and 3 weeks (W) in NTera2D1; 48 and 96 h (h) in P19) and examined together with the samples prepared from undifferentiated (UD) and differentiated (DF) cells by *Genopal*®-DNA chips. The expression levels of miRNAs are represented by their hybridization signal intensities and indicated by arbitrary units. Markedly increased and decreased miRNAs during neuronal differentiation are indicated by solid and open marks, respectively. The data indicated that the expression of the *miR-302* members, which are ES-specific miRNAs, markedly decreased after induction of neuronal differentiation by RA, and on the contrary that *miR-124a*, *miR-9*, and *miR-125*, which are brain-specific miRNAs, increased in their levels after RA treatment. The data were reprinted from (13).

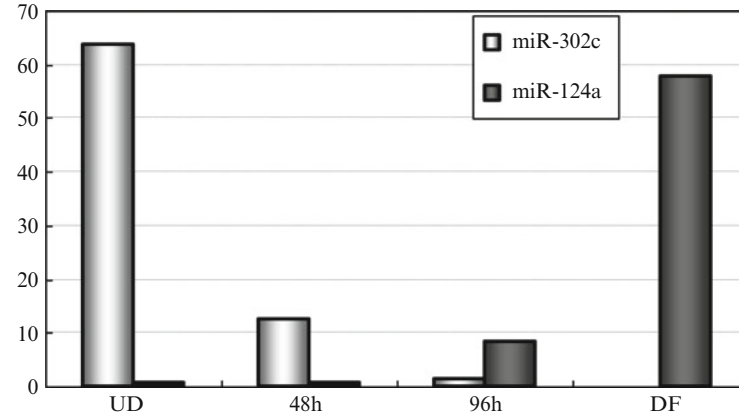

Fig. 6. RT-qPCR analysis of *miR-124a* and *miR-302c* during neuronal differentiation of P19 cells. Total RNAs were extracted from P19 cells at the indicated time points as in Fig. 5 and examined by RT-qPCR. The expression levels of *miR-124a*, *miR-302c,* and *U6RNA* examined as a control were analyzed by the cycle threshold (Ct) method and plotted when the expression level of *U6RNA* was 1. The data were consistent with the results of Fig. 5. The data were reprinted from (13).

3. cDNA synthesis is performed by the AB GeneAmp® PCR System 9700 (Applied Biosystems) with a TaqMan® MicroRNA Reverse Transcription kit together with TaqMan® MicroRNA Assays (Applied Biosystems) according to the instructions of the manufacturer.
4. Real time PCR (qPCR) analysis is subsequently carried out by means of the AB 7300 Real Time PCR System (Applied Biosystems) with a TaqMan® Universal PCR Master Mix and TaqMan® MicroRNA Assays (Applied Biosystems) according to the instructions of the manufacturer (Fig. 6).

3.9. End-Point PCR Analysis

End-point PCR analysis for *let-7a* and *5sRNA* as a control is carried out using the AB GeneAmp PCR system 9700 (Applied Biosystems) with a *mir*Vana™ qRT-PCR miRNA detection kit (Ambion) and primer sets (Ambion) according to the instructions of the manufacturer (see Note 11). The resultant PCR products are electrophoretically separated on 12% polyacrylamide gels (see Note 12) and visualized by ethidium bromide staining (Fig. 7).

4. Notes

1. Dissolve RA in DMSO and prepare 1×10^{-2} M RA stock solution. The RA solution is stored in light-blocking tubes at –20°C.
2. Dissolve Ara-C in D-PBS(–) and prepare 1 mM Ara-C stock solution. The Ara-C stock solution is stored at –20°C.

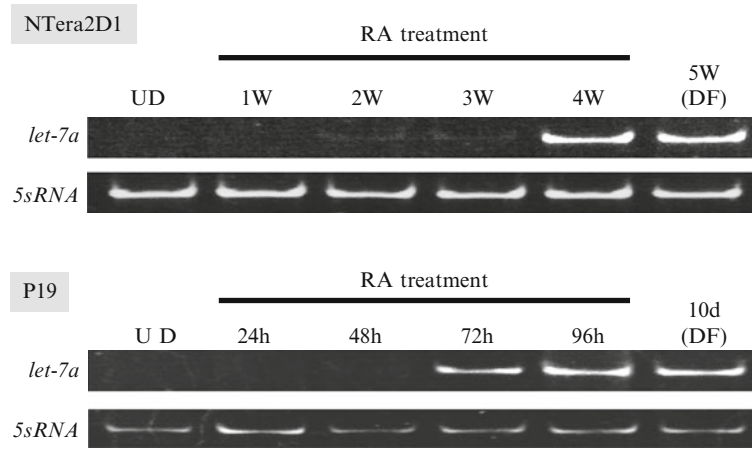

Fig. 7. Expression profile of *let-7* miRNA during neuronal differentiation of NTera2D1 and P19 cells. Total RNAs were extracted from NTera2D1 and P19 cells as in Figs. 3 and 5 and subjected to End-point PCR analysis for *let-7* and *5sRNA* as a control. The PCR products were analyzed by gel electrophoresis with 12% polyacrylamide gels followed by ethidium bromide staining. The data of P19 cells were reprinted from (14).

3. *Genopal*®- DNA chip is a novel, fibrous DNA chip that allows for real time detection of hybridization signals at every step of washing and thus provides highly reproducible expression profile data, although there is a limit to the number of probes (up to two hundred probes) in the chip (15). The list of the miRNAs, which can be detected by the *Genopal*®-MICM07 and -MICH07 DNA chips, can be found at the following address: http://www.mrc.co.jp/genome/pdf/micm07_list.pdf.
4. Ara-C is a pyrimidine analog and interferes with the synthesis of DNA, thereby destroying cells that are dividing rapidly. If Ara-C is absence, glial-like cells could occupy a majority of culture plate.
5. Disposable plastic petri dishes for bacteria can be used in place of culture plates with Ultra-Low Attachment surface.
6. P19 cells aggregate in culture plates with low attachment surface, thereby forming clumps of the cells.
7. Take care not to remove precipitated cells.
8. 3% NuSieve™ 3:1 agarose gels are prepared in 1× Tris-Acetate-EDTA (TAE) buffer.
9. When the washing is insufficient (cross hybridization is suspected), re-washing of the chips can be done. This is because hybridization signals can be examined with the chips soaking in washing buffer (15).

10. To obtain reproducible qPCR results, it is important to take and use an exact 5 μL of 2 μg/mL total RNA solution per test. Thus, I recommend using a fine automatic pipette and low retention tips.
11. To perform quantitative analysis, it is important to take and use precise amount of total RNA as a source in the PCR analysis. I recommend using a fine automatic pipette and low retention tips as in see Note 10.
12. 12% Polyacrylamide gels (19:1) are prepared in 1× TBE buffer.

Acknowledgments

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Chapter 21

Isolation and Identification of Gene-Specific MicroRNAs

Shi-Lung Lin, Donald C. Chang, and Shao-Yao Ying

Abstract

Computer programming has identified hundreds of genomic hairpin sequences, many with functions remain to be determined. Because direct transfection of hairpin-like miRNA precursors (pre)-miRNAs in mammalian cells is not always sufficient to trigger effective RNA-induced gene silencing complex (RISC) assembly, a key step for RNA interference (RNAi)-related gene silencing, we developed an intronic miRNA-expressing system to overcome this problem by inserting a hairpin-like pre-miRNA structure into the intron region of a gene and successfully increased the efficiency and effectiveness of miRNA-associated RNAi induction in vitro and in vivo. This intronic miRNA biogenesis has been found to depend on a coupled interaction of nascent precursor messenger RNA transcription and intron excision within a specific nuclear region proximal to genomic perichromatin fibrils. The intronic miRNA was transcribed by RNA type II polymerases, coexpressed with a primary gene transcript, and excised out of its encoding gene transcript by intracellular RNA splicing and processing mechanisms. Currently, some ribonuclease III endonucleases have been found to be involved in the processing of spliced introns and probably facilitating the intronic miRNA maturation. Using this miRNA generation system, we have shown for the first time that the intron-derived miRNAs were able to induce strong RNAi effects in not only human and mouse cells but also zebrafishes, chicken embryos, and adult mice. We have also developed an miRNA isolation protocol, based on the complementarity between the designed miRNA and its target gene sequence, to purify and identify the mature miRNAs generated by the intronic miRNA-expressing system. Several intronic miRNA identities and structures are currently confirmed to be active in vitro and in vivo. According to this proven-of-principle method, we now have full knowledge to design pre-miRNA inserts that are more efficient and effective for the intronic miRNA-expressing systems.

Key words: microRNA biogenesis, Gene cloning, RNA interference, RNA-induced gene silencing complex, Asymmetric assembly, Zebrafish

1. Introduction

More than 90 intronic miRNAs have been identified using the bioinformatic approaches to date (1), but the functions of the vast majority of these intronic molecules remain to be determined.

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DOI 10.1007/978-1-62703-083-0_21,

According to the strictly expressive correlation of intronic miRNAs to their encoded genes, one may speculate that the levels of condition-specific, time-specific, and individual-specific gene expression are determined by interactions of distinctive miRNAs on single or multiple gene modulation. This interpretation accounts for more accurate genetic expression of various traits and any dysregulation of the interactions will thus result in genetic diseases. For instance, monozygotic twins frequently demonstrate slightly, but definitely distinguishing, disease susceptibility and physiological behavior. Such as, a long CCTG expansion in the intron 1 of a zinc finger protein ZNF9 gene has been correlated to type 2 myotonic dystrophy in either one of the twins with higher susceptibility (2). Since the expansion motif obtained high affinity to certain RNA-binding proteins, the interfering role of intron-derived expansion fragments remains to be elucidated. Another more established example involving intronic expansion fragments in its pathogenesis is fragile X syndrome, which represents about 30% of human-inherited mental retardation. Intronic CGG repeat (rCGG) expansion in the 5′-UTR of *FMR1* gene is the causative mutation in 99% of individuals with fragile X syndrome (3). *FMR1* encodes an RNA-binding protein, FMRP, which is associated with polyribosome assembly in an RNP-dependent manner and capable of suppressing translation through an RNAi-like pathway. FMRP also contains a nuclear localization signal (NLS) and a nuclear export signal (NES) for shuttling certain mRNAs between the nucleus and cytoplasm (4). Jin et al. proposed an RNAi-mediated methylation model in the CpG region of *FMR1* rCGG expansion, which is targeted by a hairpin RNA derived from the 3′-UTR of the *FMR1* expanded allele transcript (3). The Dicer-processed hairpin RNA triggers the formation of RNA-induced initiator of transcriptional gene silencing (RITS) on the homologous rCGG sequences and leads to heterochromatin repression of the *FMR1* locus. These examples suggest that natural evolution gives rise to more complexity and more variety of introns in higher animals and plants for coordinating their vast gene expression volumes and interactions; therefore, any dysregulation of miRNAs derivation from introns may thus lead to genetic diseases involving intronic expansion or deletion, such as myotonic dystrophy and fragile X mental retardation.

To understand the disease caused by dysregulation of intronic miRNAs, an artificial expression system is needed to recreate the function and mechanism of the miRNA in vivo. The same approach may be used to develop and test therapies for the disease. Using artificial introns carrying hairpin-like miRNA precursors (pre-miRNA), we have successfully generated mature miRNA molecules with full function in triggering RNAi-like gene silencing in zebrafish (5). We introduced hairpin-like pre-miRNAs into 2-week-old zebrafish larvae and successfully tested the processing and functional significance of different miRNA–miRNA* structures, using

an intronic miRNA expression system described previously (6, 7). The pre-miRNA expression was driven by a cytomegalovirus (CMV) IE promoter, which has been previously established as a viable approach for manipulation of mRNA expression in zebrafish (8). Based on conventional reasoning, the stem-loop structure located in either end of the miRNA-miRNA* duplex should be equally cleaved by Dicer in order to form siRNA; therefore, functional bias would not be observed in the stem-loop. However, we observed different gene silencing responses when the transfection results from a pair of symmetric hairpin constructs between 5′-miRNA*-stem-loop-miRNA-3′ and 5′-miRNA-stem-loop-miRNA*-3′ pre-miRNAs were compared; both contained the same perfectly matched siRNA-like duplex stem-arm (shown in Fig. 1a). Different mature miRNAs were identified from the transfections of the pre-miRNAs, suggesting that the stem-loop structures of these pre-miRNAs can affect Dicer recognition and result in different asymmetry of the siRNA-like stem-arm construct. This type of asymmetry leads to strand selection of the mature miRNA for effective RISC assembly.

To determine the structural preference of the hairpin pre-miRNAs, we have isolated the small RNAs in zebrafish using *mir*Vana miRNA isolation columns (Ambion, Austin, TX) and then precipitated all potential miRNAs complementary to the target *EGFP* region using latex beads containing the target RNA sequence. The designed pre-miRNA constructs were directed against the target green fluorescent protein (EGFP) mRNA sequence nucleotides 280–302 in the transgenic (*UAS:gfp*) zebrafish, of which the *EGFP* expression was constitutively driven by the β-actin promoter in almost all cell types. As shown in Fig. 1b, two major miRNA identities were verified to be active (gray-shading sequences). Because of the fast turn-over rate of small RNAs in vivo (9, 10), the shorter miR-EGFP(282/300) is likely to be a stably degraded form of the miR-EGFP(280–302). The first 5′-cytosine (labeled by an asterisk *) of the miR-EGFP(280–302) was not included in the designed target region and probably provided by the original intron sequence because the cytosine (C*) is the most adjacent nucleotide to the 5′-end of the designed pre-miRNA structure in the intron. Because the effective miR-EGFP(280–302) miRNA was detected only in the zebrafish transfected by the 5′-miRNA-stem-loop-miRNA*-3′ construct (❷), the stem-loop of the construct ❷, rather than the construct ❶, pre-miRNA is able to determine the correct antisense EGFP domain for microRISC (miRISC) assembly. Given that Dicer cleavage resulted in distinct mature miRNAs from both pre-miRNA constructs (Fig. 2), switching the pre-miRNA stem-loop differed in orientation. One possibility for this preference is that the structure and/or sequence of the stem-loop preferably facilitates miRNA maturation from one orientation than the other. Alternatively, the stem-loop may change the Dicer recognition

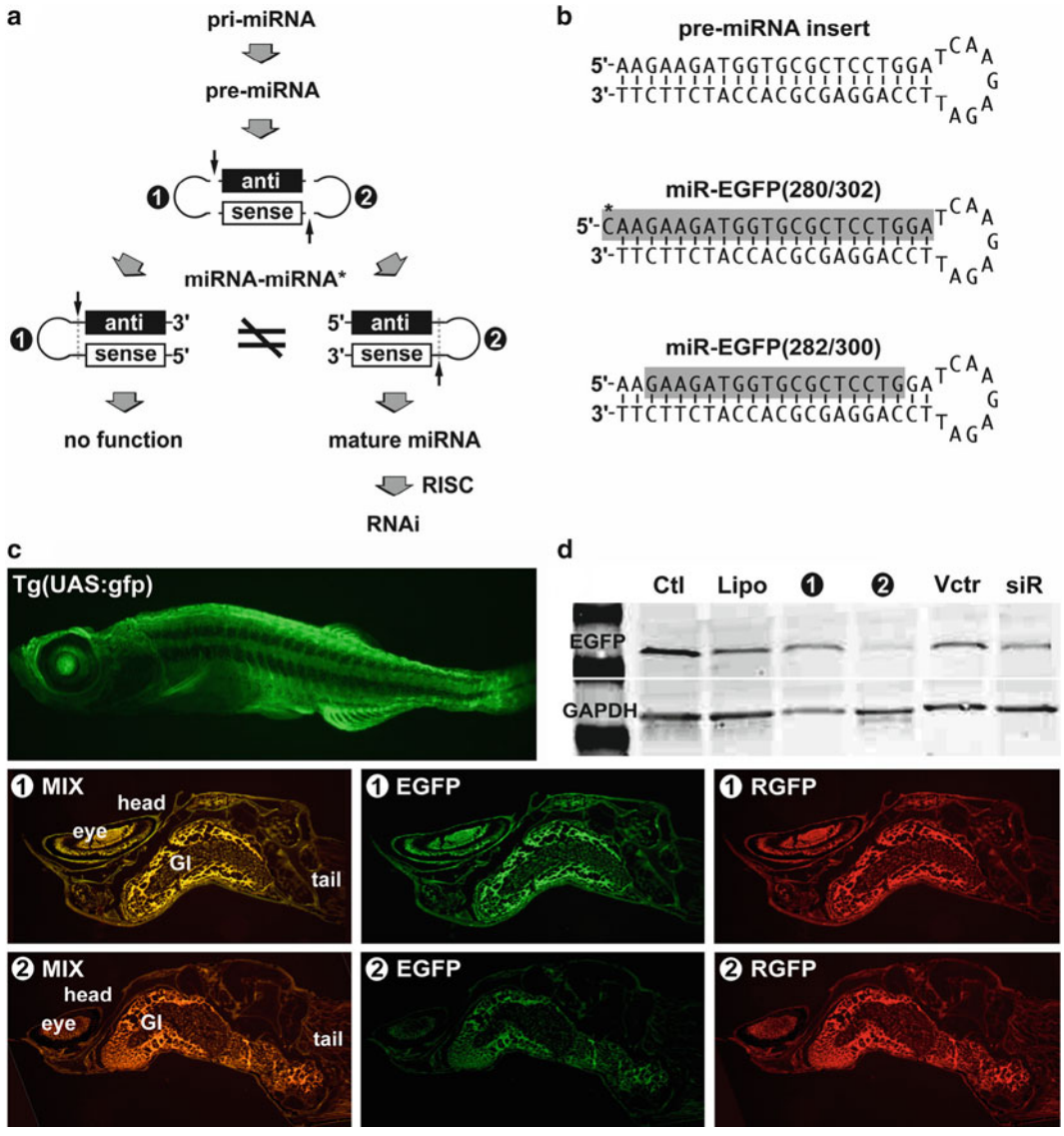

Fig. 1. Structural preference of miRNA–miRNA* asymmetry in miRISC in vivo. (**a**) We have demonstrated that only the ❷ construct was used in effective miRISC assembly. Because the miRNA is predicted to be complementary to its target messenger RNA, the "antisense" (*black bar*) refers to the miRNA and the "sense" (*white bar*) refers to its complementarity, miRNA*. (**b**) Two designed mature miRNA identities were found only in the ❷-transfected zebrafishes, namely miR-EGFP(280/302) and miR-EGFP(282/300). (**c**) In vivo gene silencing efficacy was only observed in the transfection of the ❶ pre-miRNA construct, but not the ❶ construct. Since the mixture color of EGFP and RGFP displayed more *red* than *green* (as shown in *deep orange*), the expression level of target EGFP (*green*) was significantly reduced in ❷, while miRNA indicator RGFP (*red*) was evenly present in all vector transfections. A strong strand selection was observed in favor of the 5′-stem-loop strand of the designed pre-miRNAs in RISC. (**d**) Western blot analysis of protein expression levels confirmed the specific EGFP silencing result of (**c**). No detectable gene silencing was observed in fishes without (Ctl) and with liposome only (Lipo) treatments. The transfection of either a U6-driven siRNA vector (siR) or an empty vector (Vctr) without the designed pre-miRNA insert resulted in no gene silencing significance.

Fig. 2. Bias of miRNA–miRNA* asymmetry in miRISC in vivo. Different preferences of RISC assembly were observed in the transfections of 5′-miRNA*-stem-loop-miRNA-3′ (❶) and 5′-miRNA-stem-loop-miRNA*-3′ (❷) pre-miRNA constructs in zebrafish, respectively. Based on the assembly rule of siRISC, the processing of both ❶ and ❷ pre-miRNAs should result in the same siRNA duplex for RISC assembly; however, the present experiments demonstrate that only the ❷ construct was able to silence target *EGFP*. An effective mature miRNA, namely miR-EGFP(280/302), was detected in the ❷-transfected zebrafishes directed against target *EGFP*, whereas transfection of the ❷ construct produced a different mature miRNA, miR*-EGFR(301–281), which was partially complementary to the miR-EGFP(280/302) and possessed no gene silencing effects on EGFP expression.

and, thus, may generate differently asymmetric profiles to the pre-miRNA stem-arm. In either way, the cleavage site of Dicer in the pre-miRNA stem-arm determines the strand selection of a mature miRNA, and the pre-miRNA stem-loop likely functions as a determinant for the recognition of the special cleavage site. Based on this proven principle of the intronic pre-miRNA structures, we are now able to design correct and effective pre-miRNA inserts for the intronic miRNA-generating systems.

2. Materials

2.1. Small RNA Isolation

1. *mir*Vana miRNA isolation kit (Ambion, Austin, TX).
2. Incubation chamber: 65°C and 4°C.
3. 1× Nuclease-free hybridization buffer: 100 mM KOAc, 30 mM HEPES KOH, 2 mM, pH 7.4 at 25°C (see Note 1).
4. Microcentrifuge: 17,900 × *g* (see Note 2).

2.2. Complementary Affinity Precipitation And Poly(A) Tailing

1. Synthetic 5′-fluorescein-linked oligonucleotides homologous to the target gene sequence (Sigma-Genosys), e.g., as shown here a synthetic anti-EGFP oligonucleotide 5′-fluorescein-AGAAGATGGT GCGCTCCTGG A-3′ (100 pmol/μL in DEPC-treated ddH_2O).
2. Anti-fluorescein monoclonal antibody, biotin-conjugated (Invitrogen, Carlsbad, CA).
3. Streptavidin bead suspension (Invitrogen).

4. Incubation shaker: 25°C, 120 rpm.
5. Incubation chamber: 65°C and 4°C.
6. Microcentrifuge: 17,900 × *g*.
7. DEPC-treated H_2O: Stir double distilled water with 0.1% DEPC for longer than 12 h and autoclave twice at 120°C under approx 1.2 kgf/cm² for 20 min twice.
8. RNA tailing: Poly(A) tailing kit (Ambion, Austin, TX).

2.3. Complementary DNA Generation and Polyacrylamide Gel Purification

1. Oligo$(dT)_{20}$ primer: 5′-dephosphorylated TTTTTTTTTT TTTTTTTTTT-3′ (100 pmol/μL in DEPC-treated ddH_2O).
2. 100 U/μL SuperScript II MMLV reverse transcriptase and 5× reverse transcription buffer (250 mM Tris–HCl, pH 8.3, 250 mM KCl, 15 mM $MgCl_2$, and 10 mM dithiothreitol).
3. Reverse transcription mix: 8 μL DEPC-treated ddH_2O, 6 μL of 5× reverse transcription buffer, 2 μL of 10 mM deoxyribonucleoside triphosphate mix (10 mM each for deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytosine triphosphate, and deoxythymidine triphophate), 1 μL of 25 U/μL RNase Inhibitor, 2 μL SuperScript II MMLV reverse transcriptase; prepare the reaction mix just before use.
4. Incubation chamber: 94°C, 65°C, 42°C, and 4°C.
5. Electrophoresis system for polyacrylamide gel (Bio-Rad, Hercules, CA).
6. 15% Tris-borate-ethylenediaminetetraacetic acid/urea polyacrylamide gel for oligonucleotides (Bio-Rad).
7. Mini whole gel eluter (Bio-Rad).

3. Methods

3.1. Small RNA Isolation

The small intracellular RNAs (fewer than 200 nucleotides) are isolated and collected on a glass-fiber filter using the *mir*Vana miRNA isolation kit. These small RNAs include 5 S ribosomal RNA, transfer RNA, small nucleolar RNA (snoRNA), small nuclear RNA, small mitochondrial noncoding RNA (smnRNA), miRNA, and probably siRNA.

1. Small RNA isolation: Apply 100–10^7 cells to a *mir*Vana miRNA isolation reaction, following the manufacturer's protocol. Collect the final RNAs in 30 μL of 1× nuclease-free hybridization buffer.

3.2. Complementary Affinity Precipitation and Poly(A) Tailing

Small RNAs complementary to the target sequence are recovered by binding to fluorescein-linked target oligonucleotides and then precipitated by further binding to biotin-conjugated anti-fluorescein

monoclonal antibodies and streptavidin beads. The resulting small RNAs are protected by adding poly(A) tails in their 3′-termini using *Escherichia coli* poly(A) polymerase I.

1. Complementary annealing: Add 2 μL of synthetic 5′-fluorescein-linked oligonucleotides to the isolated small RNAs and mix well. Incubate the mixture in an incubation chamber at 65°C for 5 min, then switch the mixture to an incubation shaker at 25°C for 30 min.
2. Precipitation: Add 10 μL of the biotin-conjugated anti-fluorescein monoclonal antibodies to the mixture and incubate the mixture in an incubation shaker at 25°C for 30 min. Add 10 μL of streptavidin bead suspension to the mixture and continue to incubate the mixture in an incubation shaker at 25°C for 30 min. Precipitate the bound RNAs by centrifugation at 17,900 × *g* for 10 min and discard the supernatant. Dissolve the pellet in 10 μL of DEPC-treated ddH_2O.
3. Poly(A) RNA tailing: Add poly(A) tails to the 3′-termini of the purified RNAs using *E. coli* poly(A) polymerase I, following the manufacturer's suggestions.
4. Reaction stop: Heat the reaction at 94°C for 2 min and then cool on ice immediately. Precipitate the beads by centrifugation at 17,900 × *g* for 10 min and transfer the supernatant to a clean new tube.

3.3. Complementary DNA Generation and Polyacrylamide Gel Purification

The starting material is approx 1 μg of the poly(A)-tailed small RNAs. The complementary DNAs are synthesized by reverse transcription from the poly(A)-tailed small RNAs with the oligo(dA) primer. The use of MMLV reverse transcriptase also adds a short poly(dC) tail in the 3′-end of each cDNA sequence, which is used for DNA sequencing in conjunction with a poly$(dG)_{10}$ primer.

1. Primer annealing: Mix 10 μL of the RNA supernatant with 1 μL oligo$(dT)_{20}$ primer, heat to 65°C for 5 min to minimize secondary structures, and then cool on ice.
2. Complementary DNA (cDNA) synthesis: Add 14 μL of the reverse transcriptase mix to the above hybrids, heat to 42°C for 20 min and then cool on ice.
3. Denaturation of RNA-cDNA hybrids: Heat the reaction at 94°C for 2 min and then cool on ice immediately.
4. Polyacrylamide gel electrophoresis: Load and run the denatured cDNAs on a 15% Tris-borate-ethylenediaminetetraacetic acid/urea gel and recover each cDNA band using the mini whole gel eluter system, following the manufacturer's suggestions. The cDNAs thus obtained are ready for DNA sequencing using the poly$(dG)_{10}$ primer. The resulting cDNA sequences are perfectly complementary to the miRNAs, from which the cDNAs are reverse-transcribed (see Note 3).

4. Notes

1. Autoclave the 1× hybridization buffer twice at 120°C under about 1.2 kgf/cm² for 20 min.
2. Relative centrifugal force (RCF) (g) = (1.12×10^{-5}) $(\text{rpm})^2 r$, where r is the radius in centimeters measured from the center of the rotor to the middle of the spin column, and rpm is the speed of the rotor in revolutions per minute.
3. Although most of the native pre-miRNAs contain mismatched area in their stem-arms, it is not necessary for us to construct an imperfect paired stem-arm in order to trigger RNAi-related gene silencing. Previous studies have demonstrated that a mature miRNA can be generated by placing a perfectly matched siRNA duplex in the miR-30 pre-miRNA structure (11, 12). Furthermore, there are many genes not subjected to the regulation of native miRNAs, in particular, *EGFP*, which can be otherwise silenced by intracellular transfection of the pre-miRNA containing a perfectly matched stem-arm construct. Therefore, we define a mature miRNA based on its biogenetic function and mechanism, rather than the structural complementarity of its precursor. In this view, any small hairpin RNA can be a pre-miRNA if a mature miRNA is successfully processed from the small hairpin RNA and further assembled into miRISC for target gene silencing.

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Chapter 22

Transgene-Like Animal Models Using Intronic MicroRNAs

Shi-Lung Lin, Shin-Ju E. Chang, and Shao-Yao Ying

Abstract

Transgenic animal models are valuable tools for testing gene functions and drug mechanisms in vivo. They are also the best similitude for a human body for etiological and pathological research of diseases. All pharmaceutically developed drugs must be proven to be safe and effective in animals before approval by the Food and Drug Administration to be used in clinical trials. To this end, the transgenic animal models of diseases serve as the front line of drug evaluation. However, there is currently no transgenic animal model for microRNA (miRNA) research. miRNAs, small single-stranded regulatory RNAs capable of silencing intracellular gene transcripts (mRNAs) that contain either complete or partial complementarity to the miRNA, are useful for the design of new therapies against cancer polymorphism and viral mutation. Recently, varieties of natural miRNAs have been found to derived from hairpin-like RNA precursors in almost all eukaryotes, including yeast (*Schizosaccharomyces pombe*), plant (*Arabidopsis* spp.), nematode (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*), fish, mouse, and human, involving intracellular defense against viral infections and regulation of certain gene expressions during development. To facilitate the miRNA research in vivo, we have developed a state-of-the-art transgenic strategy for silencing specific genes in zebrafish, chicken, and mouse, using intronic miRNAs. By insertion of a hairpin-like pre-miRNA structure into the intron region of a gene, we have found that mature miRNAs were successfully transcribed by RNA polymerases type II (Pol II), coexpressed with the encoding gene transcript, and excised out of the encoding gene transcript by natural RNA splicing and processing mechanisms. In conjunction with retroviral transfection systems, the designed hairpin-like pre-miRNA construct was further tested to insert into the intron regions of a cellular gene for tissue-specific expression regulated by the gene promoter. Because the retroviral vectors were randomly integrated into the genome of its host cell, the most effective transgenic animal can be selected and propagated to be a stable transgenic line for future research. Here, we have shown for the first time that transgene-like animal models were generated using the intronic miRNA-expressing system described previously, which has been proven to be useful for both miRNA research and in vivo evaluation of miRNA-associated target gene functions.

Key words: MicroRNA, RNA interference, Transgenic animal, Type II RNA polymerases type, RNA splicing, Intron, Embryonic development

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1. Introduction

Conventional transgenic animal models rely on the utilization of antisense oligonucleotide and dominant-negative technologies to generate loss-of-function mutants in vivo. Antisense oligonucleotides complementary to a gene transcript (messenger RNA (mRNA)) directly bind to the mRNA and either degrade the target-mRNA or to suppress the translation of the gene, whereas dominant-negative methods generate defective proteins to negatively compete with a targeted normal protein function. Both methods contain problems for transgenic animal research. Single-stranded antisense DNA or RNA, including phosphothio-, methylthio-, and morpholino-oligonucleotides, are inefficient—effective only for a short time, and usually required for pharmacological (nearly toxic) dosage to be effective. However, dominant-negative proteins cannot completely eliminate normal protein function and frequently generate inconsistent results because of variable cellular conditions.

To circumvent these problems, recent approaches using RNA-interference (RNAi) mechanisms provide a stable, effective, and highly specific alternative (1, 2). RNAi is a posttranscriptional gene-silencing phenomenon triggered by small regulatory RNAs, such as small interfering RNA (siRNA) and microRNA (miRNA) (3, 4). These small RNA molecules usually function as a gene silencer, interfering with intracellular expression of genes complementary to the small RNAs. miRNAs, small single-stranded regulatory RNAs capable of interfering with intracellular mRNAs that contain either complete or partial complementarity, are useful for the design of new therapies against cancer polymorphism and viral mutation (1, 4). This flexible characteristic is different from double-stranded siRNAs because a much more rigid complementarity is required for siRNA-induced RNAi gene silencing. Currently, varieties of natural miRNAs have been found to be derived from hairpin-like RNA precursors in almost all eukaryotes, including yeast (*Schizosaccharomyces pombe*), plant (*Arabidopsis* spp.), nematode (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*), fish (*Danio rerio*), avian, mouse, and human, and have been found to be involved intracellular defense against viral infections and regulation of life-essential gene expressions during development (5–15). Because of these advantages, we used miRNA as a tool for transgenic animal research.

Most of protein-coding genes are transcribed by RNA polymerases type II (Pol II), which are very inefficient in generating short RNA sequences fewer than 100 nt; thus, the minimal mRNA size in eukaryotes is usually greater than 300 nt (16, 17). To generate the short transcripts of siRNA (19–23 bp), current vector-based

RNAi systems used Pol III promoters to transcribe the siRNA. The application of Pol III-directed siRNA-expressing vectors has been found to offer better efficacy and stability for RNAi induction in many cell lines in vitro (2, 18–20); nevertheless, several in vivo studies (21, 22) using the Pol III-mediated siRNA system have failed to provide tissue-specific effectiveness in the targeted cell population because of the ubiquitous existence of Pol III activity in almost all cell types. Moreover, because the read-through effect of Pol III frequently occurs on a short transcription template if lacking the proper codon termination, long siRNA could be synthesized and caused unexpected cytotoxicity (23, 24). Such a problem can also result from the competitive conflict between the Pol III promoter and another viral promoter of the vector (i.e., long terminal repeat and cytomegalovirus promoters).

Furthermore, Sledz et al. and we have noted that high dose siRNA (e.g., >250 nM in human T cells) can trigger interferon-induced toxicity similar to that of long double-stranded RNA (dsRNA) (25, 26). It is known that interferon-induced dsRNA-dependent protein kinase R (PKR) can trigger cell apoptosis, whereas activation of interferon-induced 2′,5′-oligoadenylate synthetase system leads to extensively nonspecific cleavage of single-stranded RNAs (i.e., mRNAs) (27). Both the PKR and 2′,5′-oligoadenylate synthetase systems contain dsRNA-binding motifs that are highly conserved for binding to dsRNAs, but these motifs are relatively insensitive to single-stranded RNAs. Because miRNAs are single-stranded RNA molecules, a Pol II-mediated miRNA generation system provides a much safer and less nontoxic means for both in vitro and in vivo applications of gene regulation (28). These findings indicate the advantages of Pol II-mediated intronic miRNA generation and its related gene regulation, which can be used as a tool for analysis of gene functions, improvement of current RNAi technology, and development of gene-specific therapeutics and transgenic animals.

We are the first research group who discovered the biogenesis of miRNA-like precursors (pre-miRNAs) from the 5′-proximal intron regions of primary gene transcripts (pre-mRNAs) produced by the mammalian Pol II (29) (Fig. 1a). Depending on the promoter of the miRNA-encoded gene transcript, intronic miRNA is coexpressed with its encoding gene in the specific cell population, which activates the promoter and expresses the gene. Using artificial introns carrying pre-miRNA structures, we have successfully generated mature miRNA molecules with full function in triggering RNAi-related gene silencing in human prostate cancer (LNCaP), human cervical cancer (HeLa), and rat neuronal stem cell lines in vitro and zebrafish in vivo (29, 30).

The artificial intron (SpRNAi) was placed in a mutated HcRed1 red fluorescent membrane protein (rGFP) gene to form a recombined SpRNAi-rGFP gene cassette, in which the functional

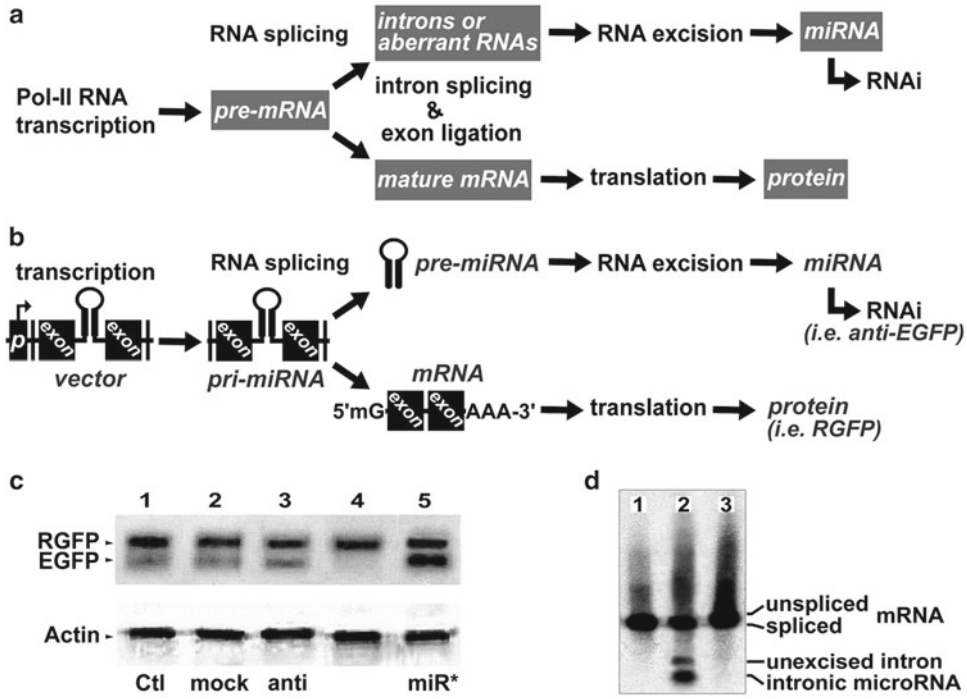

Fig. 1. Biogenesis and function of intronic miRNAs. (**a**) The native intronic miRNA is co-transcribed with a precursor messenger (pre)-mRNA by Pol II and cleaved out of the pre-mRNA by an RNA splicing machinery, spliceosome. The spliced intron with hairpin-like secondary structures is further processed into mature miRNAs capable of triggering RNA interference (RNAi) effects, whereas the ligated exons become a mature mRNA for protein synthesis. (**b**) We designed an artificial intron containing pre-miRNA, namely SpRNAi, mimicking the biogenesis processes of the native intronic miRNAs. (**c**) When a designed miR-EGFP(280-302)–stem-loop RNA construct was tested in the enhanced green fluorescent protein (EGFP)-expressing transgenic (UAS:gfp) zebrafishes, we detected a strong RNAi effect only on the target EGFP (*lane 4*). No detectable gene-silencing effect was observed in other lanes. From *left* to *right*: *1* blank vector control (Ctl); *2* miRNA-stem-loop targeting human immunodeficiency virus-p24 (mock); *3* miRNA without stem-loop (anti); and *5* stem-loop-miRNA* complementary to the miR-EGFP(280-302) sequence (miR*). The off-target genes such as vector red fluorescent membrane protein (rGFP) and fish actin were not affected, indicating the high target specificity of miRNA-mediated gene silencing. (**d**) Three different miR-EGFP(280-302) expression systems were tested for miRNA biogenesis. From *left* to *right*: *1* vector expressing intron-free rGFP, no pre-miRNA insert; *2* vector expressing rGFP with an intronic 5′-miRNA-stem-loop-miRNA*-3′ insert; and *3* vector similar to the construct in *lane 2*, but with a defected 5′-splice site in the intron. In Northern bolt analysis probing the miR-EGFP(280-302) sequence, the mature miRNA was released only from the spliced intron that resulted from the vector construct in *lane 2* in the cell cytoplasm.

fluorescent structure was disrupted by the intron insertion. Therefore, we were able to determine the occurrence of intron splicing and miRNA maturation through the appearance of red fluorescent emission on the cell membranes. The red rGFP here serves as a visual indicator for the generation of intronic miRNAs. This intron-derived miRNA system is activated under the control of specific Pol II promoters.

As shown in Fig. 1b, after Pol II RNA processing and splicing excision, some of the intron-derived miRNA fragments form mature miRNAs and effectively silence the target genes through

the RNAi mechanism, whereas the exons of the encoding gene transcript are ligated together to form a mature mRNA for protein synthesis (e.g., rGFP). Based on this miRNA generation model, we have tested various pre-miRNA constructs, and observed that the production of intron-derived miRNA fragments was originated from the 5′-proximity of the intron sequence between the 5′-splice site and the branching point. These miRNAs were able to trigger strong suppression of genes possessing more than 70% of complementarity to the miRNA sequences, whereas nonhomologous miRNAs, i.e., an empty intron without the pre-miRNA insert, an intron with an off-target miRNA insert (negative control), and a splicing-defective intron, showed no silencing effects on the targeted gene. Similar results can be reproduced in the zebrafish directed against target-enhanced green fluorescent protein (EGFP) expression (Fig. 1c), indicating the consistent preservation of the intronic miRNA biogenesis system in vertebrates. Further, no effect was detected on off-target genes, such as rGFP and β-actin, suggesting the high specificity of miRNA-directed RNAi. We have confirmed the identity of the intron-derived miRNAs, which were sized approximately 18- to 25-nt, approximately similar to the newly identified intronic miRNAs in *C. elegans* (31). Moreover, the intronic small RNAs isolated by guanidinium-chloride ultracentrifugation can elicit strong, but short-term gene-silencing effects on the homologous genes in transfected cells, indicating their temporary RNAi effects (1). Thus, the long-term (>1 month) gene-silencing effect that we observed in vivo, using the Pol II-mediated intronic miRNA system, is likely maintained by constitutive miRNA production from the vector rather than from the stability of the miRNAs.

We have successfully tested the feasibility of localized gene silencing in chicken embryos in vivo using the intronic miRNA approach and discovered that the interaction between pre-mRNA and genomic DNA may be essential for the miRNA biogenesis. The in vivo model of chicken embryos has been widely used in the research of developmental biology, signal transduction, and flu vaccine development. As an example, the β-catenin gene was selected because it plays a critical role in the biological development and ontogenesis (32). β-catenin is known to be involved in the growth control of skin and liver tissues in chicken embryos. The loss-of-function of β-catenin is lethal in many transgenic animals.

As shown in Fig. 2, experimental results demonstrated that the miRNAs derived from a predesigned intronic pre-miRNA construct transfected in the cell nucleus were capable of inhibiting β-catenin gene expression in the liver and skin of developing chicken embryos. This is because of the mechanism by which the intronic miRNA generation relies on a coupled interaction of nascent Pol II-directed pre-mRNA transcription and intron

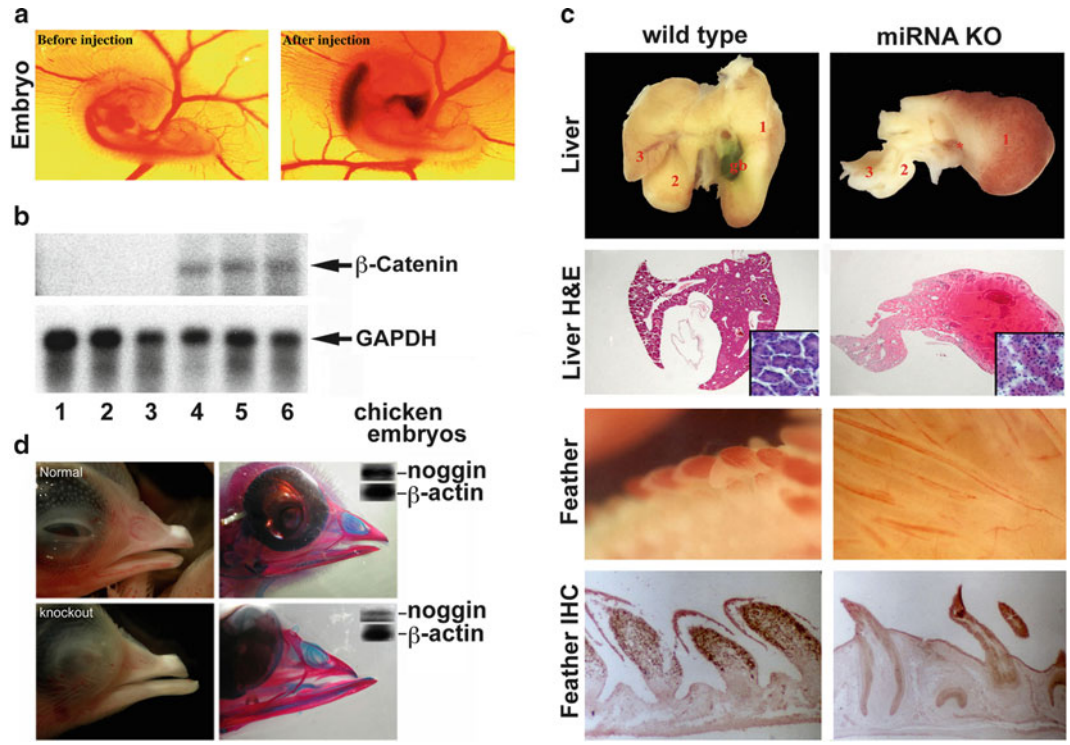

Fig. 2. In vivo gene-silencing effects of anti-β-catenin miRNA and anti-noggin miRNA (**d**) on special organ development in embryonic chicken. (**a**) The pre-miRNA-expressing construct and fast green dye mixtures were injected into the chicken embryos near the liver primordia below the heart. (**b**) Northern blots of extracted RNAs from chicken embryonic livers with (*lanes 1–3*) and without (*lanes 4–6*) anti-β-catenin miRNA treatments were shown. All three knockouts (KO) showed a greater than 98% silencing effect on β-catenin mRNA expression but housekeeping genes, such as *glyceraldehyde phosphate dehydrogenase*, was not affected. (**c**) Liver formation of the β-catenin KOs was significantly hindered (*upper right two panels*). Microscopic examination revealed a loose structure of hepatocytes, indicating the loss of cell–cell adhesion caused by breaks in adherins junctions formed between β-catenin and cell membrane E-cadherin in early liver development. In severely affected regions, feather growth in the skin close to the injection area was also inhibited (*lower right two panels*). Immunohistochemistry for β-catenin protein expression (*brown*) showed a significant decrease in the feather follicle sheaths. *H&E* Hematoxyline and eosin staining. (**d**) The lower beak development was increased by the mandible injection of the anti-noggin pre-miRNA construct (*down panel*) in comparison with the wild type (*upper panel*). *Right panels* showed bone (alizarin red) and cartilage (alcian blue) staining to demonstrate the outgrowth of bone tissues in the lower beak of the *noggin* KO. Northern blot analysis (inserts) confirmed a 60–65% decrease of *noggin* mRNA expression in the lower beak area.

excision occurring proximal to genomic perichromatin fibrils. This observation also indicates that the pre-mRNA–genomic DNA recombination may facilitate new miRNA generation by Pol II RNA excision for relatively long-term gene silencing. Alternatively, Pol II may function as an RNA-dependent RNA polymerase for producing more siRNAs, because mammalian Pol II has been reported to possess RNA-dependent RNA polymerase activities (33, 34). Taken together, the data suggest that Pol II-mediated RNA generation and excision is involved in intronic miRNA biogenesis, resulting in single-stranded small RNAs of approximately

20 nt comparable to the general sizes of Dicer-processed miRNAs frequently observed in the regulations of numerous developmental events.

To examine the transgenic model of intronic miRNAs, we transfected chicken embryos with the *SpRNAi-rGFP* construct containing a hairpin anti-β-catenin pre-miRNA, which was directed against the protein-coding region of the chicken β-catenin gene sequence (NM205081) with perfect complementarity. A perfectly complementary miRNA theoretically directs target-mRNA degradation more efficient than translational repression. Using embryonic day 3 chicken embryos, a dose of 25 nM of the *SpRNAi-rGFP* construct was injected into the body region close to where the liver primordia would form (Fig. 2a). For efficient delivery into target tissues, the construct was mixed with the FuGENE liposomal transfection reagent (Roche Biomedicals, Indianapolis, IN). A 10% (v/v) fast green solution was concurrently added during the injection as a dye indicator. The mixtures were injected into the ventral side, near the liver primordial, below the heart, using heat-pulled capillary needles. After injection, the embryonic eggs were sealed with sterilized scotch tapes and incubated in a humidified incubator at 39–40°C until day 12, when the embryos were examined and photographed under a dissection microscope. Several malformations were observed, however the embryos still survived and there was no visible overt toxicity or overall perturbation of embryo development. The liver was the closest organ to the injection site and, thus, was most dramatically affected in its phenotypes. Other regions, particularly the skin close to the injection site, were also affected by the diffused miRNA effects. As shown in Fig. 2b, Northern blot analysis detecting the target β-catenin mRNA expression in the dissected livers showed that β-catenin expression in the wild-type livers remained normal (lanes 1–3), whereas those of the miRNA-treated samples was decreased dramatically (lanes 4–6). The miRNA-silencing effect degraded more than 98% of β-catenin mRNA expression in the embryonic chicken, but had no influence in the housekeeping gene, glyceraldehydes phosphate dehydrogenase (GAPDH) expression, indicating its high target specificity and very limited interferon-related cytotoxicity in vivo.

Ten days after the primordial injection with the anti-β-catenin pre-miRNA construct, the embryonic chicken livers showed an enlarged and engorged first lobe, but the size of the second and third lobes of the livers were dramatically decreased (Fig. 2c). Histological sections of normal livers showed hepatic cords and sinusoidal space with few blood cells. In the anti-β-catenin miRNA-treated embryos, the general architecture of the hepatic cells in lobes 2 and 3 remained unchanged; however, there were islands of abnormal regions in lobe 1. The endothelium development seemed to be defective, and blood leaked outside of the blood vessels. Abnormal types of hematopoietic cells were also observed between

the space of hepatocytes, particularly dominated by a population of small cells with round nuclei and scanty cytoplasm. In severely affected regions, hepatocytes were disrupted (Fig. 2c, inserts) and the diffused miRNA effect further inhibited the feather growth in the skin area close to the injection site. The results discussed in Subheading 4.1 showed that the anti-β-catenin miRNA was very effective in knocking out the targeted gene expression at a very low dose and was effect over a long period of time (≥10 days). Furthermore, the miRNA gene-silencing effect seemed to be very specific, because off-targeted organs seemed to be normal, indicating that the small, single-stranded composition of miRNA used possessed no overt toxicity.

In another attempt to silencing *noggin* expression in the mandible beak area using the same approach (Fig. 2d), it was observed that an enlarged lower beak morphology is reminiscent of the previously reported bone morphogenetic protein (BMP4)-overexpressing chicken embryos (35, 36). Skeleton staining showed the outgrowth of bone and cartilage tissues in the injected mandible area (Fig. 2d, right panels), and Northern blot analysis further confirmed that approximately 60% of *noggin* mRNA expression was knocked out in this region (inserts). Because BMP4, a member of transforming growth factor-β superfamily, is known to promote bone development and the *noggin* is an antagonist of *BMP2/4/7* genes, it is not surprised to discover that our miRNA-mediated *noggin* knockouts created a morphological change resemble to the *BMP4*-overexpressing results as previous reports in chicken and other avian models. Thus, the gene silencing in chicken by the pre-miRNA transfection presents a great potential of localized transgene-like approach in creating animal models for developmental biology research.

To test the intronic miRNA effect on adult mammals (Fig. 3), we used the vector-based miRNA delivery approach, as previously reported in zebrafish. Patched albino (white) skins of melanin-knockout mice (W-9 black) were created by a succession of intracutaneous transduction of 50 μg of anti-tyrosinase (Tyr) pre-miRNA construct for 4 days (total 200 μg). The Tyr, a type I membrane protein and copper-containing enzyme, catalyzes the critical and rate-limiting step of tyrosine hydroxylation in the biosynthesis of melanin (black pigment) in skins and hairs. After 13-day incubation, the expression of melanin was blocked because a loss of its intermediates resulted from the anti-Tyr miRNA-silencing effect. On the contrary, the blank control and the U6-directed siRNA-transfected mice presented normal skin color (black), indicating that the loss of melanin is specifically effective in the miRNA transfections. Moreover, Northern blot analysis using RNA-polymerase chain reaction-amplified mRNAs from hair follicles showed a 76.1 ± 5.3% reduction of Tyr expression 2-day after the miRNA transfection, consistent with the immunohistochemistry results

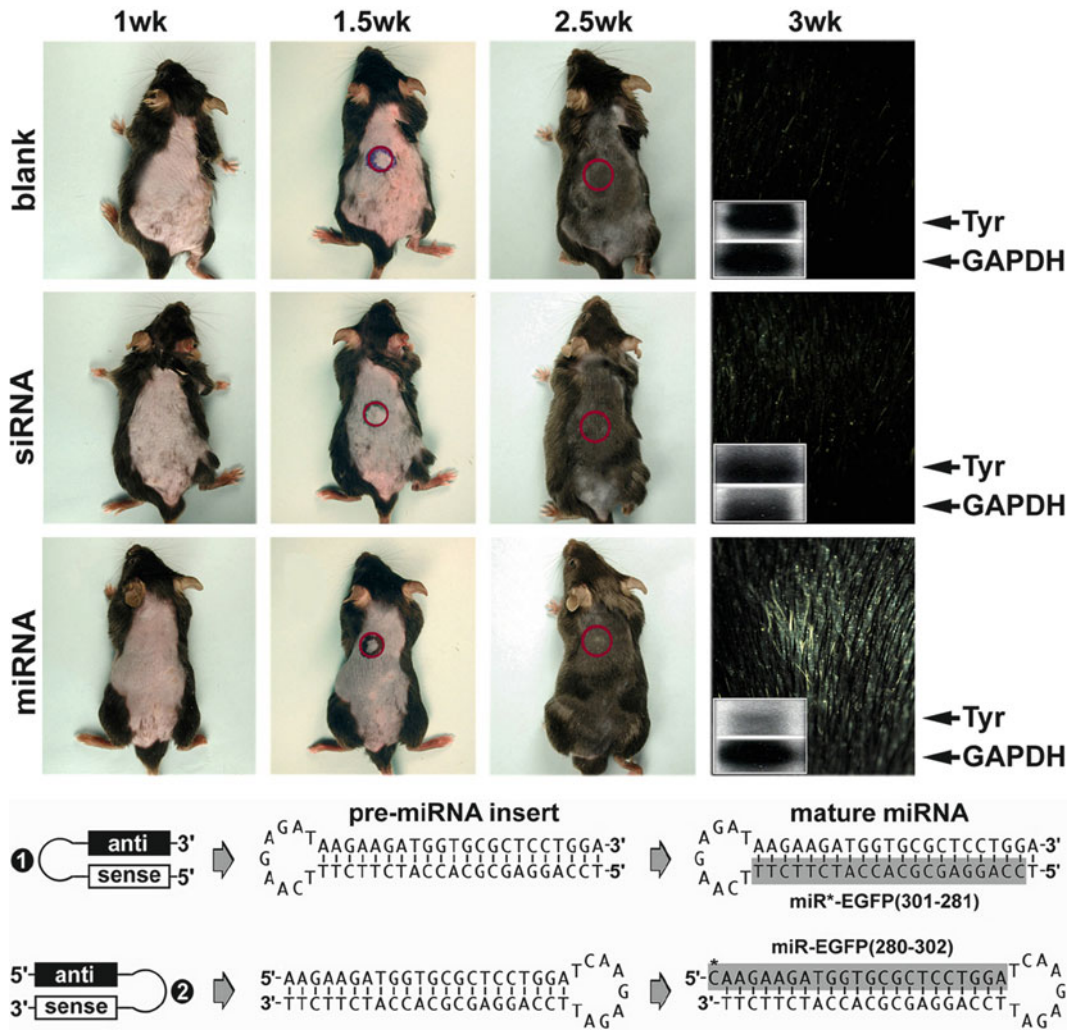

Fig. 3. In vivo effects of anti-tyrosinase (*Tyr*) miRNA on the mouse pigment production of local skins. Transfection of the miRNA-induced strong gene silencing of tyrosinase (*Tyr*) messenger RNA (mRNA) expression but not housekeeping glyceraldehyde phosphate dehydrogenase (*GAPDH*) expression, whereas expression of U6-directed small interfering RNA (siRNA) triggered mild nonspecific RNA degradation of both *Tyr* and *GAPDH* gene transcripts. Because *Tyr* is an essential enzyme for black pigment melanin production, the success of gene silencing can be observed by a significant loss of the black color in mouse hairs. The *red circles* indicate the location of intracutaneous injections. Northern blot analysis of *Tyr* mRNA expression in local hair follicles confirmed the effectiveness and specificity of the miRNA-mediated gene-silencing effect (inserts).

from the same skin area, whereas mild, nonspecific degradation of common gene transcripts was detected in the siRNA-transfected skins (seen from smearing patterns of both housekeeping control GAPDH and target Tyr mRNAs). Thus, the results show that use of intronic miRNA vectors provides a powerful new strategy for in vivo gene therapy, potentially for melanoma treatment. Under the same dosage, the miRNA transfections did not cause detectable

cytotoxicity effect, whereas the induced siRNA transfections showed nonspecific mRNA degradation, as previous reports (25, 26). This underscores the fact that the miRNA is effective even under in vivo systems, without the side effects of dsRNA. The results also indicate that this gene-silencing effect is stable and efficient in knocking down the target gene expression during a relatively long period, because the hair regrowth requires at least 10-day recovery. Further, it was observed that nontargeted skin hairs seemed normal, which implies that the intronic miRNA composition possess high specificity and no overt toxicity. Thus, the intronic miRNA approach offers relatively long-term, effective, and safe gene manipulation in local animal tissues or organs, preventing the lethal effects of certain genes in the conventional transgenic animal model.

To date, more than 135 miRNAs have been identified to be highly conserved between mammals and other animals (37). This may be an underestimation because the information on unknown 3′-UTR sequences in both genomes, non-untranslated region targets in the internal regions of gene transcripts, and new ortholog annotation, such as intronic miRNAs, were not considered. Approximately 10–30% of a spliced intron is exported into cytoplasm, with moderate half-life (38). Several types of intronic miRNA have been identified in *C. elegans*, mouse and human genomes (29, 31, 37). Therefore, it is understandable that the current miRNA computing programs do not fully predict the potential miRNA-like molecules. The finding of intronic miRNAs has opened new avenues for predicting miRNA varieties. Although there may be more than one type of new miRNAs to be identified and many new parameters to be generated from different miRNAs, the similarities and differences among these different types of miRNAs will provide better understanding of the small regulatory RNA world. Indeed, a broader definition for various miRNAs in different animal species is needed.

2. Materials

2.1. Preparation of Pre-miRNA Inserts

1. Sense pre-miRNA sequence: 5′-GTCCGATCGTC ATCAAG TCAGCTTGGGTTGCCA TCAAGAGAT TGGCAACCCA AGCTGACTTGAT CGACGCGTCAT-3′ (100 pmol/μL in autoclaved ddH_2O).
2. Antisense pre-miRNA sequence: 5′-ATGACGCGTCG ATCAA GTCAGCTTGGGTTGCCA ATCTCTTGA TGGCAACCC AAGCTGACTTGAT ACGATCGGAC-3′ (100 pmol/μL in autoclaved ddH_2O).

3. 2× Hybridization buffer: 200 mM KOAc, 60 mM HEPES KOH, 4 mM MgOAc, pH 7.4 at 25°C.
4. Incubation chamber: 94, 65, and 4°C.

2.2. Change of Pre-miRNA Inserts Located in the SpRNAi-rGFP Gene Cassette

1. SpRNAi-rGFP gene vector (University of Southern California File #3443).
2. 10× H buffer: 500 mM Tris–HCl, pH 7.5 at 37°C, 1 M NaCl, 100 mM $MgCl_2$, 10 mM dithioerythritol (DTT).
3. Restriction enzymes, including *PuvI* and *MluI* (5 U/μL for each enzyme).
4. *PuvI/MluI* digestion reaction mix: 12 μL autoclaved ddH_2O, 4 μL 10× H buffer, 2 μL *PuvI*, and 2 μL *MluI*; prepare the reaction mix just before use.
5. 10× Ligation buffer:660 mM Tris–HCl, pH 7.5 at 20°C, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP.
6. T4 DNA ligase (5 U/μL).
7. Ligation reaction mix: 4 μL autoclaved ddH_2O, 4 μL 10× ligation buffer, and 2 μL T4 ligase; prepare the reaction mix just before use.
8. Incubation chamber: 65, 37, and 16°C.
9. 1% Agarose gel electrophoresis.
10. Gel extraction kit (Qiagen, Valencia, CA).
11. Low salt Luria-Bertani (LB) culture broth.
12. Expand cloning kit (Roche Diagnostics, Indianapolis, IN).
13. DH5α transformation competent *Escherichia coli* cells (Roche).
14. 10× $MgSO_4$ solution: 1 M $MgSO_4$.
15. 1× $CaCl_2$ solution: 0.1 M $CaCl_2$.
16. 10× Glucose solution: 1 M Glucose.
17. Incubation chamber: 42 and 4°C.
18. Incubation shaker: 37°C, 285 rpm vortex.
19. Luria-Bertani agar plate containing 50 μg/mL kanamycin.
20. Spin Miniprep kit (Qiagen).
21. Microcentrifuge: 17,900×*g*.

2.3. Cloning of the SpRNAi-rGFP Gene Cassette into a Viral Vector

1. Retroiral vector: e.g., replication-competent avian sarcoma virus (RCAS) vector.
2. 10× H buffer: 500 mM Tris–HCl, pH 7.5 at 37°C, 1 M NaCl, 100 mM $MgCl_2$, 10 mM DTT.
3. Restriction enzymes, including *XhoI* and *XbaI*.

4. *XhoI/XbaI* digestion reaction mix: 2 μL autoclaved ddH_2O, 4 μL 10× H buffer, 2 μL *XhoI*, and 2 μL *XbaI*; prepare the reaction mix just before use.
5. 10× Ligation buffer:660 mM Tris–HCl, pH 7.5 at 20°C, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP.
6. 5 U/μL T4 DNA ligase.
7. Ligation reaction mix: 4 μL autoclaved ddH_2O, 4 μL 10× ligation buffer, and 2 μL T4 ligase; prepare the reaction mix just before use.

2.4. Viral Packaging and Titer Quantitation

1. Serum-free RPMI 1640 cell-culture medium.
2. FuGENE transfection reagent (Roche).
3. Chicken embryonic fibroblast (CEF) cell culture.
4. Cell-culture incubator.
5. Lentivirus purification kit (Cell Biolabs).
6. Microcentrifuge: 17,900 × *g*.

3. Methods

3.1. Preparation of Pre-miRNA Inserts

The intronic pre-miRNA insert is formed by hybridization of the sense and antisense pre-miRNA sequences, which are synthesized to be perfectly complementary to each other. Both of the SpRNAi sequences must be purified by polyacrylamide gel electrophoresis (PAGE) before use and stored at –20°C.

1. Hybridization: Mix the sense and antisense pre-miRNA sequences (5 μL for each sequence) in 10 μL of 2× hybridization buffer, heat to 94°C for 3 min, and cool to 65°C for 10 min. Stop the reaction on ice.

3.2. Change of Pre-miRNA Inserts Located in the SpRNAi-rGFP Gene Cassette

Because the intronic insert region of the SpRNAi-rGFP vector is flanked with a *PvuI* and an *MluI* restriction site at its 5′- and 3′-end, respectively, the primary pre-miRNA insert can be easily removed and replaced by various gene-specific inserts (e.g., anti-EGFP) possessing cohesive ends (see Note 1).

1. Cleavage by *MluI* and *PvuI*: Add the *PuvI/MluI* digestion reaction mix to the 20 μL of SpRNAi-rGFP vector and the above pre-miRNA hybrid, respectively. Incubate the reaction at 37°C for 4 h and stop the reaction on ice.
2. Purification of *MluI* and *PvuI*-digested sequences: Load and run the above reactions in 1% agarose gel electrophoresis and cut out the *MluI* and *PvuI*-digested SpRNAi-rGFP sequence

and the pre-miRNA fragment, respectively, using a clean surgical blade. Recover the two oligonucleotide sequences in one tube of 30 μL autoclaved ddH_2O using the gel extraction column and following the manufacturer's suggestions.

3. Ligation: Add the ligation reaction mix to the above extraction. Incubate the reaction at 16°C for 16 h and stop the reaction on ice.
4. Plasmid amplification: Transfect the above ligation product into the DH5α transformation competent *E. coli* cells using the expand cloning kit and following the manufacturer's suggestions.
5. Plasmid recovery: Isolate and collect the amplified SpRNAi-rGFP plasmid from the DH5α transformation competent *E. coli* cells into a tube of 30 μL autoclaved ddH_2O using a spin Miniprep filter following the manufacturer's suggestions (see Note 2).

3.3. Cloning of the SpRNAi-rGFP Gene Cassette into a Viral Vector

To express the SpRNAi-rGFP gene in chicken embryos, transfer the SpRNAi-rGFP gene cassette from the pHcRed1-N1/1 plasmid vector to the RCAS vector. Because the functional fluorescent structure of HcRed is disrupted by the SpRNAi intron insertion, one can determine the occurrence of intron splicing and miRNA maturation through the appearance of red fluorescent rGFP emission on the cell membranes. The rGFP protein also serves as a quantitative marker for measuring the titer activity of the pre-miRNA-expressing RCAS virus using flow cytometry. This intron-derived miRNA system is activated under the control of the *Xenopus* elongation factor 1-α enhancer-promoter located in the RCAS vector.

1. Cleavage by *XhoI* and *XbaI*: Add the *XhoI/XbaI* digestion reaction mix to the pHcRed1-N1/1 plasmid vector containing an SpRNAi-rGFP gene cassette and the RCAS retroviral vector, respectively. Incubate both of the reactions at 37°C for 4 h and stop the reaction on ice.
2. Purification of *XhoI/XbaI*-digested sequences: Load and run the above reactions in 1% agarose gel electrophoresis and cut out of the *XhoI/XbaI*-digested SpRNAi-rGFP sequence and the big RCAS fragment, respectively, using a clean surgical blade. Recover the two oligonucleotide sequences in one tube of 30 μL autoclaved ddH_2O using the gel extraction column and following the manufacturer's suggestions.
3. Ligation: Add the ligation reaction mix to the above extraction. Incubate the reaction at 16°C for 16 h and stop the reaction on ice.

3.4. Viral Packaging and Titer Quantitation

To increase transfection efficiency, we use liposomal reagents to facilitate the delivery of the SpRNAi-rGFP vector into the packaging cells.

1. Preparation of FuGENE: Add 6 μL of the FuGENE reagent into 100 μL of RPMI 1640 medium in a clean tube and gently mix the solution, following the manufacturer's suggestions. And add 20 μg (in less than 50 μL) of the SpRNAi-rGFP vector into the liposomal dilution and gently mix the solution, following the manufacturer's suggestions. Store the mixture at 4°C for 30 min.
2. Transfection: Add the above mixture into the center of the CEF cell culture and gently mix the cell-culture medium. Grow the transfected cells in a cell-culture incubator at 37°C, 5% CO_2 for 48–72 h.
3. Purification of viral particles: Harvest and concentrate the viral particles from the medium suspension using a lentivirus purification filter, following the manufacturer's protocol (see Note 3).

4. Notes

1. The synthetic pre-miRNA sequences that we show here are directed against the 5′-coding region of the chicken β-catenin gene sequence (NM205081, sense110-131 nts) with perfect complementarity. The principal rule for designing an intronic pre-miRNA insert is to synthesize two mutually complementary oligonucleotides, including one 5′-GTCCGATCGTC—19- to 27-nt antisense target gene sequence—TCAAGAGAT (stem-loop)—19- to 27-nt sense target gene sequence—CGACGCGTCAT-3′ and another 5′-ATGACGGTCG—19- to 27-nt antisense target gene sequence—ATCTCTTGA (stem-loop)—19- to 27-nt sense target gene sequence—GACGATCGGAC-3′. The hybridization of these two oligonucleotide sequences forms the intronic pre-miRNA insert, which contains a 5′-*PuvI* and a 3′-*MluI* restriction site for further ligation into the intron region of an *SpRNAi-rGFP* gene cassette. All synthetic oligonucleotides must be purified by PAGE to ensure their highest purity and integrity.
2. The sequence of the final *SpRNAi-rGFP* gene cassette and its pre-miRNA insert must be confirmed by DNA sequencing.
3. The rGFP protein can serve as a quantitative marker for measuring the titer levels of the pre-miRNA-expressing RCAS virus using either flow cytometry or the lentivirus quantitation kit (Cell Biolabs). The rGFP-specific monoclonal antibody can be purchased from BD Biosciences (Palo Alto, CA).

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Chapter 23

Mechanism and Method for Generating Tumor-Free iPS Cells Using Intronic MicroRNA miR-302 Induction

Shi-Lung Lin and Shao-Yao Ying

Abstract

Today's researchers generating induced pluripotent stem cells (iPS cells or iPSCs) usually consider the pluripotency first, then, the potential tumorigenicity. Oncogenic factors such as c-Myc and Klf4 were frequently used to boost the survival and proliferative rates of iPSCs, creating the inevitable problem of tumorigenicity that hindered the therapeutic usefulness of the iPSCs. To prevent tumorigenecity in stem cells, we have examined mechanism(s) by which the cell cycle genes of embryonic cells were regulated. Naturally occurring embryonic stem cells (ESCs) possess two unique stemness properties: pluripotent differentiation into almost all cell types and self-renewal with no risk of tumor formation. These two features are also important for the use of ESCs or iPSCs in therapy. Currently, despite overwhelming reports describing iPSC pluripotency, there have been no observations of tumor prevention mechanism(s) that suppresses tumor formation similar to that in naturally occurring ESCs. Our recent studies have revealed, for the first time, a ESC-specific microRNA (miRNA), miR-302, which was responsible for regulating human iPSC tumorigenicity through co-suppression of both cyclin E-CDK2 and cyclin D-CDK4/6 cell cycle pathways during G1–S phase transition. Additionally, miR-302 also silenced BMI-1, a cancer stem cell marker gene, to promote the expression of two senescence-associated tumor suppressor genes, p16Ink4a and p14/p19Arf. Together, the combinatory effect of reducing G1–S cell cycle transition and increasing p16/p14(p19) expression resulted in a relatively attenuated cell cycle rate similar to that of 2–8-cell-stage embryonic cells in early mammalian zygotes (20–24 h/cycle), as compared to the fast proliferation rate of iPSCs induced by four defined factors Oct4-Sox2-Klf4-c-Myc (12–16 h/cycle). These findings provide a means to control iPSC tumorigenicity and improve the safety of iPSCs in the therapeutic use. In this chapter, we reviewed the mechanism underlying miR-302-mediated tumor suppression and then applied this mechanism to generate tumor-free iPSCs. The same strategy can also be used to prevent ESC tumorigenicity.

Key words: miR-302, MicroRNA, Tumor suppressor, iPSC, ESC, Stem cell, Cell cycle, CDK2, CDK4/6, Cyclin D, BMI-1, p16Ink4a, p14/p19Arf, p21Cip1/Waf1, CDKN1A, RNAi

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1. Introduction

Somatic cell reprogramming (SCR) is an epigenetic mechanism necessary for the generation of induced pluripotent stem cells (iPSCs). The process of SCR is initiated by global DNA demethylation, which erases most of genomic methylation sites and resets the cellular gene expression pattern to an embryonic stem cell (ESC)-like profile. In human iPSCs, we have found functional roles of miR-302 in SCR (1, 2). miR-302, the most abundant microRNA (miRNA) in human ESCs (hESCs), not only silenced lysine-specific histone demethylase 1/2 (LSD1/2, AOF2/1, or KDM1/1B), and DNA (cytosine-5-)-methyltransferase 1 (DNMT1) to induce global DNA demethylation but also stimulated cellular Oct4-Sox2-Nanog expression to promote the reprogramming of somatic cells to ESC-like iPSCs (2). As a result, miR-302 can replace all four previously defined factors (either Oct4-Sox2-Klf4-c-Myc or Oct4-Sox2-Nanog-Lin28) for iPSC induction. Accompanying this miR-302-mediated SCR mechanism, we further identified a parallel tumor suppression mechanism, in which miR-302 attenuated both cyclin E-cyclin-dependent kinase 2 (CDK2) and cyclin D-CDK4/6 cell cycle pathways to inhibit iPSC tumorigenicity (3). This novel property provides a breakthrough advantage in the preparation of tumor-free iPSCs for clinical trials and therapies because tumorigenicity is still one of the major obstacles hindering stem cell applications. Based on the dual function of miR-302 in SCR induction and tumor suppression, we have successfully developed and tested an inducible miR-302 expression system for generating tumor-free iPSCs derived from various human normal and cancerous cells (1–3). To distinguish the miRNA-mediated iPSCs from the previously described iPSCs induced by four defined protein factors (Oct4, Sox2, Klf4, and c-Myc), we referred to miR-302-induced tumor-free iPSCs as mirPSCs.

Each individual mirPSC could grow into a colony or embryoid body with a relatively slow cell cycle rate (20–24 h/cycle). Due to miR-302-mediated cell cycle attenuation, mirPSCs often presented a quiescent cell-like morphology and form three-dimensional sphere colonies similar to that of an early zygote before the morula stage (Fig. 1a). The mirPSCs strongly and homogeneously expressed Oct4, Sox2, Nanog, Lin28, and many other major hESC markers (Fig. 1b, c) resembling the gene expression pattern of hESCs, as determined by immunocytochemical staining and western blotting analyses. The transcriptome of mirPSCs contained an average of >92% similarity to hESCs as determined by microarray analysis of global gene expression (1, 2). Further, genomic DNA demethylation, the first sign of SCR, was detected in the mirPSCs (1, 2). Particularly, we noted that mirPSCs were pluripotent but

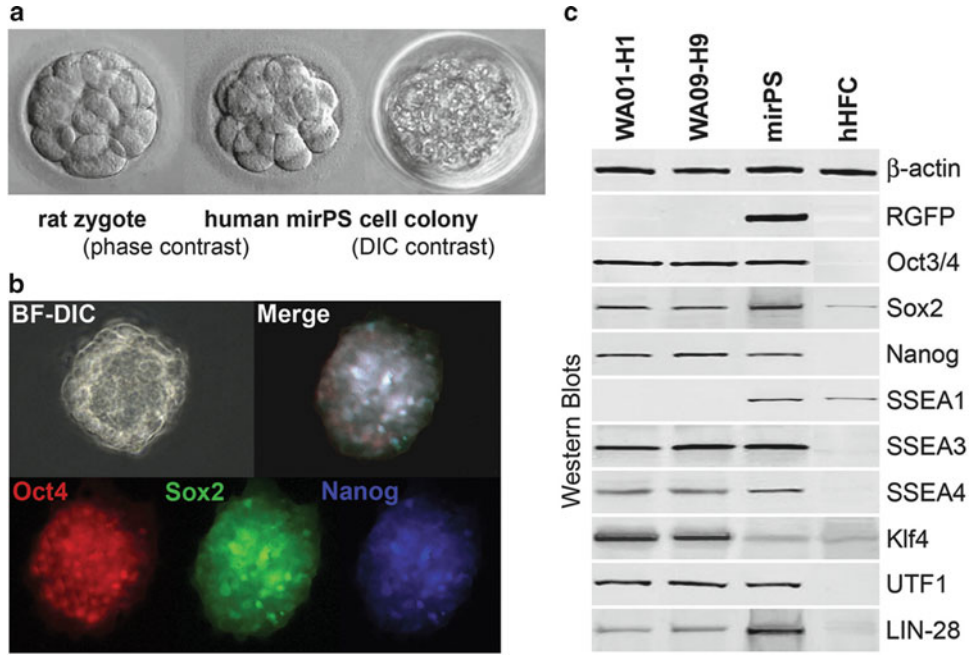

Fig. 1. Morphological and genetic properties of mirPSCs. (**a**) A morphological comparison between a morula-staged rat embryo and an mirPSC colony at 16–32-cell stage. *BF-DIC* bright field with differential interference contrast. (**b**) Fluorescent microscope examination showing the homogeneous expression of the core reprogramming factors Oct3/4, Sox2 and Nanog in an mirPSC-derived embryoid body. (**c**) Western blots confirming the expression patterns of major human embryonic stem cell (hESC)-specific markers in mirPSCs compared to those found in hESCs H1 and H9 ($n=4$, $p<0.01$).

not tumorigenetic, for they preferentially formed teratoma-like tissue cysts in the uterus or peritoneal cavity of pseudopregnant immunocompromised SCID-beige mice (1, 2). These tissue cysts, not similar to teratomas, contained various but relatively organized tissue regions derived from all three embryonic germ layers. Furthermore, the presence of partial homeobox (HOX) gradient expression was frequently observed in these tissue cysts (Lin et al. unpublished data). When xenografted into normal male mice, the mirPSCs were differentiated and assimilated by the surrounding tissues, indicating a potential application for regenerative medicine (3). Taken together, our findings have established that miR-302 reprogrammed human somatic cells to form hESC-like iPSCs with a high level of pluripotency but less or no tumorigenicity.

miR-302-mediated gene silencing is dose-dependent. The tumor suppression by miR-302 only occurred when its cellular concentration reached 1.1–1.3-folds of that in hESC H1 or H9 cells, which was corresponding to an optimal level of miR-302 for iPSC reprogramming (2, 3). When miR-302 levels, introduced by the miR-302 expression system described in Fig. 2, were equal to or lower than those in hESCs, only large tumor suppressor homolog

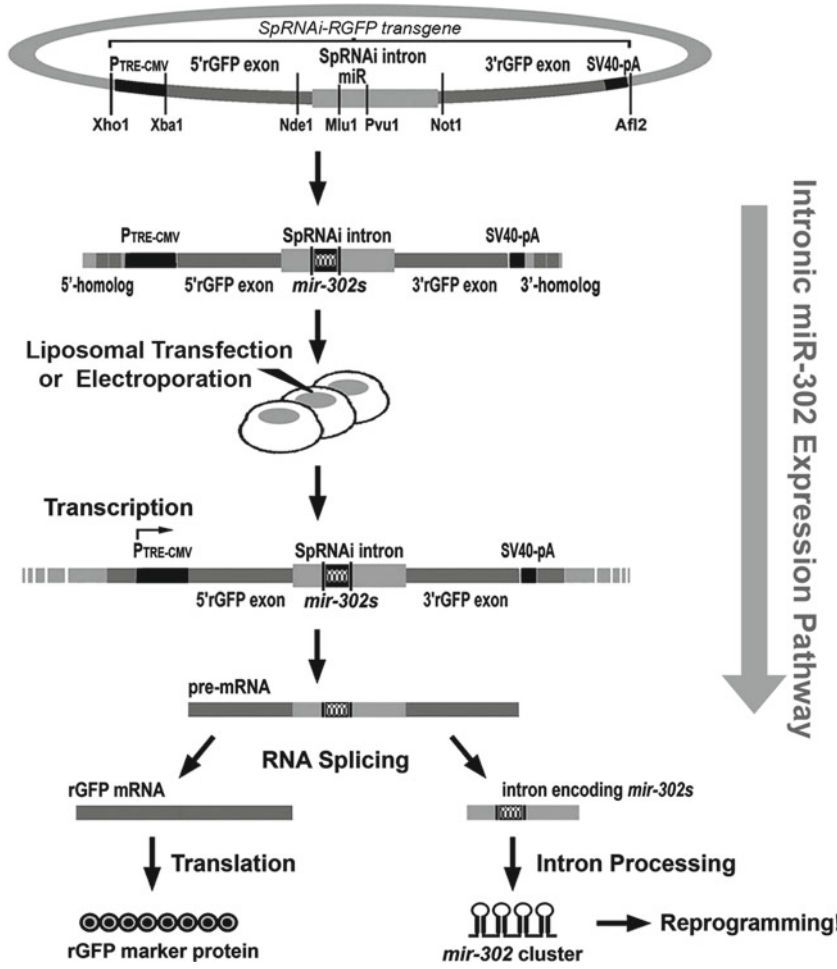

Fig. 2. Schematic procedure for induced pluripotent stem cell (iPSC) generation using intronic miR-302 expression. The miR-302-expressing *SpRNAi-RGFP* vector is transduced into human somatic cells by either electroporation or liposomal transfection. The expression of miR-302 relies on the natural intronic microRNA (miRNA) biogenesis pathway, in which the miR-302 cluster is co-expressed with the encoded *RGFP* gene transcripts and further processed into individual miR-302 molecules by RNA splicing enzymes (spliceosomal components) and RNaseIII Dicers (24).

2 (LATS2), but not CDK2, was silenced. When miR-302 levels were higher than those in hESCs, both CDK2 and LATS2 were silenced and hence cell cycle was attenuated at the G1-phase check point (3). Given that LATS2 inhibited the cyclin E-CDK2 pathway to block G1–S cell cycle transition (4), the direct silencing of CDK2 by miR-302 could enforce this LATS2 function even in the absence of LATS2. Moreover, the CDK2 silencing effect also counteracted the suppressive effect of miR-367 on CDKN1C (p57, Kip2), a cell cycle inhibitor against both CDK2 and CDK4, subsequently leading to a reduced cell cycle rate (Lin et al. unpublished data). Through such a dose-dependent gene silencing mechanism, miR-302 fine-tuned or even altered the function of its target genes at

different rates during different stages of development (2, 5). Conceivably, early human embryonic cells prior to the morula stage may express a higher miR-302 level than that in blastocyst-derived hESCs. These findings may account for the fact that 2–8-cell-stage embryonic cells have a relatively slower cell cycle rate (20–24 h/cycle) than that of hESCs (15–16 h/cycle) (6). Even though both early zygotes and hESCs were known to display a short G1 phase, the G1 phase of 2–8-cell-stage zygotic cells is still significantly longer (4 ± 1 h) than that of hESCs. On the other hand, iPSC generation was reported to be involved either a cell-cycle-dependent (with Klf4) or cell-cycle-independent (with Nanog) reprogramming process (7), in which the former cannot be explained by the function of miR-302. In fact, this hypothesis overlooked that Klf4 is an upstream transcription factor of Nanog (8, 9). Klf4 induced not only Nanog but also many other oncogenes, which promoted fast cell proliferation unrelated to the function of Nanog in reprogramming (8–11). Therefore, fast cell cycle, although can increase iPSC number, is not essential for SCR initiation to form iPSCs.

miR-302 functions differently in normal cells from tumor/cancer cells. Our previous studies have shown that miR-302 triggered massive reprogrammed cell death (apoptosis) in fast proliferative tumor/cancer cells whereas relatively slow growing normal cells could tolerate the inhibitory effect on cell proliferation (1, 3). It is conceivable that tumor/cancer cells may not survive in such a relatively slow cell cycle state due to their high metabolism and rapid consumption rates, providing a beneficial advantage in preventing the formation of tumors.

Based on the scheme depicting the mechanism by which miR-302 suppresses tumors/cancers (Fig. 3), miR-302 not only inhibits both the cyclin E-CDK2 and cyclin D-CDK4/6 pathways to block >70% of the G1–S cell cycle transition but also silences polycomb ring finger oncogene BMI-1 to increase the expression of two cell cycle inhibitors p16Ink4a and p14/p19Arf (3). As a result, 16Ink4a inhibits cyclin D-dependent CDK4/6 activity via phosphorylation of retinoblastoma protein Rb and subsequently prevents Rb from releasing E2F-dependent transcription required for S phase entry (12, 13). Furthermore, p14/p19Arf prevents HDM2, an Mdm2 p53 binding protein homolog, from binding to p53 and permits the p53-dependent transcription responsible for G1 arrest or apoptosis (14). Through simultaneously activating multiple cell cycle attenuation and tumor suppression pathways, miR-302 induces iPSC generation while preventing stem cell tumorigenicity. In addition, since DNMT1-deficient ESCs have been observed to present a significant reduction of gene loss and mutation rates (15), miR-302 may also help to enhance the genomic stability of both ESCs and iPSCs via LSD1/AOF2/KDM1-associated DNMT1 degradation (2, 3). In fact, the online

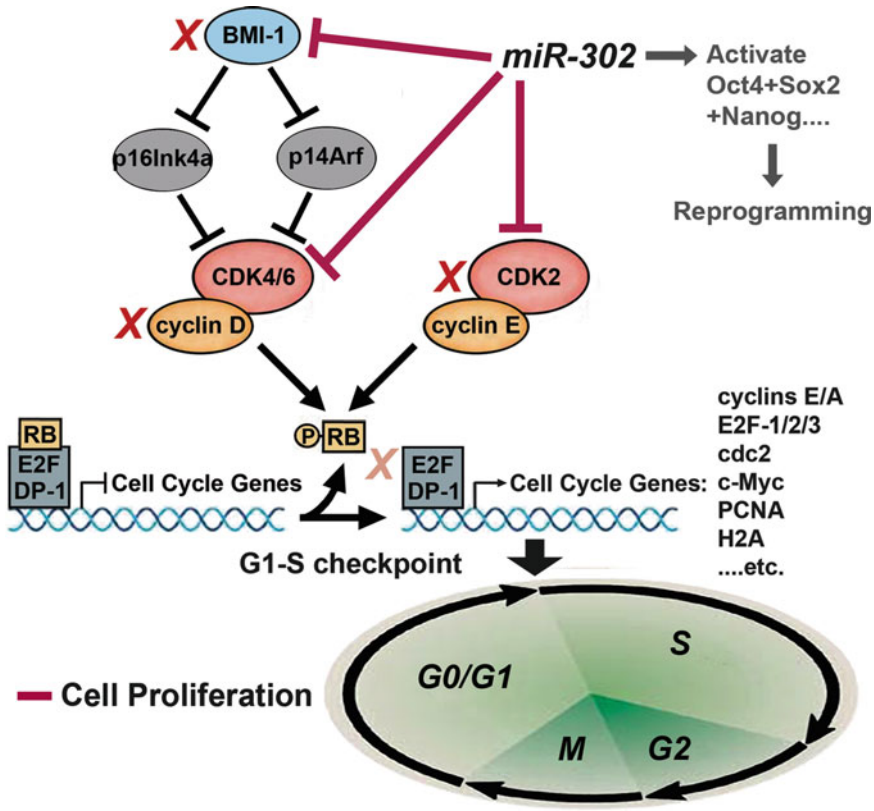

Fig. 3. Mechanism of miR-302–mediated tumor suppression in human iPSCs. miR-302 not only concurrently suppresses G1-phase checkpoint regulators cyclin-dependent kinase 2 (CDK2), cyclin D and BMI-1 but also indirectly activates p16Ink4a and p14/p19Arf to quench most (>70%) of the cell cycle activities during somatic cell reprogramming (SCR). E2F is also a predicted target of miR-302. Relative quiescence at the G0/G1 state may prevent possible random growth and/or tumor-like transformation of the reprogrammed iPSCs, leading to a more accurate and safer reprogramming process, by which premature cell differentiation and tumorigenicity are both inhibited.

miRNA-target prediction program provided by the European Bioinformatics Institute has also shown that DNMT1 is a direct target of miR-302a–d (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/detail_view.pl?transcript_id=ENST00000359526), further indicating that miR-302 can silence DNMT1 to induce global demethylation and genome stabilization. These currently identified miR-302 functions suggest that the anti-tumorigenicity and genomic stability of iPSCs and ESCs may be improved by elevating their miR-302 expression.

Over-expression of miR-302 is not always beneficial. Human iPSCs have been reported to exhibit problems including early senescence and limited expansion (16, 17). When miR-302 levels increased beyond 1.5–1.7-folds of that in hESCs, the degree of iPSC senescence was increased in proportion to the concentration of miR-302. Normal adult cells also undergo a limited number of divisions and then reach a quiescent state called replicative

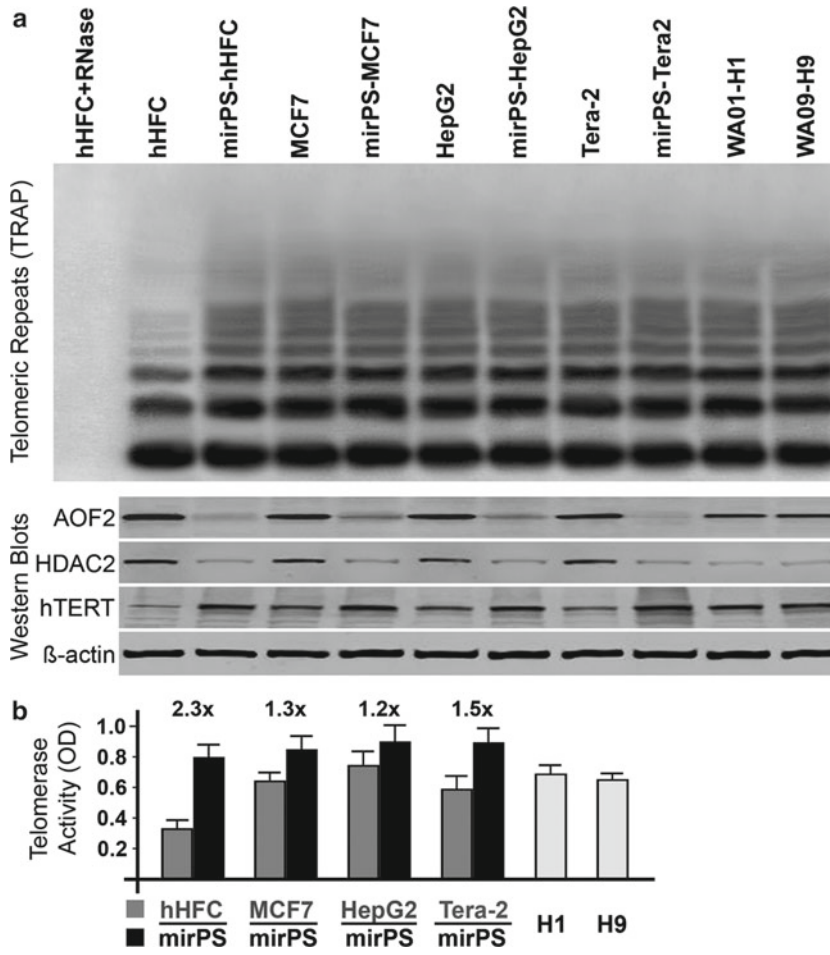

Fig. 4. Analysis of telomerase activity in various mirPSC lines in response to the miR-302 level elevated approximately 1.3-folds of the level in hESCs. (**a**) Telomerase activities shown by telomeric repeat amplification protocol (TRAP) assays ($n=5$, $p<0.01$) in the *upper panel*. Telomerase activity was sensitive to RNase treatment (hHFC + RNase). Western blotting (*lower panel*) confirming the increase of hTERT and decrease of LSD1/AOF2/KDM1 and HDAC2 expression in various mirPSC lines ($n=5$, $p<0.01$). (**b**) Telomerase activities measured by telomerase PCR ELISA assays (OD470–OD680; $n=3$, $p<0.01$).

senescence. Cells that escape replicative senescence often become immortal cells; thus, replicative senescence is a cellular defense mechanism against tumor/cancer formation. Because miR-302 increases p16Ink4a and p14/p19Arf expression, two of the major regulators in replicative senescence, the over-expression of miR-302 may exacerbate early senescence in iPSCs. Previous studies have identified several genes encoded in the Ink4a/Arf locus, such as p16Ink4a and p14/p19Arf, which pose a barrier to the success of iPSC reprogramming (16, 18–20). In particular, both of the p16Ink4a-RB and p14/19Arf-p53 activities are gradually enhanced and finally commit to the onset of early senescence after

serial passaging of iPSCs, (16, 19). In murine iPSCs, p19Arf rather than p16Ink4a is the main factor activating senescence, whereas in humans p16Inka plays a more important role than p14Arf (16, 18). Our recent observations have further ruled out the effect of telomerase in mirPSC senescence (Fig. 4). Due to forced miR-302 elevation, mirPSCs often present a higher degree of senescence than the iPSCs induced by four defined transcription factors. Under the same feeder-free conditions, the four-factor-induced iPSCs could be cultivated over 30–50 passages while mirPSCs only reach a maximum of 26 passages. However, this early senescence was not due to the shortening of telomere length. It is known that LSD1/AOF2/KDM1 and histone deacetylase 2 (HDAC2) suppress the transcription of human telomerase reverse transcriptase (hTERT) and the deficiency of both epigenetic regulator has been shown to increase hTERT expression (21, 22). Both LSD1/AOF2/KDM1 and HDAC2 are also the targets of miR-302 (2). Using telomeric repeat amplification protocol (TRAP) and telomerase activity ELISA assays, we have confirmed that miR-302 silenced both LSD1/AOF2/KDM1 and HDAC2 to enhance the hTERT activity and subsequently maintain normal or longer telomere length in mirPSCs (Fig. 4a, b). In view of this concurrent regulation of p16Ink4a, p14/p19Arf and hTERT activities by miR-302, mirPSCs may serve as a model for investigating the early senescence of iPSCs.

The balance between the mechanisms of tumorigenicity and senescence determines the safety and efficiency of iPSC generation. Our studies have shown that both mechanisms are regulated by the p16Ink4a-RB and p14/19Arf-p53 pathways (3). In mirPSCs, miR-302 and its homologues modulated the levels of p16Ink4a and p14/19Arf via fine-tuning the BMI-1 expression (3), providing important insight into the mechanism underlying stem cell self-renewal without the formation of tumors. In four-factor-induced iPSCs, Oct4, Sox2, and Klf4 were also found to repress the expression of p16Ink4a and p14/19Arf, leading to fast iPSC proliferation (18). Conceivably, the counteracting effect between miR-302 and Oct4-Sox2-Klf4 on the p16Ink4a and p14/19Arf expression may be the key in reaching the balance between tumorigenicity and senescence in iPSCs. On the other hand, we also found that miR-302 and Oct4-Sox2-Nanog formed a positive feedback cycle to fulfill the completion of iPSC reprogramming (2). In this reprogramming cycle, miR-302 and Oct4-Sox2-Nanog are reciprocally induced to constantly maintain the ESC-like pluripotent state of iPSCs. Therefore, at a certain point in the cycle, the interaction between miR-302 and Oct4-Sox2-Nanog/Klf4 may result in a stable iPSC state without the problem of either tumorigenicity or senescence. Based on this understanding, the correct combination of miR-302 and Oct4-Sox2-Nanog/Klf4 for generating tumor-free and senescence-free iPSCs will be a future challenge.

Through the natural mechanism of intronic miRNA biogenesis, we have identified the optimal miR-302 concentration for inducing tumor-free iPSCs (3, 23, 24). miR-302 is encoded in the intron region of the *La ribonucleoprotein domain family member 7* (*LARP7*, *PIP7S*) gene and expressed via an intronic miRNA biogenesis mechanism (1, 23, 25). Native miR-302 consists of four familial sense homologues (miR-302b, c, a, and d), and three distinct antisense members (miR-302b*, c*, and a*), all of which are transcribed together as a polycistronic RNA cluster along with another miRNA, miR367 (26). Many attempts were made to investigate the "miR-302" function but no complete expression of all miR-302a–d familial members was achieved. Also no studies were centered on the dose-dependent effect of miR-302, which determined the real miR-302 function. Indeed, our current approach using intronic miR-302 expression was the first successful protocol to display full expression of all native miR-302a–d familial members in human iPSCs as determined by microarray and northern blot analyses (1, 2). In this chapter, we show the step-by-step procedure of this approach.

We have first developed a *pSpRNAi-RGFP* vector mimicking the process of miRNA biogenesis from mammalian introns (23, 24, 27). As shown in Fig. 2, primary intronic miRNA precursors (pri-miRNAs) are transcribed by type-II RNA polymerases (Pol-II) and excised by spliceosomal components and/or Drosha to form miRNA precursors (pre-miRNAs), which are then exported out of the nucleus by Ran-GTP and Exportin-5 and further processed by a Dicer-like RNaseIII endoribonuclease in cytoplasm to form mature miRNAs (23, 28–31). However, the role of Drosha may be important but not required in this mechanism because the depletion of >85% Drosha with siRNA reduces less than a half of the miR-302 expression (Lin et al. unpublished data), indicating that some other nuclear RNaseIII-like enzymes may replace Drosha for processing intronic pre-miRNAs (29). Also, because mammalian intron often contains nonsense (i.e., translational stop) codons recognized by the nonsense-mediated decay (NMD) system, a cellular RNA surveillance mechanism (32, 33), the non-hairpin structures of an intron can be quickly degraded by NMD to prevent excessive RNA accumulation. RNA oversaturation has been reported to be a major problem for the direct (exonic) expression of short hairpin RNAs (shRNAs) and miRNAs in mammalian cells (34). Under this strictly coordinated regulation by Pol-II transcription, RNA splicing, and NMD mechanisms, intronic miRNA biogenesis has a great evolutional advantage in the safeguard of cellular miRNA concentration. Therefore, we have established an effective means to express the intronic miR-302 cluster, according to its own natural biogenesis mechanism, for generating tumor-free iPSCs.

2. Materials

2.1. Synthetic Oligonucleotides

1. All synthetic oligonucleotides required for the construction of the miR-302 familial cluster are listed in Table 1 (see Note 1). Each synthetic oligonucleotide was prepared in a final concentration of 200 pmol/μL.
2. 2× Hybridization buffer: 200 mM KOAc, 60 mM HEPES KOH, 4 mM MgOAc (pH 7.4 at 25°C).
3. Incubation chambers: 94–65 and 4°C.

Table 1
List of all synthetic DNA sequences required for constructing the miR-302 familial cluster

Name	Sequence
miR-302a-sense	5′-GATATCTCGA GTCACGCGTT CCCACCACTT AAACGTGGAT GTACTTGCTT TGAAACTAAA GAAGTAAGTG CTTCCATGTT TTGGTGATGG ATAGATCTCT C-3′
miR-302a-antisense	5′-GAGAGATCTA TCCATCACCA AAACATGGAA GCACTTACTT CTTTAGTTTC AAAGCAAGTA CATCCACGTT TAAGTGGTGG GAACGCGTGA CTCGAGATAT C-3′
miR-302b-sense	5′-ATAGATCTCT CGCTCCCTTC AACTTTAACA TGGAAGTGCT TTCTGTGACT TTGAAAGTAA GTGCTTCCAT GTTTTAGTAG GAGTCGTCAT ATGAC-3′
miR-302b-antisense	5′-GTCATATGAC GACTCCTACT AAAACATGGA AGCACTTACT TTCAAAGTCA CAGAAAGCAC TTCCATGTTA AAGTTGAAGG GAGCGAGAGA TCTAT-3′
miR-302c-sense	5′-GCCATATGCT ACCTTTGCTT TAACATGGAG GTACCTGCTG TGTGAAACAG AAGTAAGTGC TTCCATGTTT CAGTGGAGGC GTCTAGACAT-3′
miR-302c-antisense	5′-ATGTCTAGAC GCCTCCACTG AAACATGGAA GCACTTACTT CTGTTTCACA CAGCAGGTAC CTCCATGTTA AAGCAAAGGT AGCATATGGC-3′
miR-302d-sense	5′-CGTCTAGACA TAACACTCAA ACATGGAAGC ACTTAGCTAA GCCAGGCTAA GTGCTTCCAT GTTTGAGTGT TCGCGATCGG ATCCAC-3′
miR-302d-antisense	5′-GTGGATCCGA TCGCGAACAC TCAAACATGG AAGCACTTAG CCTGGCTTAG CTAAGTGCTT CCATGTTTGA GTGTTATGTC TAGACG-3′

Red letters indicate the sequences of each individual miR-302 precursor (pre-miR-302)

2.2. Construction of the miR-302 Cluster

1. 10× Digestion buffers for each individual restriction enzyme.
2. Restriction enzymes, such as *XhoI*, *MluI*, *BglII*, *NdeI*, *XbaI*, *PvuI*, and *BamHI*, were prepared with a stock concentration of 10 U/μL.
3. Digestion reaction mix: 10 μL of autoclaved ddH_2O, 2 μL of 10× digestion buffer, 4 μL of restriction enzyme(s), and 4 μL of each individual hybridized oligonucleotide (e.g., the hybrid of miR-302a-sense and miR-302a-antisense); the reaction mix was prepared right before use.
4. 10× Ligation buffer: 660 mM Tris–HCl (pH 7.5 at 20°C), 50 mM $MgCl_2$, 50 mM dithioerythritol, and 10 mM ATP.
5. T4 DNA ligase (5 U/μL).
6. Ligation reaction mix: 6 μL of each of the digested hybridized oligonucleotides (four kinds of hybridized oligonucleotides in total 24 μL), 3 μL of 10× ligation buffer, and 3 μL of T4 ligase; the reaction mix was prepared right before use.
7. Incubation chambers: 37, 75, 10, and 4°C.
8. 70-bp Cut-off purification filters, such as, but not limited, QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).
9. 100-bp Cut-off purification filters, such as, but not limited, QIAquick PCR Purification Kit (Qiagen).
10. Microcentrifuge ≥10,000 × *g* (13,000 rpm).

2.3. Insertion of the miR-302 Cluster into an Expression Vector

1. Expression vector (0.5 μg/μL), such as *pSpRNAi-RGFP* (see Note 2; see refs. (23, 27)) and *pLVX-AcGFP-N1* (Clontech Laboratories, Mountain View, CA). Any expression vector containing an either *MluI/PvuI* or *XhoI/BamHI* multiple cloning site for insertion of the miR-302 cluster in the 5′-untranslated region (5′-UTR) or in-frame intron of an reporter gene (i.e., EGFP or RGFP) can be used here.
2. 10× Restriction buffer, such as *MluI* and *PvuI* for digesting *pSpRNAi-RGFP* or *XhoI* and *BamHI* for digesting *pLVX-AcGFP-N1*.
3. Restriction enzymes, such as *MluI* and *PvuI* (for *pSpRNAi-RGFP*) or *XhoI* and *BamHI* (for *pLVX-AcGFP-N1*) in a stock concentration of 10 U/μL.
4. Digestion reaction mix: 13 μL of the miR-302 cluster, 2 μL of 10× digestion buffer, 4 μL of restriction enzymes (*MluI/PvuI* or *XhoI/BamHI*), and 1 μL of the expression vector; prepare the reaction mix right before use.
5. 100-bp Cut-off purification filters, such as, but not limited, QIAquick PCR Purification Kit (Qiagen).
6. Microcentrifuge ≥10,000 × *g* (13,000 rpm).

7. 10× Ligation buffer: 660 mM Tris–HCl (pH 7.5 at 20°C), 50 mM $MgCl_2$, 50 mM dithioerythritol, and 10 mM ATP.
8. T4 DNA ligase (5 U/μL).
9. Ligation reaction mix: 25 μL of the digested miR-302 cluster and expression vector mix, 3 μL of 10× ligation buffer, and 2 μL of T4 ligase; prepare the reaction mix just before use.
10. Incubation chambers: 37, 75, 10, and 4°C.
11. Luria-Bertani (LB) agar plates containing 50 mg/mL kanamycin for *pSpRNAi-RGFP* or 100 mg/mL ampicillin for *pLVX-AcGFP-N1* clone selection.
12. Low salt LB broth containing 50 mg/mL kanamycin for *pSpRNAi-RGFP* or 100 mg/mL ampicillin for *pLVX-AcGFP-N1* clone amplification.
13. Ready-to-use transformation competent *Escherichia coli* cells, such as, but not limited, Z-Competent *E. coli*-DH5α Kit (Zymo Research, Irvine, CA).
14. Incubation shaker: 37°C, >180 rpm vortex.
15. Plasmid extraction kit, such as, but not limited, QIAprep Spin Miniprep Kit (Qiagen).

2.4. Transfection or Electroporation

1. Electroporator, such as, but not limited, Neon Transfection System (Invitrogen, Carlsbad, CA) or Multiporator Electroporation System (Eppendorf, Hamburg, Germany).
2. Electroporation buffer (see Note 3).
3. Polysomal or liposomal reagents, such as, but not limited, FuGENE HD Transfection Reagent (Roche Diagnostics, Indianapolis, IN).
4. Target cells (>2,000 cells).
5. Cell culture medium, depending on the cell type of interest.
6. 1× Trypsin/EDTA solution.

2.5. Selection and Cultivation of miR-302-Positive mirPSCs

1. Feeder-free mirPSC medium: KnockOut DMEM/F-12 medium supplemented with 10% KnockOut serum replacement (Invitrogen), 1% MEM nonessential amino acids, 100 μM β-mercaptoethanol, 1 mM GlutaMax, 1 mM sodium pyruvate, 0.1 μM A83-01, 0.1 μM valproic acid (Stemgent, San Diego, CA), 10 ng/mL bFGF, 10 ng/mL FGF-4, 5 ng/mL LIF, and a mix of 50 IU/mL penicillin and 50 μg/mL streptomycin (see Note 4).
2. G418 (a final concentration ranged from 100 to 300 μg/mL for culturing *pSpRNAi-RGFP*-transfected cells) or puromycin (a final concentration ranged from 15 to 100 μg/mL for culturing *pLVX-AcGFP-N1*-transfected cells).
3. Incubator for cell culture: 37°C, 5% CO_2.

3. Methods

3.1. Construction of the miR-302 Cluster

The double-stranded DNA of each individual miR-302 precursor is formed by annealing the sense strand of each miR-302 familial member to its respective antisense strand; for example, miR-302a-sense to miR-302a-antisense, miR-302b-sense to miR-302b-antisense, miR-302c-sense to miR-302c-antisense, and miR-302d-sense to miR-302d-antisense (see Table 1). Then, the double-stranded DNAs of all four miR-302 (a, b, c, and d) precursors are separately cleaved by different restriction enzymes to generate various cohesive ends. Based on these cohesive ends, all four miR-302 precursors can be sequentially ligated into one familial cluster ready for co-expression. All synthetic strands of miR-302 members must be purified by polyacrylamide gel electrophoresis (PAGE) and stored at –20°C.

1. Hybridization: Mix the synthetic sense and antisense strands of each miR-302 member (each 1 nmol) in 10 μL of autoclaved ddH_2O; add 10 μL of 2× hybridization buffer, mix and heat to 94°C for 3 min, and then slowly cool to 65°C in 30 min. Stop the reaction on ice.
2. Restriction enzyme digestion: Prepare one digestion reaction mix for each hybridized miR-302 member (a, b, c, and d, respectively), containing 4 μL of the hybridized DNA, 2 μL of 10× digestion buffer, 4 μL of restriction enzymes, and 10 μL of autoclaved ddH_2O. Use 4 μL of *BglII* for miR-302a cleavage, 2 μL of *BglII* and 2 μL of *NdeI* for miR-302b cleavage, 2 μL of *NdeI* and 2 μL of *XbaI* for miR-302c cleavage, and 4 μL of *XbaI* for miR-302d cleavage. Incubate the reaction at 37°C for 4 h and then stop at 4°C. Purify each of the digested miR-302 member (a, b, c, and d, respectively) using a 70-bp cut-off purification filter, following the manufacturer's protocol, and recover the DNA in 30 μL of autoclaved ddH_2O.
3. Cohesive end ligation: Combine all four miR-302 members and mix well. Prepare a ligation reaction mix, containing 24 μL of the miR-302 mixture, 3 μL of 10× ligation buffer, and 3 μL of T4 ligase, and incubate the reaction at 10°C for 16 h and then stop at 4°C. This forms the miR-302 familial cluster containing miR-302a, b, c, and d in a 5′–3′ sequential order. Purify the miR-302 cluster using a 100-bp cut-off purification filter and recover it in 30 μL of autoclaved ddH_2O.

3.2. Insertion of the miR-302 Cluster into an Expression Vector

Intronic miRNA can be expressed from the 5′-UTR, 3′-UTR or in-frame intron region of a gene; hence, any expression vector containing an insertion site in these regions can be used for miR-302 expression. For example, the *pSpRNAi-RGFP* plasmid vector

(23, 27) possesses an *MluI/PvuI* insertion site in its in-frame intron, while the retroviral *pLVX-AcGFP-N1* vector (Clontech) contains a *XhoI/BamHI* cloning site in the 5′-UTR of its *AcGFP* gene. Both vectors have been tested for expressing the miR-302 cluster. However, due to the low stability of the highly structured miR-302 cluster, we have noticed that during the processes of vector amplification and extraction, the transformed *E. coli* competent cells cannot be stored at 4°C or some of the hairpin pre-miRNA structures may be lost. For storage, the vector containing the miR-302 cluster is stable at 4°C for up to 4 months and at −80°C for over 2 years.

1. Restriction enzyme digestion: Mix 1 μL of the expression vector to 13 μL of the miR-302 cluster and prepare one digestion reaction mix containing the 14 μL mixture, 2 μL of 10× digestion buffer, and either 2 μL of *MluI* and 2 μL of *PvuI* (for *pSpRNAi-RGFP*) or 2 μL of *XhoI* and 2 μL of *BamHI* (for *pLVX-AcGFP-N1*). Incubate the reaction at 37°C for 4 h and then 4°C. Purify the digested reaction using a 100-bp cut-off purification filter and recover the DNAs in 30 μL of autoclaved ddH_2O.
2. Cohesive end ligation: Prepare a ligation reaction mix, containing 25 μL of the cleaved vector and miR-302 cluster mixture, 3 μL of 10× ligation buffer, and 2 μL of T4 ligase. Incubate the reaction at 10°C for 16 h and then 4°C. This forms the miR-302-expressing vector.
3. Vector selection: Add 5 μL of the miR-302-expressing vector to the ready-to-use transformation competent *E. coli*-DH5α cells, mix and incubate the mixture at 4°C for 10 min, following the manufacturer's protocol. Next, pour and smear the mixture evenly onto an antibiotic-containing LB agar plate (50 mg/mL kanamycin for *pSpRNAi-RGFP* or 100 mg/mL ampicillin for *pLVX-AcGFP-N1*) and incubate the transformed *E. coli*-DH5α cells at 37°C, overnight.
4. Vector amplification: Pick and transfer each single cell colony, with a sterilized platinum loop or pipet tip, from the LB agar plate into 30 mL of antibiotic-containing LB broth (50 mg/mL kanamycin for *pSpRNAi-RGFP* or 100 mg/mL ampicillin for *pLVX-AcGFP-N1*), respectively. Further incubate the cell-containing LB broth on a shaker (>180 rpm) at 37°C, overnight.
5. Vector extraction: Isolate and recover the amplified vector in 30 μL of autoclaved ddH_2O using a plasmid extraction mini-prep filter, following the manufacture's protocol. To confirm the insertion of the miR-302 cluster, the isolated vector can be digested with either *MluI* and *PvuI* (for *pSpRNAi-RGFP*) or *XhoI* and *BamHI* (for *pLVX-AcGFP-N1*) to generate a ~350 bp DNA band on 2% agarose gel electrophoresis.

3.3. Transfection or Electroporation

To express the miR-302 cluster in the cells of interest, we recommend either electroporation or liposomal/polysomal transfection. Although the *pLVX-AcGFP-N1* vector can also be used for lentiviral production, this approach must be performed with extreme care due to the unknown function of miR-302 in vivo. For miR-302-induced iPSC generation, the cell types currently tested are human skin/hair-derived somatic cells, including melanocytes, keratinocytes and fibroblasts, and neural-like HEK-293 as well as several tumor/cancerous cell lines, such as human melanoma Colo-829, breast cancer MCF7, prostate cancer PC3, hepatocellular carcinoma HepG2 and embryonal teratocarcinoma Tera-2 cells. Notably, the reprogramming efficiency may vary in different cell types; for example, using electroporation, the average iPSC formation rates are >70% for HEK-293 cells, 15–20% for keratinocytes, 7–10% for melanocytes, <1–3% for adult fibroblasts, and <1% for all tested tumor/cancerous cells, respectively. The reprogramming efficiency in other cell types remains to be determined.

1. Electroporation: Add 2,000–200,000 cells and 15–40 μg of the miR-302-expressing vector in 250 μL of electroporation buffer, mix well and place into a 400 μL cuvette with aluminum electrodes. Perform electroporation tests following the manufacturer's protocol (see Note 3). After electroporation, grow the cells in the feeder-free mirPSC medium at 37°C under 5% CO_2.
2. Liposomal/Polysomal transfection (see Note 5): Grow cells to 50% confluency in a 100-mm culture dish and replace the cell culture medium by 9 mL of serum-free cell culture medium 4-h before transfection. For transfection preparation, add 15 μg of the miR-302-expressing vector into 1 mL of serum-free cell culture medium and mix well. Next, add 50 μL of FuGENE HD reagent into the center of the vector-medium mixture and vortex for 10 s. Place the mixture at room temperature for 15 min (see Note 6). After incubation, add the mixture drop-wise, covering the whole 100-mm culture dish and shake the culture dish back-and-forth and right-and-left several times to evenly distribute the mixture (see Note 7). Incubate the cells at 37°C under 5% CO_2 for 12–18 h and then replace the medium with feeder-free mirPSC medium. Continue to grow the transfected cells in the feeder-free mirPSC medium at 37°C under 5% CO_2.

3.4. Selection and Cultivation of miR-302-Positive mirPSCs

Since the *pSpRNAi-RGFP* and *pLVX-AcGFP-N1* vectors contain an antibiotic resistant gene against G418 and puromycin, respectively, the positive miR-302-transfected cells can be selected by using either G418 (for *pSpRNAi-RGFP*) or puromycin (for *pLVX-AcGFP-N1*). The positive miR-302-transfected cells also express either red fluorescent RGFP (*pSpRNAi-RGFP*) or green AcGFP

(*pLVX-AcGFP-N1*) for color identification under a fluorescent microscope or cell sorting by an FACS flow cytometry machine. Both antibiotic selection and color identification/sorting ensure the purity of the miR-302-transfected cell population.

1. Antibiotic selection: When the transfected cells start to express red or green fluorescent GFP, add either G418 (100–300 μg/mL for *pSpRNAi-RGFP*-transfected cells) or puromycin (15–100 μg/mL for *pLVX-AcGFP-N1*-transfected cells) to the cell culture medium and mix well. The optimal antibiotic concentration for mirPSC selection may vary dependent on the original cell types. Incubate the cells at 37°C under 5% CO_2 for 24–48 h and then replace the medium with fresh feeder-free mirPSC medium. Continue to grow the cells in feeder-free mirPSC medium at 37°C under 5% CO_2 for 3 more days and observe the purity of the fluorescent cells. If there are still many non-transfected (nonfluorescent) cells, repeat the steps of this section using a higher antibiotic concentration until the fluorescent cell population is relatively pure.
2. mirPSC culturing and passaging: Under the above feeder-free culture condition, mirPSCs tend to form large embryoid body-like colonies. When an mirPSC colony contains more than 2,000 cells, divide the colony into several small pieces with a scalpel and transfer the cells to a new collagen-coating culture dish in feeder-free mirPSC medium. Incubate the cells at 37°C under 5% CO_2 (see Note 8).

4. Notes

1. All synthetic oligonucleotides are purified by PAGE to ensure their highest purity and sequence integrity.
2. The detailed method for constructing the *SpRNAi-RGFP* expression vector has been described in our previous publications (23, 27).
3. Electroporation buffer varies dependent on your electroporation machine. Please follow the manufacturer's suggestions.
4. The addition of A83-01, an SMAD2 inhibitor, and valproic acid, an HDAC inhibitor, is optional, depending on the cell type of interest.
5. The protocol shown here is optimized for FuGENE HD (Roche). For other liposomal/polysomal reagents, a different protocol may be adopted. Please follow the manufacturer's protocol for this section if not using FuGENE HD. Also, the transfection volume is prepared for a 100-mm culture dish

containing cells of 50% confluency (in 10 mL of cell culture medium). If a smaller volume is desired, please reduce the amount of all materials and reagents in proportional to the volume of cell culture medium.

6. Maximal 30 min at room temperature.
7. Do not swirl the cell culture dish to mix the mixture. This results in uneven distribution of the transfection mixture.
8. When the mirPSC colony grows too big (>5,000 cells), the cells in the center of the colony may undergo apoptosis, which causes whole colony differentiation or death. The apoptotic mirPSC colony cannot be recovered for further passaging. Even under the best cell culture condition, the mirPSCs can maximally be passaged up to 26 generations, based on the current studies (2, 3).

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Chapter 24

Salivary MicroRNAs and Oral Cancer Detection

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Abstract

MicroRNAs (miRNAs) in human saliva have recently become an emerging field in saliva research for diagnostics applications and its potential role in biological implications. miRNAs are short noncoding RNA molecules that play important roles in regulating a variety of cellular processes. Dysregulation of miRNAs are known to be associated with many diseases. miRNAs were found present in the saliva of OSCC patients and could serve as potential biomarkers for oral cancer detection. Understanding the biological function of miRNAs in association with diseases is important towards utilizing miRNAs as diagnostic markers. There are currently a variety of profiling methods available for detecting miRNA expression levels. In this chapter, we overview the Applied Biosystem Stem-loop RT based Taqman MicroRNA Assay for salivary miRNA profiling. Using this highly sensitive and specific assay, miRNAs in saliva are profiled with only a few nanograms of starting RNA. This method is also applicable for studying biomarkers in other body fluids or clinical samples that contain small amounts of RNA.

Key words: Saliva, MicroRNAs, Biomarkers, Diagnostics, Oral cancer, qPCR, TaqMan Micro RNA assays

1. Introduction

Oral cancer, most commonly oral squamous cell carcinoma (OSCC) in ~90% of oral cancer patients, is the sixth most common cancer in the United States. If OSCC is detected at the early stage (T-1 stage), the 5-year survival rate is close to 80%. If OSCC is detected at the later stages (T-3 or T-4 stage), the 5-year survival rate decreases to 20–40%, indicating early detection methods are necessary for increasing long-term patient survival. Previously, protein, mRNA, and DNA extracted from saliva have been used to detect OSCC (1–4). By using transcriptomic and proteomic technology, mRNA and protein salivary biomarkers, respectively, were discovered and validated to be highly discriminatory for oral cancer detection (2, 5–7).

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The presence of microRNAs (miRNAs) in human saliva has recently become an emerging field for monitoring oral diseases using salivary diagnostics. miRNAs are short noncoding RNA molecules 19–24 nt in length that were first identified in 1993 as small RNAs in *Caenorhabditis elegans* (8). Since then, miRNAs have been categorized according to mass (9–11) and the biogenesis of miRNAs and its mode of action have been well characterized (12, 13). miRNAs binding to complementary sequences in the 3′-untranslated region (3′-UTR) of mRNAs to regulate gene expression by inhibiting protein translation and/or causing mRNA degradation (14). miRNAs play important roles in regulating various cellular processes such as cell growth, differentiation, apoptosis, and immune response (15–17). Due to imperfect complementary binding by miRNAs, a single miRNA can potentially bind to >100 different mRNAs. To date, there are over 1,000 known miRNAs in the human genome and over 30% of human mRNAs are post-transcriptionally regulated by miRNAs (14, 18, 19). In saliva, miRNAs were found present in both whole saliva and saliva supernatant. At the time of study, 314 of the 708 human miRNAs registered in the miRBase version 12.0 were profiled (20, 21). Combined with transcriptomic and proteomic approaches, miRNA represents the third diagnostic alphabet in saliva.

Dysregulated expression of miRNAs is known to affect cell growth and can function as tumor suppressors or oncogenes in various cancers (22–24). In oral cancer, miRNAs have been shown to affect cell proliferation (25), apoptosis (26), and even chemotherapy resistance in OSCC patients (27). miRNAs have also been shown in OSCC to be epigenetically regulated by DNA methylation (28, 29). miRNAs have distinct expression profiles due to being differentially expressed in cancer cells in comparison to normal cells (15). These distinct miRNA expression profiles have also appeared between OSCC and normal tissue (26, 29). Additionally, the expression level of many miRNAs in cancer cells and normal cells exhibit fold changes tens to hundreds of times higher than the expression levels of mRNAs (30). This data suggest that miRNAs can be potentially used as biomarkers to detect early-stage diagnosis of oral cancer and lead to the development of miRNA-based cancer-treatment and therapies.

In the past few years, several research papers have been published that showed the presence of miRNAs in saliva and their potential as noninvasive biomarkers in oral cancer detection. Park and colleagues compared the expression level of approximately 50 miRNAs in whole saliva and saliva supernatant between OSCC patients and normal controls. Two saliva miRNAs, miR-125a and miR-200a, were significantly decreased ($P < 0.05$) in OSCC patients than in normal controls (20). A preliminary analysis from another group showed miR-31 over-expressed in the saliva of OSCC patients (31). A group studying oral rinses from patients with

squamous cell carcinoma of the head and neck (SCCHN) detected miR-137 promoter methylation associated with gender and body mass index (32).

How salivary mRNA and miRNA biomarkers are protected and shuttled from the source of the tumor to the saliva remains a current interest. Salivary mRNAs were discovered to be protected from ribonucleases present in the saliva by macromolecules called exosomes (33). These exosomes are small cell-secreted vesicles around 30–100 nm in length that are known to package and transport mRNAs and miRNAs (34). By being localized inside exosomes, salivary mRNAs were found to be remarkably stable (33). This stability has also been shown with endogenous salivary miRNAs degrading at a much slower rate than exogenous miRNAs (20). Exosomes have been shown to transfer mRNA from different cell types and activate or modulate gene expression in oral keratinocytes (33). Recently, miRNAs were extracted from exosomes in human whole saliva from both normal controls and Sjögren's syndrome patients (21). These findings of salivary exosomes and how they regulate cell-to-cell interaction and gene expression may allow us to understand the molecular basis of oral diseases.

Since 2004, there are now a variety of profiling methods that are available for detecting miRNA expression levels. The main principal methods include quantitative PCR (qPCR) (35, 36), microarray hybridization (37, 38), and next-generation sequencing (NGS) (39). For detecting salivary miRNA expression, there are currently several challenges. miRNAs present in saliva are relatively low, typically in the nanogram range upon extraction. Profiling arrays typically require several micrograms or more of input RNA, causing problems with sensitivity and specificity. miRNAs are short and similar in sequence, making it difficult to design RT-qPCR assays or hybridization probes. There are also three different forms of miRNA (pre-miRNAs, pri-miRNAs, and mature miRNAs) so the profiling method needs to be able to detect and distinguish between each type. While there have been challenges in detecting miRNA expression, there have also been numerous advances as sequence technology continues to improve each year as the discovery of new miRNAs and modifications to existing miRNAs expands (40).

For miRNA expression profiling in saliva, we focused particularly on the Applied Biosystem Stem-loop RT based Taqman® MicroRNA Assay. This method measures quantitation for mature miRNA expression and is believed to be the gold-standard method with a large dynamic range, high specificity, and high sensitivity (35, 41, 42). Far less input amount of RNA is required as compared to microarray and other technologies, making Taqman® MicroRNA assays extremely suitable for salivary miRNA analysis.

In this chapter, we provide a detailed method for the isolation and profiling of salivary miRNAs. The first part describes a modified

protocol for isolating total RNA from human saliva and the second part describes the method for reverse transcribing and profiling the miRNAs of saliva on Taqman® miRNA arrays. A pre-amplification step between reverse transcription of the RNA to cDNA and miRNA array analysis during the second part is required due to the low amount of total RNA present in the saliva sample. This approach is also applicable for studying biomarkers in other body fluids or clinical samples that contain a few nanograms of starting total RNA.

2. Materials

2.1. Saliva Collection and Processing

1. 50 mL Sterile tube and Styrofoam cup.
2. Crushed ice.
3. Distilled water.
4. Laboratory vortex mixer.
5. Refrigerated bench top centrifuge that can accommodate 50 mL tubes.
6. SUPERase Inhibitor (Ambion).

2.2. Total RNA Isolation

1. mirVana PARIS Kit (Ambion).
2. Crushed ice and container.
3. 100% Ethanol, ACS quality or better.
4. DNase I stock solution (QIAGEN).
5. RDD buffer (QIAGEN).
6. Elution solution.
7. Nuclease-free water.
8. Heat block.
9. Microcentrifuge.

2.3. Taqman® MicroRNA Array

1. TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems).
2. Megaplex RT Primers for array platform: Human Pool A and B (Applied Biosystems).
3. TaqMan® PreAmp Master Mix (Applied Biosystems).
4. Megaplex PreAmp Primers for array platform: Human Pool A and B (Applied Biosystems).
5. Nuclease-free water.
6. GeneAmp PCR System 9700 Thermal Cycler.
7. Taqman® 2× Universal Master Mix II, No UNG (Applied Biosystems).

8. Taqman® Human MicroRNA Arrays Card Set v3 (Applied Biosystems).
9. Applied Biosystems 7900HT Fast Real-Time PCR Instrument with special card holder.
10. PCR tubes or 96-well PCR plates.

2.4. MicroRNA Array Data Processing

1. RQ Manager 1.2.1 Enterprise Software.

3. Methods

3.1. Saliva Collection and Processing

1. Un-stimulated saliva samples should be collected from patients between 9 and 11 a.m. following standard operating procedures. Patients should refrain from eating, drinking, smoking, or using oral hygiene procedures for at least 1 h prior to collection.
2. Ask patients to rinse their mouth well with distilled drinking water for 1 min. Subjects can either expectorate or swallow the water.
3. Five minutes after the oral rinse, ask subjects to spit ~5 mL of saliva into a 50 mL sterile tube placed on ice. It is encouraged that the tube remains on ice while collecting saliva.
4. Following collection, the saliva samples should be briefly vortexed (~20 s). Centrifuge the saliva samples at 2,600 × *g* for 15 min at 4°C. Collect the saliva supernatant that has separated from the cellular phase (see Notes 1 and 2).
5. For every milliliter of saliva supernatant collected, add 1 μL (20 U) of SUPERase Inhibitor. Unless proceeding to next step, freeze at –80°C immediately.

3.2. Total RNA Isolation from Saliva Supernatant

1. Total RNA is isolated from the saliva supernatant according to a modified protocol from the manufacturer (mirVANA PARIS kit) (see Notes 3 and 4).
2. If saliva supernatant is frozen, thaw on ice until liquid and start protocol immediately. In the meantime set heat block at 95°C and put a microcentrifuge tube with elution buffer or nuclease-free water into a well. Warm 2× Denaturing Solution at 37°C if it appears solid.
3. To 300 μL of saliva supernatant, add 1.5× amount of 2× Denaturing Solution (450 μL). Mix thoroughly and incubate solution on ice for 5 min.
4. To the saliva supernatant/2× Denaturing Solution mix, add 1.25 volume of room temperature 100% ethanol (937.5 μL). Mix thoroughly (see Note 5).
5. Add 700 μL of the saliva supernatant/2× Denaturing/Ethanol mix onto a Filter Cartridge placed in a collection tube. Spin for

60 s at 8,000 × *g*. Discard flow-through and repeat until rest of mix has been processed.

6. Wash the Filter Cartridge with 350 µL miRNA Wash Solution 1. Spin for 60 s at 8,000 × *g*. Discard flow-through.
7. Combine 10 µL DNAse I stock solution to 70 µL RDD buffer. Mix by gently pipetting solution several times in tube. Pipet the DNase I mix (80 µL) directly onto the filter membrane. Incubate sample on the bench top at room temp for 15 min (see Note 6).
8. Add 350 µL miRNA Wash Solution 1 to the Filter Cartridge. Spin for 60 s at 8,000 × *g*. Discard flow-through.
9. Wash the Filter Cartridge with 500 µL Wash Solution 2/3. Spin for 60 s at 8,000 × *g*. Discard flow-through. Repeat process again. After flow-through has been discarded a second time, spin Filter Cartridge for 1 min at 10,000 × *g* to remove any residual fluid from the filter.
10. Transfer the Filter Cartridge to a new collection tube. Apply 100 µL of elution solution or nuclease-free water heated at 95°C to the filter membrane. Let sit for 1 min. Spin for 60 s at 10,000 × *g*. One hundred microliters of total RNA elute collected. If 100 µL is not collected, spin again for another 60 s. Store at −80°C (see Notes 7 and 8).

3.3. Taqman® MicroRNA Arrays

Isolated total RNA undergoes reverse transcription, pre-amplification, and real-time PCR using a modified protocol from the manufacturer.

3.3.1. Megaplex RT

1. Thaw the following reagents on ice: Megaplex RT primers, $MgCl_2$ (included with Megaplex RT primers), and all Taqman® MicroRNA Reverse Transcription components. Spin down components. Do not vortex the RT enzyme mix.
2. In a microcentrifuge tube, prepare the RT Enzyme mix by combining the following reagents listed below. A stock solution is recommended if several RT reactions are to be prepared.

Components	Megaplex RT reaction volume (µL)
Megaplex RT primer mix A or B (10×)	0.75
dNTPs with dTTP (100 mM total)	0.15
MultiScribe Reverse Transcriptase (50 U/µL)	1.50
RT Buffer (10×)	0.75
$MgCl_2$ (25 mM)	0.90
RNase Inhibitor (20 U/µL)	0.09
Nuclease-free water	0.36
Total	*4.5*

3. Mix RT Enzyme mix gently and spin briefly. Aliquot 4.5 μL of RT Enzyme mix per well in a 96-well PCR plate or PCR tubes.
4. Add 3 μL of total RNA (1–350 ng) into each well. Mix thoroughly by pipetting up and down several times. Seal the plate or cap the PCR tubes and spin down briefly.
5. Incubate on ice for 5 min.
6. Set up the RT protocol in the 9700 Thermocycler, incubating (16°C for 2 min, 42°C for 1 min, and 50°C for 1 s) for 40 cycles, inactivating the RT reaction at 85°C for 5 min and then holding at 4°C.
7. Load plate or tubes in thermocycler and start the RT run.
8. Proceed to pre-amplification step immediately or store samples at –20°C.

3.3.2. Megaplex Pre-amplification

1. Thaw the following reagents on ice: Megaplex Pre-amplification primers and 2× Taqman® PreAmp Master Mix. Spin down components. Do not vortex.
2. In a microcentrifuge tube, prepare the pre-amplification mix by combining the following reagents listed below. A stock solution is recommended if several RT reactions are to be prepared.

Components	Megaplex Pre-amplification reaction volume (μL)
Megaplex Preamp Primer Mix A or B (10×)	4.0
Taqman® Preamp Master Mix (2×)	20.0
Nuclease-free water	8.5
Total	*32.5*

3. Combine Preamp mix with RT product (40 μL total). Mix thoroughly by pipetting up and down several times. Seal the plate or cap the PCR tubes and spin down briefly.
4. Set up the Preamp protocol in the 9700 Thermocycler, incubate at (95°C for 10 min, 55°C for 2 min and 72°C for 2 min), then (95°C for 15 s and 60°C for 4 min) for 14 cycles, 99.9°C for 10 min to inactivate reaction, and then hold at 4°C.
5. Do not dilute the product. Directly proceed to real-time PCR immediately or store samples at –20°C for up to 1 week.

3.3.3. Real-Time PCR

1. Prepare RT-PCR reaction at room temperature. Let all reagents sit at room temperature for at least 30 min.

2. In a microcentrifuge tube, prepare the RT-PCR reaction mix by combining the following reagents listed below.

Components	RT-PCR reaction volume (μL)
Pre-Amplification product (no dilution)	9.0
TaqMan® Master Mix, no UNG (2×)	450.0
Nuclease-free water	441.0
Total	*900.0*

3. Mix thoroughly and spin down briefly.
4. Load 105 μL of the RT-PCR reaction per port in the MicroRNA Array Card.
5. Spin down and seal the array card.
6. Create plate document on the 7900HT Fast Real-Time PCR Instrument with the following conditions, cycling at 95°C for 10 min, and then (95°C for 15 s, and 60°C for 60 s) for 40 cycles. Use default TLDA setting and FAM as reporter.
7. Load card into instrument. Start run. More detailed information on how to run the arrays can be found at the Applied Biosystem Taqman® Array User Bulletin.

3.4. Data Analysis

For RT-PCR, there are two common methods used for data analysis: absolute quantification and relative quantification. Absolute quantification determines the copy number of a target by using a standard curve with a sample of known concentration. However, this method is usually not preferred due to variations in sample quantity, sample quality, and variable PCR efficiency. The more commonly used method is relative quantification. In relative quantification, target molecules are compared to a reference target relative to a reference group (control sample). Typically, endogenous controls are used as reference targets because the gene expression remains relatively constant across tissue and cell types. Since endogenous controls vary from each target type, the chosen endogenous control should always be validated first. In saliva, RNA polymerase III-transcribed U6 snRNA has been used as an endogenous control for RT-qPCR data analysis (20). Information on how to select endogenous controls has been mentioned elsewhere (43). Relative quantification in RT-PCR data can be calculated by using the comparative C_T method, a convenient way for determining relative changes in gene expression. The comparative C_T method is also referred to as the $2^{-\Delta\Delta C_T}$ method, the same as calculating fold change. For fold change, the standard deviation (SD) is calculated to determine if the expression fold change between groups is significant.

Normalization of expression data is necessary due to many factors in the RT-PCR reaction (e.g., RNA quality, RNA quantity, efficiency) that can contribute to the variation in the expression level. Data normalization is performed by using endogenous controls to correct for variation. We performed data normalization with RQ Manager 1.2.1 from Applied Biosystems (see Note 9). Detailed information on data analysis using Taqman® Arrays has been previously published (43).

3.4.1. Calculating Fold Change

1. First determine the most appropriate endogenous controls for your samples. Several endogenous controls are recommended for data normalization when small differences in expression levels are involved.
2. Determine which of your sample will be your reference sample (calibrator). Reference samples are usually the untreated sample (control or normal) or a sample that is to be compared.
3. Using the endogenous controls selected for your experiment, normalize the C_T values with the average C_T of the endogenous controls: $\Delta C_T = C_T$ miRNA $- C_T$ endogenous control.
4. Calculate the standard deviation (SD) for each ΔC_T.
5. Calculate $\Delta\Delta C_T$: $\Delta\Delta C_T = \Delta C_T$ experimental sample $- \Delta C_T$ of reference sample.
6. Calculate fold change: fold change $= 2^{-\Delta\Delta C_T}$.
7. Incorporated the SD into fold change as a range: $2^{-\Delta\Delta C_T + SD}$ and $2^{-\Delta\Delta C_T - SD}$.

4. Notes

1. Some saliva samples are very viscous, so vortexing helps in collecting the saliva supernatant after centrifuging. However, do not vortex too much because mechanical rupture of cellular elements that may come from the pellet would interfere with RNA collected from the cell-free supernatant (6).
2. If there is no clear separation of the saliva supernatant from the pellet after the first spin, you may spin again at $2{,}600 \times g$ for 10 min. It is critical that only the cell-free supernatant is collected.
3. Other miRNA extraction kits are commercially available for saliva miRNA extraction. While the mirVANA miRNA isolation kit is only for extracting miRNAs, the mirVANA PARIS kit contains a lysis buffer that is used for saliva optimization.
4. It is recommended to follow the procedure for extracting total RNA from saliva than for only small RNAs so that the quality

of RNA can be verified for array analysis and the quantity can be measured.

5. The acid-phenol: chloroform step listed in the original protocol is skipped. An on-column DNase treatment step is included for removing DNA.
6. Another on-column DNase treatment that can be used instead of DNase 1 is TURBO DNase (Ambion). Close to 80 U of Turbo DNase would be required per sample. The wash steps after will inactivate the DNase.
7. Elution solution or nuclease-free water heated at 95°C and directly put on the column for elution results in the highest quantity of total RNA. If samples need to be concentrated in a speed-vacuum, elute samples using only nuclease-free water.
8. There are several quality controls test that can be performed on the extracted total RNA before proceeding to the Taqman Microarray RT step:
   (a) Run a 260/280 reading of your total RNA on a spectrophotometer. Clean RNA has a ratio of approximately 1.8–2.2.
   (b) Run samples on a gel to make sure rRNA bands are intact.
   (c) Run samples on Agilent Bioanalyzer or Biorad Experion.
   (d) Run a qPCR of your samples with a known reference gene.

   Even if rRNA is somewhat degraded, this does not indicate your miRNA quality is bad. miRNAs are small so they usually will not be affected. Samples with mRNA and rRNA compromised by fragmentation have been shown to give good miRNA data. If you want an estimate on how much miRNA is in the sample, the Agilent Bioanalyzer Small RNA gel measures the quantity of small RNAs and miRNAs present.
9. Global normalization is an alternative method for data normalization that does not use endogenous controls (44). Global normalization takes the median C_T of C_Ts common among all samples. The median C_T is used for normalizing the $\Delta\Delta C_T$ calculation on a per sample basis. This method follows the assumption that even though genes are differentially expressed, the amount of transcription remains similar across all samples. Global Normalization can be performed with Data Assist™ Software from Applied Biosystems.

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Conflict of Interest: D.T.W. Wong is cofounder of RNAmeTRIX, a molecular diagnostic company.

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Chapter 25

Application of Intronic MicroRNA Agents in Cosmetics

Jack S.K. Chen and David T.S. Wu

Abstract

MicroRNAs (miRNA) are short, single-stranded, noncoding RNAs that regulate a number of cellular processes through their capability to silence the expression of target proteins. Using miRNAs to target and reduce the expression of tyrosinase, the key enzyme involved in the synthesis of skin pigment melanin, presents a novel and feasible approach for achieving skin whitening. We have developed an artificial miRNA expression system that synthesizes miRNAs that downregulate tyrosinase expression by binding and degrading tyrosinase messenger RNAs. We used this anti-tyrosinase miRNA expression system (miR-Tyro) to successfully demonstrate the feasibility of miRNA-mediated skin whitening in vitro and in vivo. In light of bans of previously proven and popular skin whitening cosmetic ingredients such as hydroquinone and kojic acid, use of miRNAs that target and silence tyrosinase expression may present a novel and promising cosmetic approach to achieve skin whitening. Our finding on mirTyro-mediated skin whitening in mice is the first miRNA application proven to be feasible in vivo.

Key words: Skin whitening, MicroRNA, miR-Tyr, miR-Tyro, Tyrosinase, Melanin, Hyperpigmentation, Depigmentation

1. Introduction

Thanks in large to media propagation, we live in an era where the idea/concept of lighter skin is instilled in our subconscious. People were led to believe that having lighter skin tone symbolizes youthfulness, innocence, wealth, and elegance. In certain parts of the world, particularly Asian and African countries, women consider having a lighter skin tone as esthetically pleasing, and this enhances their degree of self-esteem and confidence. The obsession with attaining lighter, more pale skin tone is the main reason that the cosmetic industry generate an astrological amount of annual revenues from selling skin whitening/lightening products, and the demand for these products is expected to increase (see Note 1).

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The degree of one's skin tone is determined by the amount of the skin pigment, melanin, produced by melanocytes located in the basal epidermis. Even though the pigments that make up each individual's skin varies, they can be generally broken into two parts: *Constitutive skin color* (CSC) and *Facultative skin color* (FSC). CSC denotes the naturally occurring pigment generation in each individual while FSC designates the auxiliary, reversible pigmentation on top of the CSC that are induced by UVR and/or hormones (1). While FSC is easier to regulate via molecules with antioxidant or UV protection activity (i.e., Vitamin C, Sun screen), alterations in CSC may be made possible by the use of a genetic-based approach in targeting tyrosinase (2).

The rate-limiting step during melanin synthesis, or melanogenesis, is the conversion of tyrosine to dopaquinone catalyzed by the enzyme tyrosinase on the surface of melanosomes, organelles residing in melanocytes (3, 4). Melanins are stored in melansomes and then transported to neighboring keratinocytes via dendrites produced by these melanocytes (5).

One of the most common approaches to achieve skin whitening is to inhibit tyrosinase, which interrupts melanogenesis and leads to a reduction of melanin production and the eventual manifestation of a lighter skin tone. A number of compounds have been used as ingredients in various skin lightening products in the cosmetic industry thus far, including hormone-derived inhibitory oligopeptides, hydroxytetronic acid derivatives, benzoyl- and hydroquinone-based compounds, kojic acid, arbutin and plant extracts (6). Among these compounds, only hydroquinone has been demonstrated to mediate effective skin whitening effects in human trials and thus skin lightening products containing hydroquinone have enjoyed enormous success and popularity. However, due to high risks of side effects such as exogenous ochronosis and permanent depigmentation after long-term use, hydroquinone was banned for use in cosmetic products in Europe in 2001 and now faces possible similar ban in the United States by the FDA (7). On the other hand, data from animal studies suggest that kojic acid may cause toxicity to the embryo, liver, and kidney cells and increases the chances of liver cancer. Even though these findings have not been confirmed in humans, the use of kojic acid in skin lightening products was promptly banned in Japan in 2003 and subsequently in Korea and Switzerland (8).

MicroRNAs (miRNAs) are short single-stranded noncoding RNAs (20- to 25-nucleotide (nt) long) that represent a class of small regulatory RNAs. By inhibiting the translation of target mRNAs, miRNAs regulate gene expression posttranscriptionally and thus play an important role in a wide range of cellular processes (9, 10). Currently, two types of miRNAs are known: intergenic or exonic miRNAs and intronic miRNAs. Biogenesis of an intergenic miRNA starts with the synthesis of a primary miRNA

transcript (pri-miRNA) catalyzed by types-II or -III RNA polymerase (Pol-II/III). Pri-miRNAs are processesd in the nucleus by ribonuclease Drosha to a precursor miRNA (pre-miRNA) approximately 60-nt in length. After being transported into the cytoplasm, pre-miRNAs are further processed into mature and functional miRNAs by cytoplasmic ribonuclease Dicer (11). MiRNAs associate with a number of proteins to form RNA-induced silencing complex (RISC) that bind with target mRNAs having complementary sequences to the miRNAs and inhibit the subsequent protein translation of the mRNA. The process by which miRNAs bind to target mRNAs and suppress target protein translation is known as RNA interference (RNAi) (10, 12).

Following DNA transcription, a premature messenger RNA (pre-mRNA) strand consisting of introns and exons is synthesized. The introns are removed from the pre-mRNA molecule via RNA splicing and the exons are ligated together to form the mature mRNA for subsequent protein translation. Introns, on the other hand, were long considered to be unimportant sequences whose function was to be removed from pre-mRNA to form the mature mRNA. This view on introns turned out to be incorrect as they have been identified to undergo processing to form functional miRNAs (13). Following RNA splicing, intronic fragments serve as pri-miRNAs which are converted into pre-miRNAs in the cell nucleus by *Drosha*. As in the case with intergenic miRNAs, these intronic pre-miRNAs are then exported to the cytoplasm and processed into mature and functional intronic miRNAs that incorporate into RISC and mediate gene silencing effects via RNAi. Figure 1 provides a schematic description of the biosynthesis of both intergenic and intronic miRNAs. In general, intronic miRNA-mediated RNAi is more tightly regulated and target-specific than those mediated by exonic miRNAs (14).

Based on the principal mechanisms underlying the synthesis of intronic miRNAs, we designed and constructed an artificial expression system to produce miRNAs that target tyrosinase mRNAs and downregulate its expression. Initially, we designed an artificial splicing-competent intron that contains a pre-miRNA sequence for miRNA-434-5P (mir-434-5P), a native tyrosinase-targeting miRNA. We inserted this intron into a recombinant DNA molecule which is then cloned into a plasmid DNA serving as an expression vector. Using methods such as electroporation and lipid transfection, these plasmids can be delivered into target cells such as melanocytes where Pol-II catalyzes the transcription of pre-mRNA molecules. The intron portion is co-transcribed during this process and subsequently spliced out during RNA processing and further processed into mature and functional mir-434-5P miRNA molecules in the cell's cytoplasm. Figure 2a shows the structural composition for a typical intronic miRNA expression vector system. Given that mir-434-5P also targets five other genes, this expression

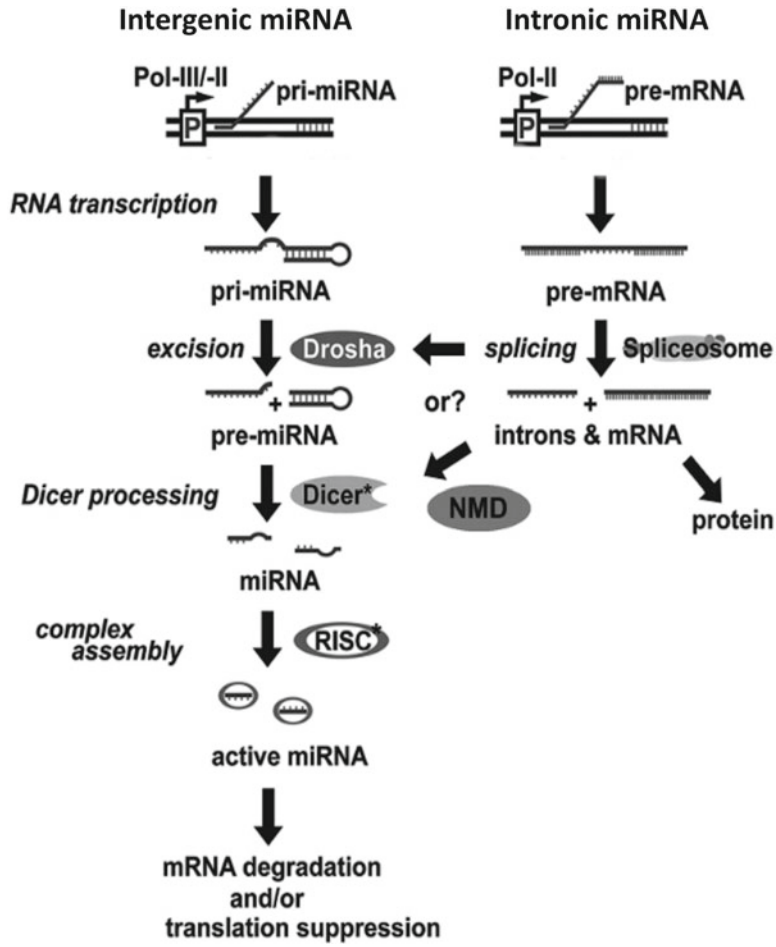

Fig. 1. Mechanisms underlying intergenic and intronic microRNA (miRNA) biosynthesis. Intergenic miRNAs are directly transcribed as a long single-strand noncoding RNA precursor (pri-miRNA) by either Pol-II or Pol-III RNA promoter, whereas intronic miRNAs are transcribed strictly via Pol-II promoter of its encoded gene and coexist within the intron region of the encoded gene transcript premature messenger RNA (pre-mRNA). After pre-mRNA splicing, the spliced intron functions as a pri-miRNA to form intronic miRNA. In the nucleus, the pri-miRNA is excised by either the RNase *Drosha* (intergenic miRNA) or spliceosomal/NMD components (intronic miRNA) to form hairpin-like miRNA precursors (pre-miRNA) that are then exported to cytoplasm for further processing by Dicer to form mature and functional miRNAs. Both intergenic and intronic miRNAs are incorporated into an RNA-induced silencing complex (RISC) that bind with target mRNAs exhibiting complementary sequences to the miRNAs. Subsequent protein translation from these target mRNAs is suppressed via RNA interference (RNAi) as a result.

system presents potential target specificity and safety concerns and thus may not be ideal for cosmetic application. To address these concerns, we redesigned the mir-434-5P pre-miRNA sequence to one that matches specifically to a region corresponding to nucleotides 3–25 of tyrosinase mRNA (15). We designated the redesigned anti-tyrosinase pre-miRNA sequence miR-Tyr and the

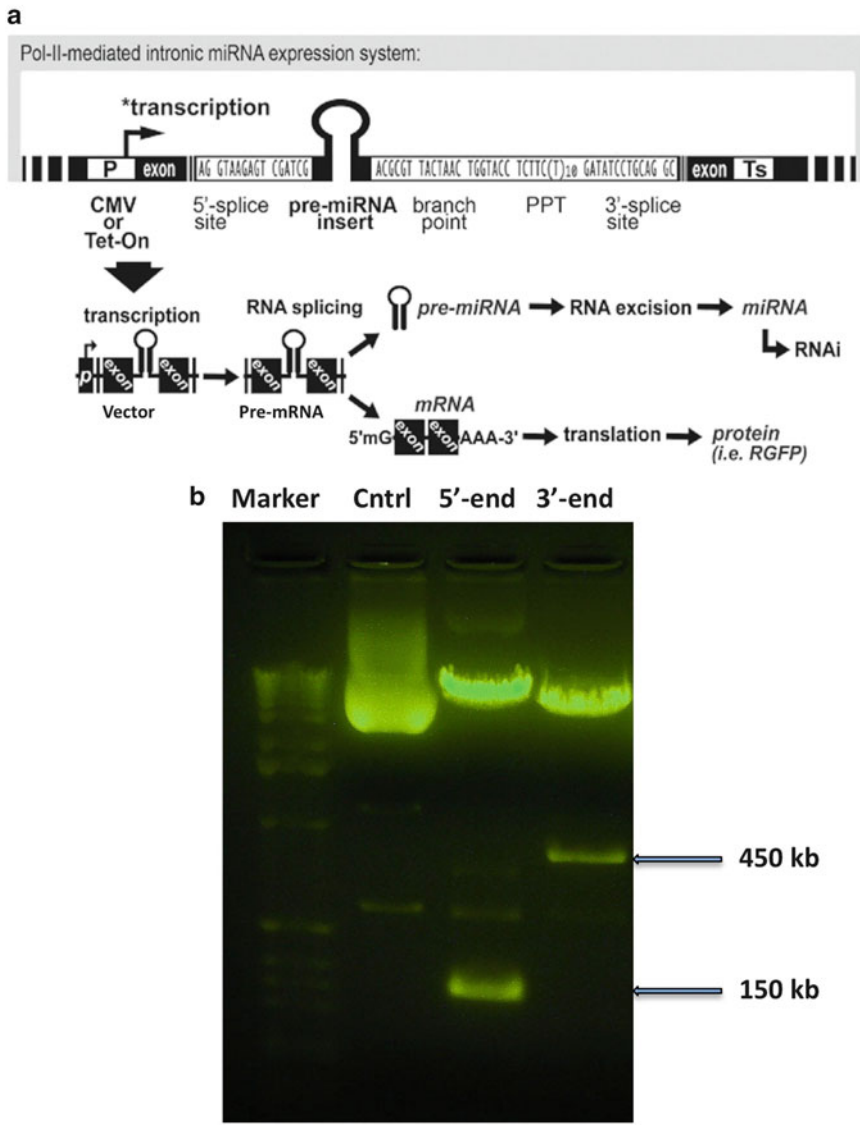

Fig. 2. Structural composition of an intronic miRNA expression vector (**a**) and restriction analysis of pHcRed vector-based miR-Tyro (**b**). MiR-Tyr pre-miRNA insert and pHcRed mammalian expression vector were digested with MluI/PvuI and ligated together to generate pHcRed miR-Tyro. Detection of 150 bp MluI/XhoI-digested band and 450 bp PvuI/BfrI-digested band confirms pHcRed miR-Tyro's sequence integrity at 5′ and 3′ ends of miR-Tyr insertion points, respectively.

resulting intronic expression vector containing the miR-Tyr sequence insert miR-Tyro.

In this chapter, we show in detail the steps in constructing an miR-Tyro expression vector and assessed its in vitro as well as in vivo effects using human melanocyte cells and nude mice. Taken together, our data suggest that miR-Tyro treatment efficiently suppressed tyrosinase expression, reduced melanin accumulation, and achieved detectable skin whitening effect.

2. Materials

2.1. Hybridization of MiR-Tyr Pre-miRNA Insert Oligonucleotides

1. Sense miR-Tyr sequence: 5′-GCTCGAGTGT CGCCCTACTC TATTGCCTAA GCCGCTAAGC CAGGCGGCTT AGGCAA TAGA GTAGGGCCGA GGATCCAT-3′ (100 pmol/μL in autoclaved ddH_2O).
2. Antisense miR-Tyr sequence: 5′-ATGGATCCTC GGCCCTA CTC TATTGCCTAA.
3. GCCGCCTGGC TTAGCGGCTT AGGCAATAGA GTAGG GCGAC ACTCGAGC-3′ (100 pmol/μL in autoclaved ddH_2O).
4. 2× Hybridization Buffer: 200 mM KOAc, 60 mM HEPES-KOH, 4 mM MgOAc, pH 7.4 at 25°C.

2.2. Restriction Enzyme Digestion and Sequential Ligation

1. Mammalian expression plasmid vector: pHcRed.
2. 10× Buffer H: 500 mM Tris–HCl, pH 7.5 at 37°C, 100 mM $MgCl_2$, 1 M NaCl, and 10 mM dithioerythritol (DTE).
3. Restriction enzymes: MluI (10 U/μL) and PvuI (5 U/μL).
4. MluI/PvuI digestion reaction mix: 9 μL autoclaved ddH_2O, 2 μL MluI, 2 μL PvuI, 2 μL 10× Buffer H; prepare the reaction mix immediately before use.
5. 10× Ligation Buffer: 660 mM Tris–HCl, pH 7.5 at 20°C, 50 mM $MgCl_2$, 50 mM dithiothreitol (DTT), and 10 mM adenosine triphosphate (ATP).
6. 5 U/μL T4 DNA ligase.
7. Ligation reaction mix: 4 μL autoclaved ddH_2O, 4 μL 10× Ligation Buffer, and 2 μL T4 ligase; prepare the reaction mix immediately before use.
8. 10× M buffer: 100 mM Tris–HCl, pH 7.5 at 37°C, 500 mM NaCl, 100 mM $MgCl_2$, and 10 mM DTT.
9. 10 U/mL T4 polynucleotide kinase.
10. Ethidium bromide (EtBr): prepare 10 mg/mL stock solution in ddH_2O and store at 4°C in dark.
11. 3% Agarose gel: Add 1.5 g agarose to 60 mL 1× TBT buffer and put in microwave for 1 min to dissolve. Cool for 10 min at room temperature, add 5 μL EtBr solution, and pour onto gel apparatus. Place in a sealed plastic bag and store at 4°C in dark before use.
12. Gel extraction kit from Qiagen.
13. Table-top microcentrifuge.

2.3. MiR-Tyro Amplification

1. Luria-Bertani (LB) growth medium: NaCl (10 g/L), Tryptone (10 g/L), Yeast Extract (5 g/L), $MgSO_4$ (10 mM), Ampicillin (100 μg/mL), or kanamycin (50 μg/mL). Prepare $MgSO_4$, Ampicillin, and kanamycin as 100× stock solutions (1 M, 10 mg/mL, and 5 mg/mL, respectively) and store them at 4°C.
2. Bacterial transformation: Z-competent *Escherichia coli* cells from Zymo Research (see Note 2).
3. Plasmid extraction reagents from Qiagen (see Note 3).
4. High-speed centrifuge.
5. Plasmid Resuspension Buffer: 10 mM Tris–HCl, pH 8.5.

2.4. MiR-Tyro Quality Control (QC)

1. Restriction enzymes: BfrI, PvuI, MluI, and XhoI.
2. 10× Buffer M: 100 mM Tris–HCl, pH 7.5 at 37°C, 500 mM NaCl, 100 mM $MgCl_2$, and 10 mM DTT.
3. 5′ end QC reaction mix: 9 μL autoclaved ddH_2O, 2 μL 10× Buffer H, 2 μL MluI, and 2 μL XhoI; prepare the reaction mix immediately before use.
4. 3′ end QC reaction mix: 9 μL autoclaved ddH_2O, 2 μL 10× Buffer M, 2 μL PvuI, and 2 μL BfrI; prepare the reaction mix immediately before use.
5. 6× Gel Loading Dye: 15% Ficoll 400, 66 mM EDTA, 20 mM Tris–HCl, 0.1% SDS, 0.09% Bromophenol Blue, pH 8.0 at 25°C.
6. 3% Agarose gel: Weigh 1.5 g of Certified Molecular Biology Agarose and transfer to a 500 mL flask. Add 50 mL 1× TBE buffer to the flask and microwave at high power for 1 min to dissolve the agarose. After cooling down for 10 min at room temperature, add 5 μL of 10 mg/mL EtBr and pour onto an agarose gel apparatus.
7. UV imager and camera.

2.5. In Vitro Testing of MiR-Tyro: Transfection

1. Cell line: Human epidermal melanocytes derived from darkly pigmented donors (HEMnDp; purchased from Invitrogen).
2. Culturing media and supplements: Medium 254 and Human Melanocytes Growth Supplements (HMGS).
3. Trypsin Solution (0.25%).
4. 1× Defined Trypsin Inhibitor.
5. Cell number determination: 0.4% Trypan Blue Solution and Hemacytometer.
6. Cell observation: any inverted microscope suitable for routine and laboratory applications. We use Leica DM IL inverted microscope mounted with DFC 295 camera.
7. Transfection reagent: X-tremeGENE HP DNA Transfection Reagent (purchased from Roche Diagnostics).

8. Cell lysis: CelLytic™ M Cell Lysis Reagent from Sigma.
9. Protein concentration determination: *RC DC* Protein Assay Kit II from Bio-Rad.
10. Immunodetection of tyrosinase: Mouse Monoclonal Anti-Tyrosinase antibody and Goat Anti-Mouse Secondary Antibody from Sigma.
11. Western Blot reagents from BioRad.

2.6. Melanin Determination

1. Solubilization Buffer: 1 N NaOH with 10% DMSO.
2. Melanin stock solution: Prepare 1 mg/mL melanin (Sigma) stock solution using Solubilization Buffer and store it at room temperature in dark. Prepare melanin standard solutions of 4, 10, 20, 40, 100, 200, 400, 600, and 800 μg/mL from diluting stock solution using Solubilization Buffer.
3. Spectrophotometer.

2.7. In Vitro Skin Whitening Experiment

1. Test articles: miR-Tyro, Arbutin, Ascorbic Acid, Tranexamic Acid, Kojic Acid.
2. Functional human skin model (MelanoDerm™) tissues: MEL-300-B tissue inserts containing NHM (normal human melanocytes) from African donors (MatTek Corporation; Ashland, MA). The structure and viability of MelanoDerm™ tissues can be retained for 2–3 weeks provided that appropriate culture conditions are utilized.
3. Culturing medium for MelanoDerm™ tissues: EPP-100-NMM Maintenance Medium.
4. Melanin Assay: Solvable™, 500 mL.
5. Macroscopic darkening observation: Any camera capable of capturing high resolution images. We use Nikon D200 digital single lens reflex (DSLR) camera, set at 10 megapixel image resolution.

2.8. In Vivo Skin Whitening Experiment

1. Animal models: male beige nude mice deficient in T-cell, B-cell, and Natural Killer (NK) cells purchased from Harlan Laboratories, Inc. The animals are maintained and monitored in accordance to the eighth edition of the Guide for the Care and Use of Laboratory Animals set by NIH.
2. Glycerol–Fructose infusion solution: 8% glycerol (v/v), 5% fructose (w/v), and 0.9% NaCl (w/v). Make 4 M NaCl stock solution and store it at room temperature after autoclaving it. Make 62.5% fructose (w/v) stock solution and filter-sterilize it before storing at 4°C (see Note 4). Prepare glycerol–fructose infusion solution by adding the appropriate amounts of dH_2O, autoclaved glycerol, and stock solutions of NaCl and fructose. Mix well and store at 4°C.

3. Methods

3.1. Construction of miR-Tyro

Both sense and antisense miR-Tyr pre-miRNA sequences were purified via polyacrylamide gel electrophoresis (PAGE) and then hybridized to form the miR-Tyr pre-miRNA insert. PHcRed and PVGV mammalian expression plasmid vectors and mirTyro pre-miRNA insert were then digested using MluI and PvuI restriction enzymes and ligated together to generate miR-Tyro.

1. Mix the sense and antisense miR-Tyr sequences (5 μL of each) with 10 μL of 2× hybridization buffer.
2. Heat the mixture to 94°C for 3 min and subsequently cool to 65°C for 10 min. Stop the reaction on ice.
3. Generation of restriction ends via MluI/PvuI digestion: add 5 μL each of pHcRed vector and miR-Tyr pre-miRNA insert to MluI/PvuI digestion reaction mix in two separate tubes. Incubate the reactions at 37°C for 4 h and stop the reaction on ice.
4. Purification of MluI/PvuI-digested constructs: Load and run the digested samples in 3% agarose gel and carefully cut out the portion of gel containing the MluI/PvuI-digested pHcRed vector and miR-Tyr pre-miRNA fragments. Recover and transfer them into separate eppordorf tubes and resuspend in 30 μL autoclaved ddH_2O. Extract the constructs using Qiagen's gel extraction kit according to manufacturer's instructions.
5. Ligation: Add ligation mix to the tube containing the purified MluI/PvuI-digested pHcRed vector and miR-Tyro pre-miRNA insert. Incubate the reaction at 16°C for 16 h and stop the reaction on ice.

3.2. Clonal Selection and Purification of miR-Tyro

Z-Competent cells were transformed with miR-Tyro and streaked over an LB agar plate. A number of clones were selected and further expanded by growing in LB media. The optical density and purity of each mirTyro clone was determined following extraction. To ensure the sequence integrity of miR-Tyro, each clone was treated with specific restriction enzymes and analyed by agarose gel electrophoresis. The clone(s) with the highest intensity of the correct restriction band as visualized under UV is then selected and stored at 4°C for further experimentation.

1. Prepare agar plates: LB media (10 g/L NaCl, 10 g/L Tryptone, and 5 g/L yeast extract) with agar (1.5% w/v) and 50 μg/mL kanamycin.
2. Bacterial transformation: Mix 5 μL miR-Tyro in 1 vial of partially thawed Z-competent *E. coli* cells and place it at 4°C for 10 min. Dilute the transformed cells 25× using LB media

and add 50 μL to an LB agar plate. Carefully spread the contents over the entire surface of the plate without damaging the agar. Incubate the plate overnight at 37°C.

3. Pick select numbers of colonies using sterile disposable pipet tips. Inoculate each colony into appropriate volumes of LB media containing appropriate selection antibiotic and incubate overnight at 37°C
4. Plasmid extraction: Pellet the overnight LB cultures via centrifugation at 3,000 rpm (700×*g*) for 10 min. Extract miR-Tyro using the appropriate plasmid extraction kit from Qiagen according to manufacturer's instructions (see Note 5). Resuspend the miR-Tyro pellet using appropriate volumes of Plasmid Resuspension Buffer.
5. MiR-Tyro concentration and purity: Determine the concentration of miR-Tyro using a spectrophotometer. The ratio of absorbance at 260 and 280 nm is used to assess miR-Tyro's purity. A pure dsDNA sample has an $A_{260\ nm}/A_{280\ nm}$ ratio of approximately 1.8–1.9 (see Note 6).
6. Sequence integrity: Add 5 μL miR-Tyro each to 5′ end and 3′ end QC reaction mixes and incubate at 37°C for 4 h. Add 4 μL 6× Gel Loading Dye to each sample and load the samples on 3% agarose gel and run the electrophoresis procedure at 106 V. Stop the procedure after approximately 20–25 min. Observe the gel under UV and take pictures. The presence of 150 bp band (5′ end) and 450 bp band (3′ end) confirms the presence of miR-Tyro pre-miRNA insert in miR-Tyro (Fig. 2b).

3.3. Transfecting HEMnDp Cells Using miR-Tyro

Effect of miR-Tyro expression on melanogenesis was assessed using human epidermal melanocyte cells. First, these cells were transfected with miR-Tyro and assayed for melanin content and tyrosinase expression via Western Blot.

1. Cell preparation: Plate 1×10^6 HEMnDp cells in 10 mL Medium 254 per 100-mm cell culture dish 2 days prior to transfection (total volume of media in each dish is 10 mL). Make sure the cells are approximately 60–70% confluent at the time of transfection. Use four plates of cells for each experimental sample (untreated control and miR-Tyro transfected).
2. Preparation of transfection complex: Add 12 μg VSVG-based miR-Tyro in 1 mL of Medium 254. Add 36 μL X-tremeGENE HP reagent into medium containing the miR-Tyro (1:3 ratio of μg miR-Tyro to μL transfection reagent). Vortex gently and incubate the HP:miR-Tyro complex for 15 min at room temperature. Thereafter, add the transfection complex solution to the cells in a dropwise manner and shake the culture plate gently to ensure even distribution of the transfection complex over the entire culture plate surface (see Note 7).

3. Post transfection: Replace the transfection solution with fresh media the following day. Incubate the cells for additional 48 h before collecting them for assessing tyrosinase expression (Western Blot) and melanin content (melanin assay).

3.4. Melanin Assay

1. Cell collection: Following transfection, add 1 mL Trypsin Solution to each cell culture dish. Gently tap the side of each dish after 1 min to detach the cells followed by adding 1 mL Trypsin Inhibitor (see Note 8). Combine cells from two culture dishes and collect 50 μL for cell number determination. Centrifuge the remaining cells for 5 min at 3,000 rpm to pellet the cells.
2. Cell number determination: Add 50 μL Trypan Blue solution to the 50 μL cells collected from step 1. Mix well and add 20 μL to a hemacytometer. View the cells under an inverted microscope and count the number of viable cells (see Note 9).
3. Add 250 μL of Solubilization Buffer to each cell pellet and mix well.
4. Incubate samples at 60°C overnight along with melanin standards.
5. Reading assay: Transfer 250 μL of sample solution to a new tube. Measure OD at 490 nm using a spectrophotometer. Repeat with other samples.
6. Statistical analysis: Evaluate differences in the average values of melanin concentration by Student's *t*-test. Figure 3a shows the effect of miR-Tyro transfection on melanin content in HeMnDp cells.

3.5. Western Blot

1. Total cell extract preparation: Remove cells using trypsin and pellet by centrifugation. Resuspend cell pellet using CelLytic™ M (200 μL per sample). Determine lysate protein concentration using the Bio-Rad protein assay reagent (Hercules, CA), according to manufacturer's instructions.
2. Separate cell lysate proteins by SDS/PAGE. Transfer proteins to a nitrocellulose membrane using a semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA). Following overnight blocking, incubate the membrane with monoclonal anti-tyrosinase antibody (1:500). Incubate the membrane with goat anti-mouse peroxidase-conjugated secondary antibody (1:80,000) and detect tyrosinase bands using the Amersham-Pharmacia ECL system according to the manufacturer's instructions. Figure 3b shows the level of tyrosinase expression in HeMnDp cells following miR-Tyro transfection.

3.6. In Vitro Skin Whitening Experiments Using 3-D Human Skin Model

MatTek's MelanoDerm™ artificial skin model consists of normal, human-derived epidermal keratinocytes (NHEK) and melanocytes (NHM) cocultured to form a multilayered, highly differentiated model resembling the human epidermis. Mel-300-B tissues contain

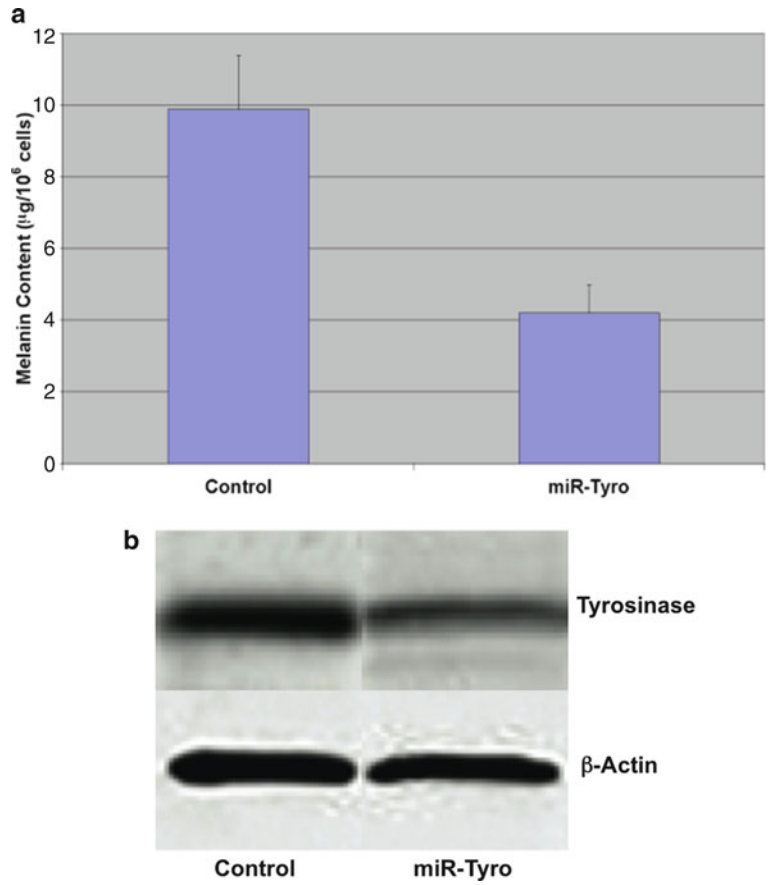

Fig. 3. In vitro effects of miR-Tyro transfection. (**a**) Effects of miR-Tyro transfection on melanin content in human melanocyte cells. Melanin level was normalized to total cell number and expressed as μg per 10^6 viable cells. (**b**) Effects of miR-Tyro transfection on tyrosinase expression. Total cell extract of HEMnDp cells were separated via SDS-PAGE and probed with Monoclonal Anti-Tyrosinase antibodies.

NHMs derived from African donors and become increasingly pigmented when cultured for up to 3 weeks (post-shipment). Thus, Mel-300-B provides a useful in vitro model to evaluate cosmetic and pharmaceutical agents designed to modulate skin pigmentation. In this experiment, Mel-300-B tissues were treated with miR-Tyro and a number of known skin-whitening compounds.

1. Preparation of MelanoDerm samples: store Mel-300-B at 4°C upon receipt. On the day of experiment, add 5 mL of pre-warmed medium (EPI-100-NMM) into the wells of 6-well plates. Add two sterile washers, one on top of one another, to each well. Carefully place the cell culture inserts on top of the culture stand or two-washer pedestal.
2. Topical application of miR-Tyro and other test articles: Apply miR-Tyro (2 μg/mL), Kojic acid (1%), Arbutin (1%),

Tranexamic acid (1%), and Ascorbic acid (3%) topically into each Mel-300-B tissue insert. Add each compound in a total volume of 100 μL per insert on days 1, 6, and 12. End experiment on day 18. Add each testing compound in triplicate tissue inserts.

3. Macroscopic observation: Observe tissues under phase contrast microscopy and take pictures of the tissues under a microscope and take pictures (Fig. 4a). Then carefully remove the

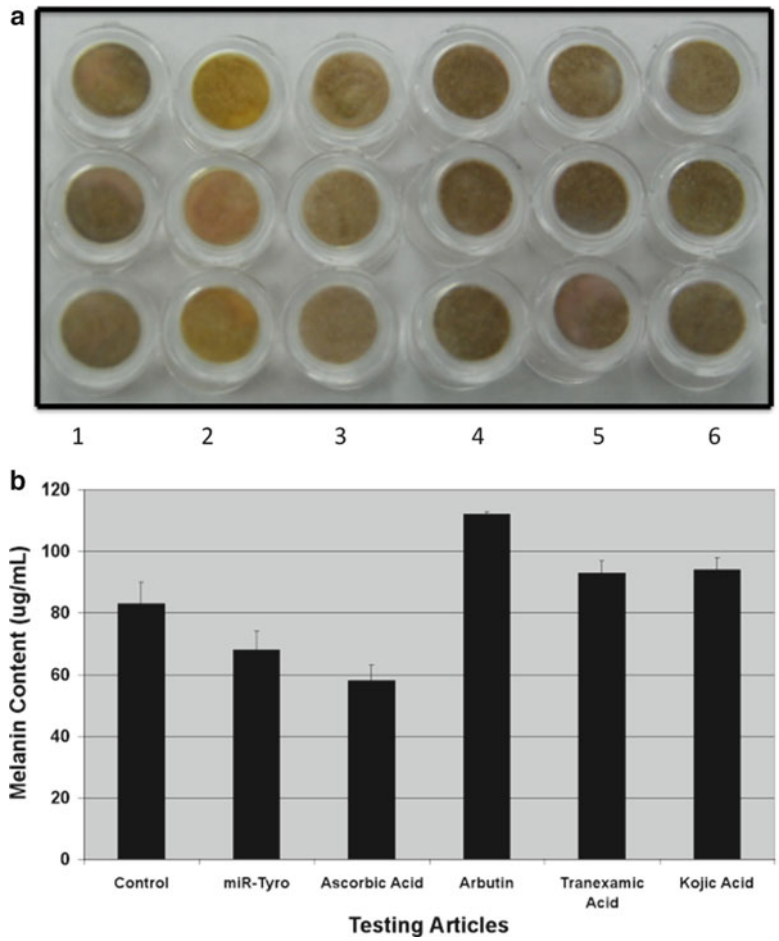

Fig. 4. Effects of topical application of miR-Tyro on melanin content in three-dimensional human skin model. MEL-300-B tissue inserts containing human melanocytes from African donors were treated with 2 μg/mL pHcRed miR-Tyro (2) and numerous known skin whitening compounds (3% Ascorbic Acid (3), 1% Arbutin (4), 1% Tranexamic Acid (5), and 1% Kojic Acid (6)) at selected time points as described in Methods. MEL-300-B inserts added with equal volume of dH_2O served as control (1). Extent of macroscopic darkening was compared among all samples and photographed on day 18 (**a**). The tissue inserts were then dissolved in Solvable™ and assayed for melanin content (**b**). Skin whitening effects of pHcRed and VSVG miR-Tyros on mice. miR-Tyro was formulated with Glycerol–Fructose infusion solution and applied to male nude mice via subcutaneous injection on days 1, 7, and 14. Whitening effects can be seen by day 6, and plateaued at Day 21. This whitening effect was reversible, as the treated skin area in both mice returned to the original condition by Day 30. Compared to mouse treated with pHcRed miR-Tyro (one miR-Tyr copy per vector), the extent of skin whitening effects in mouse treated with VSVG miR-Tyro (four miR-Tyr copies per vector) was significantly greater.

inserts containing the tissues using a sterile forcep and submerge them in PBS for 10 min to remove residual phenol red and test compound. Store the inserts at −20°C in a sealed plastic bag if they are not to be used immediately (see Note 10).

4. Melanin assay: Remove the inserts to thaw the tissues. To proceed, place the insert in a 2 mL microfuge tube and add 250 μL of Solvable™ (see Note 11). Incubate at 60°C overnight along with melanin standards. Use two inserts per test article.
5. Reading assay: Transfer 250 μL of sample solution to a new tube. Measure OD at 490 nm using a spectrophotometer. Repeat with other samples. Figure 4b shows the melanin content in Mel-300-B inserts following topical application of miR-Tyro and other known skin whitening compounds.

3.7. Skin Whitening Effects of miR-Tyro In Vivo

To determine the effects of miR-Tyro in vivo, two male nude mice with relatively dark skin tone were used. In addition to the pHcRed vector-based miR-Tyro described thus far, another miR-Tyro was generated using a different mammalian expression vector, pCMV-VSV-G (VSVG), and used for comparison in this experiment (see Note 12).

1. Preparation of miR-Tyro solution for injection: Add 300 μg of miR-Tyro to 900 μL Glycerol–Fructose infusion solution in a 2 mL microcentrifuge tube (330 μg miR-Tyro/mL concentration). Mix well and store at 4°C before use.
2. MiR-Tyro application: First withdraw 400 μL miR-Tyro solutions (containing 100 μg miR-Tyro) onto the 1 mL syringe attached with the 20G needle. Get rid of airbubbles and adjust the amount to 300 μL by dispensing excess miR-Tyro solution. Replace the 20G needle with the 26G needle and inject miR-Tyro solution subcutaneously on the dorsal side of nude mice's right hind legs. Perform the injections on days 1, 7, and 14 (see Note 13).
3. Observation: observe mice on daily basis. Take pictures at selected time points. Figure 5 shows the time course changes in nude mice's skin color following three miR-Tyro treatments.

4. Notes

1. The terms "skin whitening" and "skin lightening" are often used interchangeably, and that their difference lies in the marketing target. Skin whitening is often used by companies marketing products to Asian women, who associate light skin with beauty and wealth. On the other hand, skin lightening is often

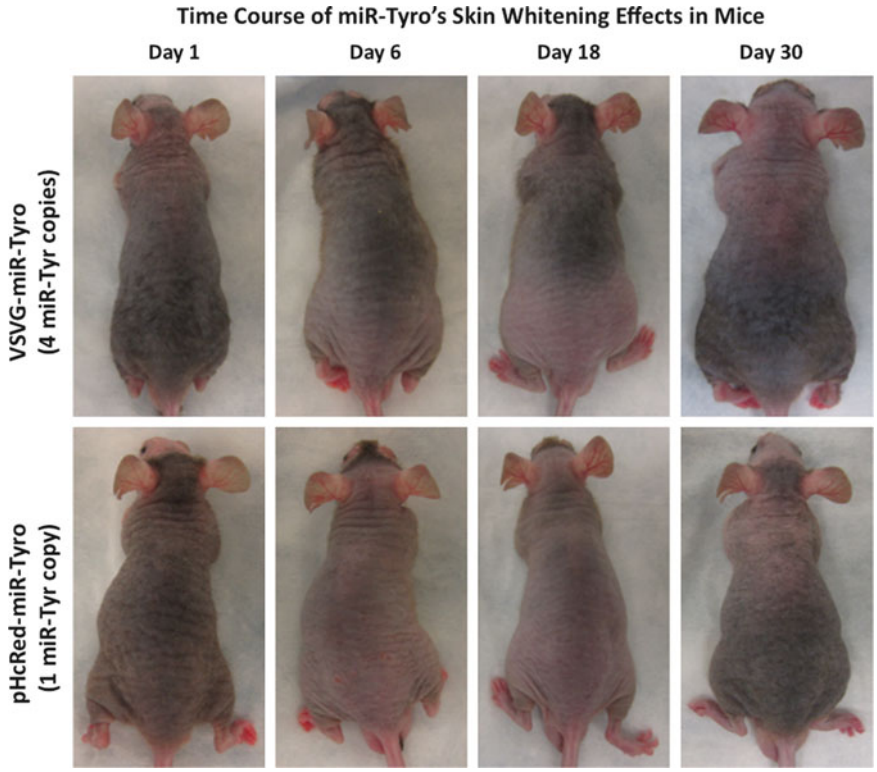

Fig. 5. Time course miR-Tyro's skin whitening effects in mice.

used to market to women seeking products that will reduce their hyperpigmented areas to even their skin tone.

2. Premade Z-Competent *E. coli* cells were used for transformation in preference over conventional competent *E. coli* cells because they are made chemically competent via a proprietary method that eliminates the need for heat shock procedure. As a result, Z-Competent cells can be used for spreading on LB agar plate or added to LB broth for overnight amplification following the addition of plasmid DNAs.
3. We have tested numerous plasmid extraction kits from various vendors and came to the conclusion that Qiagen's extraction kits are the best in terms of yield quality and consistency.
4. Fructose solution turns yellow after being autoclaved. To avoid this problem, filter-sterilize fructose solution and then store it at 4°C.
5. A number of buffers involved in plasmid extraction contain MOPS solution. When using MOPS from outside vendors (Sigma for example) to make these buffers, it is important to confirm and/or adjust its pH value to 7 in order to ensure

obtaining proper yield of plasmid DNA pellet following the high-speed centrifugation step.

6. It is common for DNA samples to be contaminated with other molecules such as RNA and proteins. An $A_{260\ nm}/A_{280\ nm}$ ratio greater than 1.9 indicates the sample is contaminated with RNA. On the other hand, an $A_{260\ nm}/A_{280\ nm}$ ratio less than 1.8 indicates the sample is usually contaminated with protein which absorb strongly at 280 nm.
7. Do not use tubes made of polystyrene for preparing the miR-Tyro:HP complex. Make sure undiluted X-tremeGENE HP transfection reagent does not come into direct contact with plastic surfaces in order to avoid significant reduction in transfection efficiency.
8. When supplemented with Human Melanocyte Growth Supplement, Medium 254 contains 0.5% of fetal bovine serum, which is insufficient for inhibiting trypsin. The Defined Trypsin Inhibitor is added at 1:1 ratio to ensure viability of HEMnDp cells following exposure to trypsin.
9. Trypan Blue is a vital dye used to distinguish between viable and dead cells. This is based on the principle that viable cells possess intact cell membranes that exclude Trypan Blue. Dead cells, on the other hand, have damaged cell membranes and therefore will take up Trypan Blue and appear as blue cells under the microscope.
10. It is preferable to keep the MEL-300-B tissue inserts in a sealed plastic bag when storing them since this prevents dehydration of the tissues.
11. Solvable™ is an aqueous-based solubilizer with an excellent capacity for solubilizing wet tissue, aqueous tissue homogenates, proteins, nucleotides, and other substances into a solution. Due to its aqueous nature, Solvable™ is safer to use than Soluene-350 for many similar applications.
12. Another mammalian expression vector, pCMV-VSV-G (VSVG for short), was used for generating a second version of miR-Tyro. Briefly, the miR-Tyr pre-miRNA insert and VSV-G were digested with BamHI/XhoI and ligated together to form VSV-G-based miR-Tyro. Based on the intensity of miR-Tyr band from electrophoresis, we estimate that the pHcRed vector-based miR-Tyro contains one copy of miR-Tyr pre-miRNA insert sequence per vector whereas the PVGV vector-based miR-Tyro contains four copies of miR-Tyr pre-miRNA insert sequence per vector.
13. We realized it would be more ideal to apply miR-Tyro topically in order to better assess its effectiveness as a cosmetic ingredient. We chose to deliver miR-Tyro via subcutaneous injection due to the fact that mice constantly lick their bodies and

therefore miR-Tyro solution will be removed from their skins if applied topically. As shown in Fig. 5, our data presented a proof-of-concept for miR-Tyro's ability to mediate skin whitening effects in vivo.

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Chapter 26

MicroRNAs in Skin and Wound Healing

Jaideep Banerjee and Chandan K. Sen

Abstract

MicroRNAs (miRNAs) are small noncoding RNA molecules ~22 nucleotides in length that can post-transcriptionally repress gene expression. MiRNAs bind to their target messenger RNAs (mRNAs), leading to mRNA degradation or suppression of translation. miRNAs have recently been shown to play pivotal roles in skin development and are linked to various skin pathologies, cancer, and wound healing. Chronic wounds represent a major health burden and drain on resources and developing more effective treatments is therefore a necessity. Increase in the understanding of the regulation of chronic wound biology is therefore required to develop newer therapies. This review focuses on the role of miRNAs in cutaneous biology, the various methods of miRNA modulation, and the therapeutic opportunities in treatment of skin diseases and wound healing.

Key words: MicroRNA, Hypoxia, Wound healing, Skin

1. Introduction

1.1. MicroRNAs: The Body's In-Built Gene Expression Regulators

MiRNAs have become important as therapeutic targets as they target a group of functionally similar genes and can be manipulated to target entire pathways as opposed to conventional practice of manipulating individual genes. The miRNAs usually bind to the 3′ untranslated region of the target messenger RNAs (mRNAs) and can repress protein expression by blocking translation or by degrading the mRNA (1). They are predicted to be regulating almost 30 % of the coding genes (2) and thus differential expression of miRNAs may result in various physiological abnormalities.

1.1.1. MicroRNA Biogenesis

MiRNAs are encoded in the human genome as miRNA genes and are then processed to mature miRNAs. RNA polymerase II transcribes primary miRNAs (pri-miRNAs), which are then capped and polyadenylated. The microprocessor complex, which is composed of the RNase III enzyme drosha and DGCR8, then cleaves

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the pri-miRNAs into shorter premature miRNAs (pre-miRNA). The resulting pre-miRNAs are then exported to the cytoplasm through the Ran-GTP-dependent nuclear export factor exportin-5. Another RNase III enzyme, dicer, then cleaves the pre-miRNAs into 18-to 24-nt double-stranded RNAs. The resulting RNA-duplex associates with the miRNA-induced silencing complex (RISC), where one of the strands is degraded while the other becomes the mature miRNA. The mature miRNAs interact with target mRNAs via complementarity binding with a particular region known as "seed sequence." The resultant complex hinders assembly of ribosome, subsequently suppressing gene expression. An overview of the key processes involved in the biogenesis of miRNA is illustrated in (see Fig 1).

miRNA research in the field of dermatology is in its early phase, but the early findings are substantial, pointing toward a vast opportunity for developing effective therapies for treatment of skin diseases and wounds.

1.2. The Skin: The Largest and the Most Varied Organ of the Body

The skin is made of three distinct layers of tissue (3).

Epidermis: The epidermis is seen on the surface of the skin. The epidermis contains five layers. From bottom to top the layers are named stratum basale, stratum spinosum, stratum granulosum, stratum licidum, and stratum corneum. The epidermis is composed mostly of keratinocytes along with dendritic cells, melanocytes,

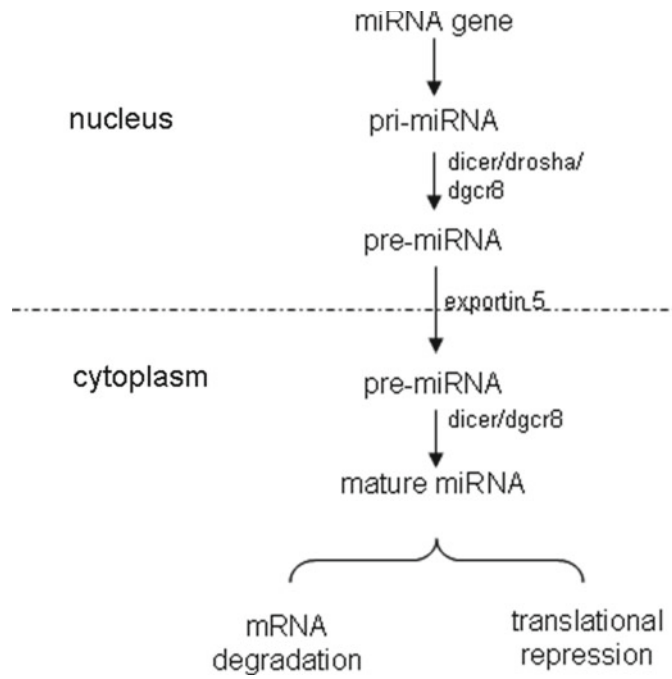

Fig. 1. miRNA biogenesis pathway.

Langerhans, and Merkel cells. The melanocytes produce a dark pigment called melanin which contributes to skin color and provides UV protection. They are located at the bottom of the epidermis. Basal cells are small cells found at the bottom of the epidermis.

Dermis: The dermis consists of collagenous and elastic fibers populated by fibroblasts, macrophages, mast cells, and lymphocytes and also consist of a glycosaminoglycan/proteoglycan fraction which functions as a supporting matrix or ground substance and makes up its base. The dermis supplies the avascular epidermis with nutrients by means of its vascular network. It contains sense organs for touch, pressure, pain, and temperature (Meissner's corpuscles, Pacinian corpuscles, free nerve endings), as well as blood vessels, nerve fibers, sebaceous, and sweat glands. The dermis also contains hair follicles which are epidermal outgrowths and have a reservoir of stem cells that may regenerate the epidermis.

Subcutaneous layer or Hypodermis: The hypodermis is composed of adipocyte lobules. This layer acts as a protective cushion and helps to insulate the body by monitoring heat gain and heat loss (see Fig. 2).

1.2.1. Skin Morphogenesis and the Role of MicroRNAs

Increasing evidence suggests that miRNAs may play important roles in regulating self-renewal and differentiation in skin stem cells. The human epidermis continuously renews itself by a process which involves generation of transient amplifying cells by basal epidermal stem cells, which move outward from the basal membrane, migrate through the epidermis, and undergo terminal differentiation (4, 5). MiRNAs play an important role during this process, as demonstrated by epithelium-specific depletion of dicer or dgcr8 genes which lead to improper barrier formation, abnormal hair follicle development, and hyper proliferation of basal interfollicular keratinocytes (6–8). Some of the most highly expressed miRNAs in the skin are miR-152, -143, -126, -21, -27a, -214, -16, -203, -125b, -34a, -205, -27b, -30b, -125a, -191, -200 family (-200a, -200b, -200c, -141, -429), -199 family (-199a, -199b), and -19/-20 family (-19b, -20, -17-5p, -93) (3). MiR-125b silences Blimp1 and VDR and represses stem cell differentiation (9). When miR-125b is sustained in stem cell progenies, stemness dominates over epidermal, oil-gland, and hair-follicle differentiation. Another microRNA (miRNA), miR-203 promotes epidermal differentiation by restricting proliferative potential and inducing cell-cycle exit by silencing p63 which is an essential regulator of stem-cell maintenance in stratified epithelial tissues (10). Nine additional miRNAs (miR-23b, miR-95, miR-210, miR-224, miR-26a, miR-200a, miR-27b, miR-328, and miR-376a) have recently been found that are associated with human keratinocyte differentiation in vitro and in vivo (11).

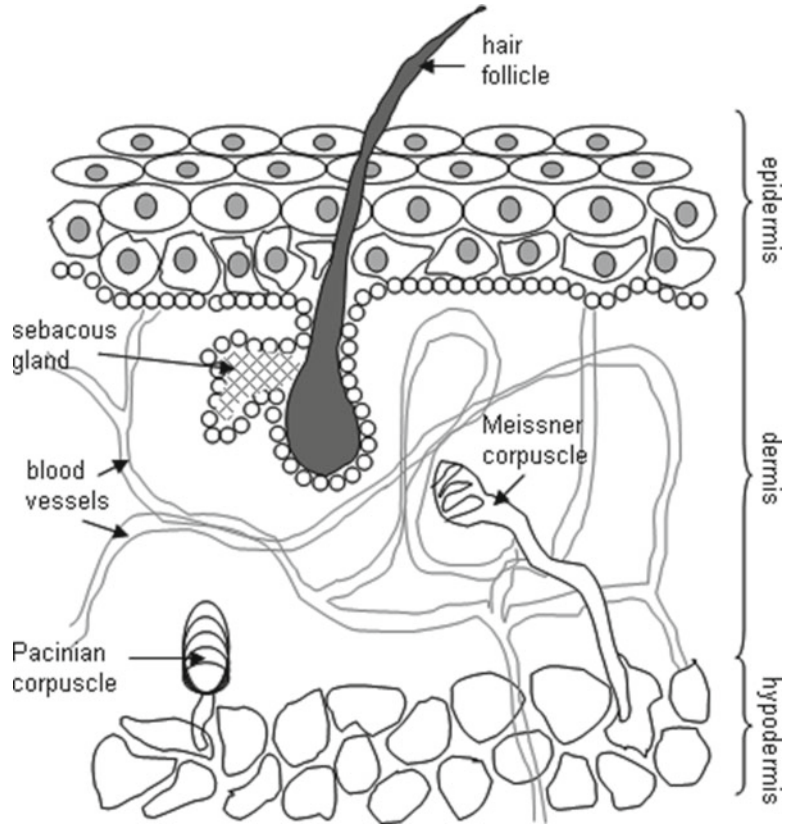

Fig. 2. Schematic diagram showing the structure of the skin.

1.3. MicroRNA Regulation of Skin Function: Wound Healing

Chronic wounds represent a major and rising socioeconomic threat affecting over 6.5 million people in the United States costing in excess of the US $25 billion annually (12). Wound healing is a physiological response to injury that ends in wound closure. In humans, wound healing proceeds along a cascade comprising of the following overlapping phases (13): hemostasis and inflammation, proliferative (granulation, vascularization, and wound closure), and remodeling (can continue from weeks to years and encompasses scarring, tensile strength, and turnover of extracellular matrix components).

1.3.1. MicroRNAs in Wound Healing

Inflammation

Mounting of a successful inflammatory response as well as resolution of the inflammation at the right time is critical to healing of a wound. Disruption of miRNA biogenesis has a major impact on the overall immune system. The inflammation response to wound is tightly regulated by proinflammatory signals as well as signals that are anti-inflammatory to resolve inflammation. An imbalance between these signals results in chronic inflammation and derails the healing cascade (12). miRNA have been shown responsive to as well as regulate some of the key mediators of inflammatory

response in the course of wound healing. Emerging studies indicate that miRNAs, especially miR-21, miR-146a/b, and miR-155, play a key role in regulating several hubs that orchestrate the inflammatory process (12, 14). miR-146 and miR-155 are induced by TNF-α and IL-1β among others. miR-21 is induced by IL-6. miR-21 silences a number of inflammatory mediators like PTEN and PDCD4; miR-146 silences IRAK and COX2 while miR-155 silences SHIP1, SOCS1, and IL-12 (12).

Angiogenesis

The first series of observations establishing key significance of miRNAs in the regulation of angiogenesis came from experimental studies involved in arresting miRNA biogenesis by Dicer depletion to deplete the miRNA pools of vascular tissues and cells (15). One of the drivers of wound angiogenesis is reactive oxygen species derived from NADPH oxidase which is subject to control by miRNAs (16). Hypoxia drives angiogenesis and hypoxia-sensitive miR-200b is involved in such induction of angiogenesis via directly targeting Ets-1 (17). Various aspects of angiogenesis, such as proliferation, migration, and morphogenesis of endothelial cells, can be regulated by specific miRNAs in an endothelial-specific manner. Some of the most well-characterized miRNA-regulated proteins involved in angiogenesis are Spred1 (miR-126) (18), c-kit (miR-221/222) (19), Tsp-1 (miR-17-92) (20), ITGB5 (miR-92a) (21), VEGF (miR-20a) (22), and TIMP-1 (miR-17-5p) (23).

1.3.2. Re-Epithelialization and Wound Closure

Restoration of an intact epidermal barrier is enabled through wound epithelialization, also known as re-epithelialization (24, 25). A wound that is not epithelialized is not considered "healed" no matter how perfectly restored the underlying dermal structures may be. Thus, wound re-epithelialization is a critical and defining feature of wound repair. Re-epithelialization of the wound occurs as the result of three overlapping keratinocyte functions: migration, proliferation, and differentiation. It begins with dissolution of cell–cell and cell–substratum contacts which is followed by the polarization and initiation of directional migration in basal and a subset of suprabasilar keratinocytes over the provisional wound matrix. A subset of keratinocytes immediately adjacent to, but not within the wound bed, then undergoes mitosis, and finally there is multi-layering of the newly formed epidermis and induction of differentiation-specific gene products to restore the functionality of the epidermis.

Problematic wounds are generally characterized by tissue ischemia (13) and mostly arise due to vascular complications. Hypoxia is a component of ischemia and is defined (13) by a lower tissue partial pressure of oxygen (pO_2) compared with the pO_2 to which the specific tissue element in question is adjusted to under healthy conditions in vivo, i.e., it represents a reduction in oxygen delivery below tissue demand. Limitations in the ability of the vasculature

to deliver O_2-rich blood to the wound tissue leads to, among other consequences, hypoxia. Three major factors may contribute to wound tissue hypoxia (13): (1) peripheral vascular diseases (PVDs) limiting the O_2 supply, (2) increased O_2 demand of the healing tissue, and (3) generation of reactive oxygen species (ROS) by way of respiratory burst and for redox signaling. Wound hypoxia may range anywhere from near-anoxia to mild–modest hypoxia. It is likely that the magnitude of wound hypoxia is not uniformly distributed throughout the affected tissue especially in large wounds. This is most likely the case in chronic wounds presented clinically as opposed to experimental wounds, which are more controlled and homogenous in nature, where the situation presents towards near-anoxic pockets. Primarily based on the tumor literature, hypoxia is generally viewed as being angiogenic. This is true with the condition that hypoxia be acute and mild to modest in magnitude. Extreme near-anoxic hypoxia, as commonly noted in problem wounds, is not compatible with tissue repair. Adequate wound tissue oxygenation is required but may not be sufficient to favorably influence healing outcomes.

Hypoxia induces specific miRNAs collectively referred to as hypoxamiRs (26). Among the known hypoxamiRs, miRNA-210 has been reported to be the most sensitive to hypoxia. miR-210 is directly under the control of hypoxia regulated genes—hypoxia-inducible factor-1 and -2 (Hif-1α and Hif-2α) and NF-κB. Hypoxia regulated miRNAs are generally considered to favor angiogenesis (27); however, in ischemic situation, they impair cell proliferation and migration. Keratinocyte proliferation and migration is essential for re-epithelialization and wound closure and miR-210 silences some key proteins like E2F3 which is required for keratinocyte proliferation. Silencing-specific hypoxamiRs may therefore represent a prudent approach to facilitate tissue repair. The iron sulfur cluster assembly proteins ISCU1/2 have also been identified as HIF-dependent and silenced by miR-210 and can potentially affect healing outcomes. ISCU1/2 are essential for iron sulfur cluster biogenesis which are incorporated into wide variety of proteins, many of which like Complex I and Aconitase are involved in mitochondrial metabolism. miR-210 can thus impair mitochondrial activity by repressing ISCU1/2 (26, 28). This will translate into compromised ATP production. In hypoxic condition, this may be compensated due to the Pasteur effect and result in a more optimized energy production. In the short term, therefore, this may result in hypoxic cellular adaptation and may improve survival. In contrast, chronic repression of mitochondrial function during hypoxia has been linked to various pathologic consequences including ischemic diseases (26). These outcomes are not compatible with the higher energy demands associated with tissue repair and so, this may be another possible mechanism for compromised healing upon HIF stabilization in ischemic wounds.

1.3.3. Remodeling Phase

Collagen deposition is an important aspect of the remodeling phase. miR-29a directly regulates collagen expression at the post-transcriptional level (29). Mammalian fetal skin can heal without a scar whereas during the late gestational stage, it transitions to a scarring phenotype (30, 31). Several miRNAs are differentially expressed between the two stages and probably contribute to this transition and miRNA-29b, miRNA-29c, and miRNA-192 have been found to be the key mediators with their levels being highly induced during the late gestation phase (32). MiRNA-29b and miR-29c targets repress several extracellular matrix proteins, anti-fibrotic TGF-β and proteins like Smads, β-catenin which are involved in the signaling pathways important for scarless healing (33, 34) while miR-192 enhanced collagen 1α2 expression by targeting the Smad-interacting protein 1 (SIP1) (35) (see Fig. 3) summarizes the miRNA involved in the different phases of wound healing.

1.4. MicroRNA Regulation of Other Functions of the Skin

The role of miRNAs in regulation of the other functions of the skin is not yet very well characterized and work is in progress to elucidate the mechanisms. A key function of the skin is to serve as a barrier and prevent loss of water from the organism. A key protein involved in the skin's barrier function is E-cadherin which is required for the maintenance of proper localization of key tight junctional proteins and its absence results in permeable tight junctions compromising epidermal barrier function of the skin (36, 37). miR-200 and miR-205 silence the transcriptional repressors of E-cadherin—ZEB1 and SIP1 (also known as ZEB2) (38–40). Thus, miR-200 and miR-205 are expected to positively regulate E-cadherin and seem to be essential in maintaining epithelial stability. These studies were however performed in cell lines and transformed cells and the significance in normal skin biology remains to be elucidated. Another important function of the skin is pigmentation. Human pigmentation involves production and dispersion of melanin by epidermal melanocytes to neighboring keratinocytes. Skin pigments are essential for absorbing the harmful ultraviolet radiations less than 310 nm and also regulate skin vitamin D production (41, 42). Among the miRNAs regulating skin color, miR-25 silenced micropthalmia-associated transcription factor (MITF) in skin melanocytes that regulates genes linked to coat color like tyrosinase (tyr) and tyrosinase-related protein 1 (43). Another miRNA, miR-434-5p has been implicated in skin whitening and lightening by targeting tyr and hyaluronidase (hyal) genes (44), which play an important role in melanin production.

1.5. MicroRNAs Involved in Skin Pathology

A number of miRNAs have also been implicated in various skin diseases.

1.5.1. Skin Cancer

Up-regulation of miR-221/222 silences p27kip1 and c-kit causing increased cell proliferation and melanogenesis respectively.

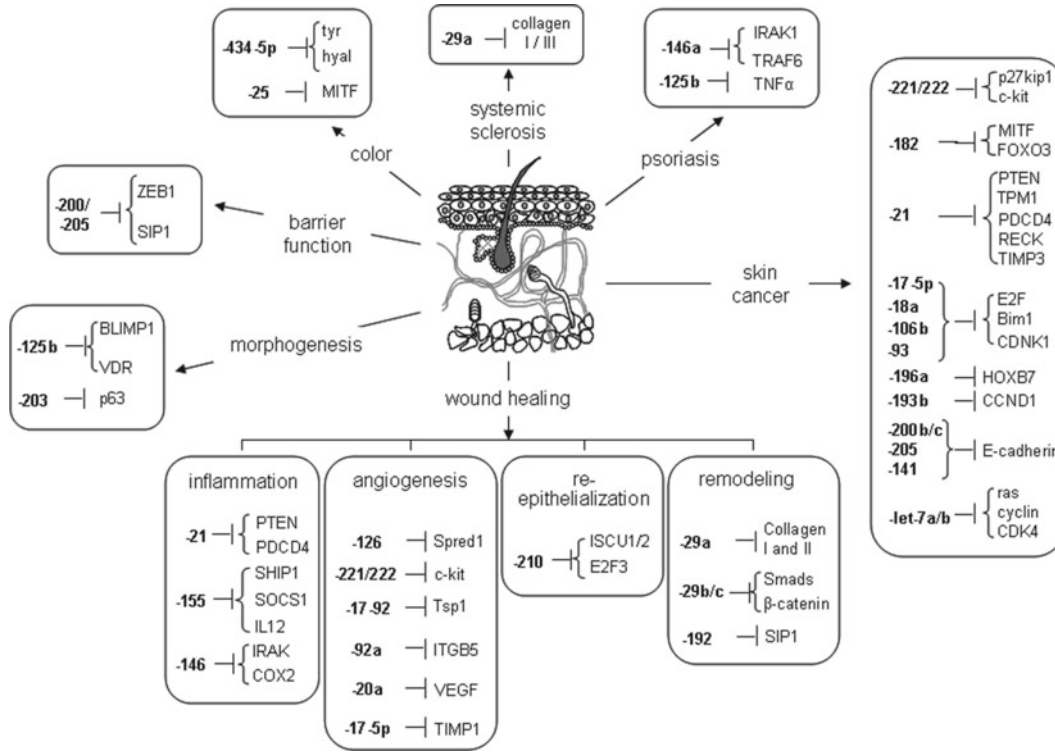

Fig. 3. MicroRNAs involved in skin morphogenesis and function.

High miR-182 silences MITF and FOXO3 resulting in increased metastasis. miR-21 silences PTEN, TPM1, PDCD4, RECK, TIMP3 and so elevated miR-21 results in metastasis. miR-17-5p, miR-18a, miR-106b, and miR-93 when high also cause cancer by repressing E2F, CDKN1, and BIm1 causing increased cell proliferation and reduced apoptosis. Release in repression of HOXB7 (miR-196a), CCND1 (miR-193b), E-cadherin (miR-200b, -200c, -141, -205), ras, cyclin, and CDK4 (let-7a and let-7b) increases migration, proliferation, invasion, and metastasis and thus results in skin cancer.

1.5.2. Psoriasis

High miR-146a silences IRAK1/TRAF6 while less miR-125b elevates TNF-α, both of which are important players in psoriasis (3).

1.5.3. Systemic Sclerosis

Elevated miR-29a silences type I and type III collagens which results in increased collagen deposition and fibrosis which are hallmarks of systemic sclerosis (3).

2. Materials

We have developed a murine and a porcine delayed healing model to study the role of miRNAs in wound healing (45, 46). Varied heterogeneity of the wounds as well as ethical challenges in repeated

biopsy collections from human patients presents a big challenge in studying the mechanisms of healing. The porcine model provides researchers with a preclinical alternative to understand the basic processes of tissue repair and to develop and validate strategies for clinical treatment. This is particularly useful because pig skin closely resembles human skin anatomically as well as physiologically (46) and this model is highly suitable for testing the clinical relevance for possible wound healing therapies. However, limited availability of genetically modified animals, antibodies, and reagents makes the porcine model less useful for study of molecular mechanisms in detail. To address this concern, a murine bipedicle flap model has been developed and characterized (45). These two models thus provide very strong tools to researchers to study molecular mechanisms of the wound healing process, develop therapies, and test their clinical relevance.

3. Methods

The potential to therapeutically regulate miRNA levels at the wound site make miRNA-based therapies an attractive possibility in wound healing. MiRNA therapeutics provides a unique advantage because by modulating a single miRNA, a group of functionally related genes in a pathway can be targeted as opposed to modulating a single gene at a time in conventional gene therapy.

MiRNAs can be up-regulated using mimics and down-regulated using anti-miRs. The over-expression of an miRNA can be achieved using synthetic short double-stranded oligonucleotide (mimics). Mimics are double-stranded with one strand called guide whose sequence is same as the mature miRNA while the other called passenger is complimentary to the mature sequence and only the guide sequence is incorporated into the RISC complex (47). Efficacy of mimics has not yet been demonstrated in vivo. Another approach, however, has been successfully tested in vivo and involves using expression vector systems (47). Anti-miRNAs essentially act as competitive inhibitors binding to the mature miRNA and also can affect miRNA maturation by binding to the pre-miRNA. Three different approaches have been used to deliver anti-miRs to mammalian tissues (47)—(a) Intravenous injection of antagomiRs (chemically modified cholesterol-conjugated single strand oligos); (b) conjugation of RNA oligos with other lipophilic molecules, i.e., high-density lipoproteins; (c) Using locked nucleic acid (LNA)-modified oligonucleotides.

3.1. In Vivo Delivery Systems

Successful delivery of the synthetic oligonucleotides will depend on their resistance to degradation in tissues, specificity, and high binding affinity to the specific miRNA in question. To achieve

these goals, chemical modifications of the oligonucleotides are often necessary. Delivery of anti-miRNAs to mammalian tissues is generally administered by using either of these approaches (47, 48): (a) Intravenous injection of antagomiRs (chemically modified cholesterol-conjugated single strand oligos); or (b) conjugation of RNA oligos with other lipophilic molecules, i.e., high-density lipoproteins.

Three forms of chemically modified oligonucleotides that have been used are (a) 2′-O-methyl group (OMe)–modified oligonucleotides; (b) 2′-O-methoxyethyl-modified oligonucleotides, and (c) Locked nucleic acid or LNA (48).

4. Notes

1. A number of miRNA therapeutic studies are currently going through clinical trials (45). A phase I clinical trial in which an LNA-based anti-miRNA targeting miR-122 was developed as hepatitis C therapy has recently been completed. A number of clinical trials involving skin carcinoma are also under way. This validates the viability of miRNAs as therapeutic targets and miRNA inhibitors and mimics as a new class of drugs. Further development of the technology into a successful therapy involves identifying new miRNA targets and developing efficient in vivo delivery system.
2. A big drawback of miRNA-based therapy lies in the fact that a single miRNA can silence a number of different proteins and so specificity of treatment becomes a problem. In order to address this issue, a number of different approaches have been adopted (45). Sponges are essentially competitive inhibitors which contain multiple, tandem binding sites to an miRNA of interest. Sponges have a bulge at the position cleaved by the Ago2. They can thus stably interact with the miRNA target that cannot be sliced. Sponges therefore have the advantage of being able to block all the miRNAs that recognize the same sequence and thus inhibit all the miRNAs of the same family resulting in a much more effective outcome. miRNA “erasers” are similar to sponges, except that they use only two copies of the perfectly complementary antisense sequence of the miRNA. The mask approach involves an oligonucleotide which is made to bind to the miRNA target sequence of the specific mRNA of interest, thus preventing the miRNA/mRNA association. Thus, using this approach, the miRNA interaction with one specific target can be modulated (see Fig. 4a, b, c).
3. In order to do basic research to understand the role of miRNAs in wound healing, a murine and porcine delayed healing

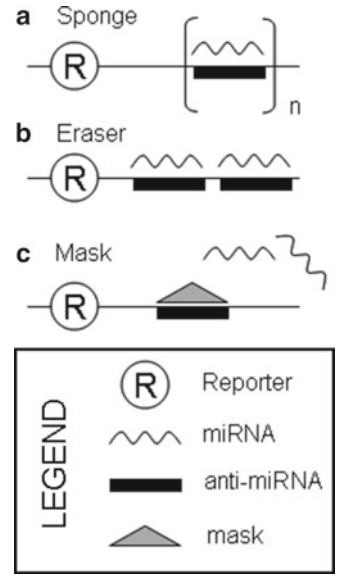

Fig. 4. Schematic diagram demonstrating the principle behind sponges, erasers, and masks.

model has now been developed (47). The porcine model provides researchers with a preclinical model to understand the basic processes of tissue repair and to develop and validate strategies for clinical treatment. This is particularly useful because varied heterogeneity of the wounds as well as ethical challenges in repeated biopsy collections from human patients present a big challenge in studying the mechanisms of healing. Since pig skin closely resembles human skin anatomically as well as physiologically, this model is highly suitable for testing the clinical relevance for possible wound healing therapies. On the other hand, while this model is of high translational significance and clinical relevance, limited availability of genetically modified animals, antibodies, and reagents makes them less useful for study of molecular mechanisms in detail. To address this concern, a murine bipedicle flap model has been developed and characterized. These two models thus provide very strong tools to researchers to study molecular mechanisms of the wound healing process, develop therapies, and test their clinical relevance. As mentioned before, the same miRNAs often have been found to have different and contrasting function in different cell types. To solve this problem, a new technology employing laser capture micro dissection can be used to perform cell type-specific miRNA studies from in vivo tissue samples. Recent publications demonstrate the feasibility of using this technique for analysis of genes captured from blood vessels from human tissues (49), prostate cancer epithelial and interstitial stromal cells, and epithelial cells from other regions (see Fig. 5).

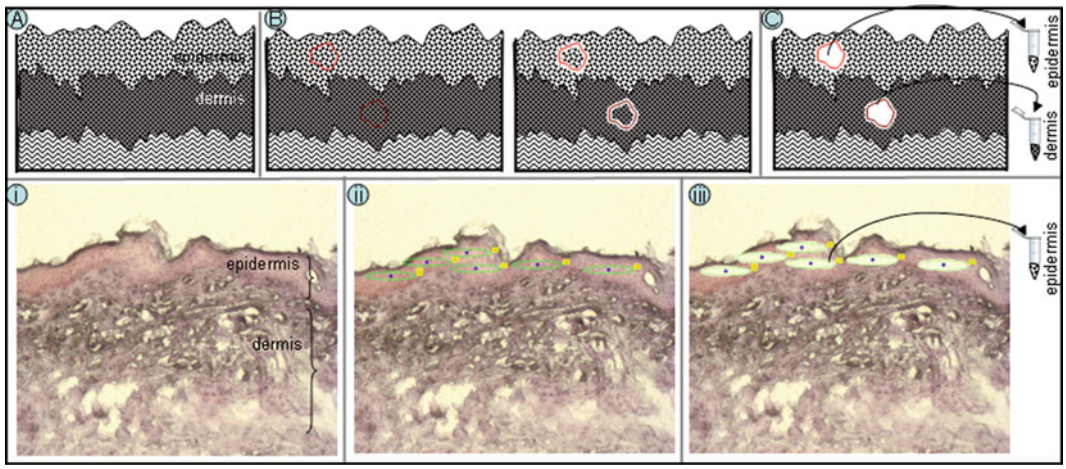

Fig. 5. (**a**–**c**) Schematic diagram demonstrating principle of laser capture microdissection. (i–iii) Representative example of laser capture microdissection from mouse skin. (**a**, i) Skin is sectioned onto a membrane slide; (**b**, ii) Specific region to be dissected is first marked. The laser cuts along the marked *lines*; (**c**, iii) Dissected sections are then catapulted into labeled tubes.

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Chapter 27

MiRNA Targets of Prostate Cancer

Jia Han Deng, Qinggao Deng, Chih-Hao Kuo, Sean W. Delaney, and Shao-Yao Ying

Abstract

Prostate cancer (PC) is the most prevalent strain of cancer in men, but it is often slow-acting or undetected. Common diagnostic tools for PC include prostate biopsy and consequent analysis by the Gleason scoring of the tissue samples, as well as tests for the presence and levels of prostate-specific antigens. Common treatments for androgen-dependent PC include prostatectomy or irradiation, which can be invasive and significantly lower the patient's quality of life. Alternative treatments exist, such as androgen ablation therapy, which, though effective, causes relapse into androgen-independent PC, which is far more invasive and likely to metastasize to other parts of the body. MicroRNAs (miRNA) are short nucleotide sequences (between 19 and 25 nucleotides long) that bind to various targeted messenger RNA (mRNA) sequences post-transcriptionally through complementary binding and control gene expression, often through silencing or leading to the degradation of targeted mRNA. Studies have shown that miRNAs are expressed abnormally in various cancers, suggesting that they play a pivotal role in cancer development and progression. Some miRNAs are oncogenes that incite cancerous growth, while others are involved in tumor suppression and cell cycle controls. MiRNA expression also differs in various types of cancers. Studies of PC-specific miRNAs show potential for their utilization in the prevention, diagnosis, and treatment of PC to more effectively target tumor growth and provide patients with better therapeutic options.

Key words: Prostate cancer, MicroRNA, Therapeutics

1. Introduction

Prostate cancer (PC) affects approximately 23% of all male cancer patients, and second only to lung cancer as the harbinger of cancer deaths (1). Despite the high prevalence of PC, most cases are slow-acting, and not fatal. However, about one third of PCs are aggressive and tend to metastasize to other parts of the body (1, 2). PC is categorized into four stages, based upon the size of the tumor and the spread of the disease: stage one would include slightly elevated prostate-specific antigen levels, and evidence of benign prostatic

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hyperplasia (generalized enlargement of the prostate), while stage two shows signs of tumor growth in the prostate. Tumor proliferation throughout the prostate gland is indicative of stage three PCs, while stage four PC has metastasized to other organs and systems (2). PC is further divided into androgen-dependent and androgen-independent forms. Androgen-independent PC, also known as hormone refractory PC (HRPC), is typically far more aggressive and difficult to combat (3). With androgen-dependent PC, hormonal deprivation therapy is common, but leads to remission and occurrence of HRPC. Other treatments include prostatectomy and radiation therapy to more aggressively target resilient cancers (1).

The classical view of epigenetics pertains to the idea that acetyl groups can regulate gene expression through altering accessibility of transcriptional machinery to DNA and histones, methyl. Modification of gene expression by such functional groups allows for the orchestration of complex temporal and spatial developmental processes. The discovery of miRNA, however, suggests that there is an additional layer of epigenetic control through which gene expression may be also modulated.

MiRNA represent a class of short (between 19 and 25 nucleotides) noncoding RNAs that negatively affect gene expression post-transcription through complementary binding to target messenger RNA (mRNA), impairing translation of the mRNA or marking it for early degradation (4). MiRNA exerts its indirect epigenetic control by negatively inhibiting the mRNA of its target genes. We are just beginning to understand how miRNAs can affect posttranscriptional gene expression by having multiple miRNAs mediate the expression of a single gene or a single miRNA regulate multiple genes (5, 6). MiRNAs inhibit translation of their target mRNAs in one of two possible ways, depending on the degree of their complementarity to their target mRNAs. The majority of miRNA inhibits translation and thereby decreases gene expression, through imperfect basepairing with the 3′-untranslated region (3′UTR) of target mRNAs (4, 7). This occurs during the initiation phase of translation and interferes with the recruitment and function of the eukaryotic translation initiation factor eIF4E (8).

Alternatively, if a miRNA shares near perfect complementarity with its target mRNA, it can introduce instability and cause direct mRNA degradation instead (9–13). Because most of miRNA suppression is mediated by only partial complementarity to its target mRNAs, it is very likely that each miRNA has the potential to regulate multiple target mRNAs and multiple miRNAs may inhibit the same mRNA.

Recent studies have shown that microRNAs (miRNA) are associated with a number of important biological processes, including, but not limited to, developmental timing, cell death, stem cell differentiation, hematopoiesis, cell proliferation, patterning

of the nervous system, longevity, fragile X syndrome, and viral infection (14). It is therefore conceivable that their deregulation contributes to the pathophysiology of human diseases. Growing evidence now suggests that epigenetic alterations, in addition to genetic mutations, are implicated in cancers (15–19). Furthermore, diseases are frequently associated with malfunction of the wide expanse of noncoding DNA. Both point to the possibility that miRNA, which is a noncoding RNA, may be involved. Highly conserved in evolution, noncoding DNA makes up nearly 95% of the human genome, and of this, a significant portion consists of miRNA sequences yet to be discovered. Many previously inexplicable diseases may potentially be explained by looking at miRNA deregulation. Although the exact contents encoded in much of this unknown expanse remains to be elucidated, what we have discovered so far is providing valuable insight into the function of miRNA. The intimate relationship between miRNA and cell differentiation, proliferation, and development makes miRNA a natural subject of interest in the study of cancer (20).

Studies by Volinia et al. have shown about 50 different miRNAs that are irregularly expressed in PC tissues when compared to normal human tissues (21). These miRNA serve varying functions depending on their targets; some are tumor suppressors, while others are oncogenes. With the discovery of the various targets and workings of PC-related miRNAs, there is potential for the development of diagnostic or therapeutic tools based on these sequences. In this review, we shall examine miRNA 15, 16, 205, 143, 145, 146, 23b, 100, 146, 221, 222, and 302. Of these, 221 and 222 (of the same miRNA family) have shown to be oncogenes, while the others, 15, 16, 143, 145,146, 205, and 23b, have shown to be tumor-suppressor miRNAs (22, 23) (Table 1).

2. MiRNAs

2.1. MiR-15a + miR-16-1

The miRNAs 15a—16-1 target BCL2, CCND1, and WNT3A genes within PC cells (24–26). Together, they mediate apoptosis in tumor cells through the BCL2 gene (29). In addition, CCND1 and WNT3A increase PC cell growth and proliferation, and WNT3A and BCL2 decrease the effectiveness of docetaxel (a common chemotherapy medication) (24). In PC cells, miR-15a and miR-16 are under-expressed (23, 27, 28), while BCL2, CCND1, and WNT3A are over-expressed. In studies by Bonci et al., transfecting miR-15a and miR-16 induced rapid regression of the tumorous cells (24).

The miR-15 family in cells is important in cell proliferation. In PC cells, miR-15 is shown to be under-expressed, with a direct correlation between tumor growth and miR-15 expression (13).

Table 1
MicroRNAs (miRNAs) on suppression and enhancement of cancer development

MiRNA	Target gene(s)	Biological manifestation	Signaling pathways
miR15 (25)	BCL2	Apoptosis/tumor suppression	Mitotic cell cycle
miR16 (30)	CDK6, CDC27, CARD10, C10orf46	Cell cycle mediation: accumulation of cells in the G1/G0 phase/ tumor suppression	Multiple pathways of cell cycle genes
miR205 (51)	ZEB2 E2F1, E2F5, IL 6, EZH2, Cε	Represses cell–cell adhesion, deters metastasis/tumor suppression	E2F Pathways
miR143 (39)	ERK5	Represses growth and cell proliferation/tumor suppression	ERK5 signaling cascade
miR145 (41)	IRS-1, RTKN	Represses invasion and metastasis	NF-κB (44, 59)
miR23b (49)	UPA, c-MET, GLS	Deters metastasis of malignant cells	Mitochondrial glutaminase/ glutamin metabolism (33)
miR100 (35)	PlK1	Reduces PlK1, leads to apoptosis	Mitotic regulation pathways
miR146a (47)	ROCK1	Silences ROCK1 oncogene	Hyaluronan (HA)-mediated HRPC transformation, PI3K-mediated Akt/TOR/eIF4E signaling pathway
miR302 (49, 51)	Various cell differentiation genes	Tumor suppressor	Cyclin E-CDK2 and cyclin D-CDK4/6 pathways (49)
miR221&222 (52)	p27Kip1	Oncogene, silences cell cycle genes	Cellular differentiation pathways

The down-regulation of miR-15 is more significant in late stage PC tumors and may be due to the GRN growth factor in cancerous cells, mediated by miR-15 (30). When miRNA-15 is expressed, it targets the BCL2 gene to enhance apoptosis and suppresses tumor growth and proliferation (25).

MiR-16 family is usually under-expressed in many different types of PC cells (23, 27, 28); it has been shown that miR-16-1 is under-expressed in 22Rv1, Du145, PPC-1, PC-3M, and with decreased expression as the cancer advances (30). One anomaly in Takeshita et al.'s study is that LNCaP PC cells show slight over-expression of miR-16, and when such tumors were treated with

additional miR-16, showed no change in growth or proliferation (32). miR-16 has been shown to regulate the genetic expression of CDK1 and 2, which regulate the G1/S phase transition in the cell cycle. It also increases apoptosis of tumor cells with expression of genes that regulate DNA metabolism and the mitotic cycle (30). Studies by Takeshita et al. have shown that treating prostate tumors, which had initial under-expression of miR-16 in mice models with injections of miR-16, decreased the cancer growth and proliferation (30).

2.2. miR-23b

MiR-23b has been associated with the control of endothelial cell growth in the blood vessels, as certain types of blood flow cause an up-regulation of miR-23b, which leads to cell death (31). Such observations are logical, as in many cancers, including PC; miR-23b is down-regulated and contributes to cell proliferation and invasion (27).

In liver cancer, hepatocellular carcinoma, there was an inverse trend between the expression of miR-23b and the expression of urokinase-type plasminogen activator (uPA) and c-met, an oncogene. Decreased levels of miR-23b lead to higher levels of uPA and c-met, which has been shown to be associated with cell migration and proliferation. When miR-23b was transfected into liver cancer cells, there was a drastic decrease in uPA and c-met expression, followed by decreased proliferative ability and mobility. However, inactivation of miR-23b did not cause cells to have an increase in mobility or metastasis (32).

In PC, miR-23b is also down-regulated, and the studies have indicated that the targets are in glutamine metabolism, which is essential in cancer cell metabolism. The Myc oncogene is shown to target and repress miR-23b and lead to an up-regulation of glutaminase, which converts glutamine to glutamate and consequently used for anaerobic ATP generation in cancer cells. When miR-23b was transfected into MCF-7 breast cancer cells, glutaminase activity was inhibited and is partially associated with possibly decreasing the Myc expression as well (33).

2.3. miR-100

MiR-100 has been shown to be drastically up-regulated in PC tissue and cell lines (34). In particular, there seems to be noted overexpression in localized, high Gleason scoring PC tissue when compared to metastasized or lower grade tumors (34). This suggests that miR-100 may promote metastasis of PC cells to other regions of the body. This suggested target has not yet been tested or confirmed (35). In nasopharyngeal cancers, with under-expression of miR-100, it has been shown to target the P1k1 gene, which regulates the mitotic cycle, disruption of which often leads to apoptosis. In addition, an increase in miR-100 in nasopharyngeal cancer cells led to an increase in the P1k1 expression (36). In PC, P1k1 has shown to cause increases in Cyclin B1 mRNA level, and

silencing of Plk1 does cause increased rate of apoptosis in cells, thus there may be other targets in PC that regulates Plk1 (35).

Another suggested target of miR-100 is the SWF/SNF mRNA, which regulates chromatin. SWI/SNF complexes allow cells to regulate complex mechanisms with regard to its genes, including but not limited to DNA replication, expression, and maintenance (37). Many cancer cell lines lack SWI/SNF complexes, and under such conditions, tumor progression increases aggressively. It is suggested that SWI/SNF complexes act in conjunction with various tumor-suppressor genes and proto-oncogenes and are far more over-expressed in androgen-independent PC than in androgen-dependent PC (37).

Because of the confusing nature of miR-100, being under-expressed or over-expressed in various cancers, and targeting multiple targets, it may not be a definitive potential resource for PC diagnosis and treatments.

2.4. miR-143

With regard to PC, miR-143 expression is inversely proportional to the progression of PC, as defined by the Gleason scale, and has been shown to have decreased expression in PC cell lines such as LNCaP in comparison to normal prostate cells. When miR-143, miR-143 inhibitors, and nonrelevant miR-143 were introduced to different PC cell colonies, the number of cells decreased when cells were introduced to miR-143. In addition, there was an increase of cells in G0–G1 phase with increased miR-143 levels (38, 39). The ERK5 protein, the target of miR-143 in colon cancer, is also affected by miR-143 in PC: the ERK5 protein levels were significantly increased in the cancer cells, and injections of miR-143 into animal models with grafted LNCaP and C4-2 tumors showed decrease in tumor growth and lowered ERK5 levels (39).

Such studies by Clape et al. have suggested that because miR-143 is under-expressed gradually, depending on the severity and aggressiveness of the PC (39). It may be a novel diagnostic tool to target fast spreading, aggressive strains of the cancer. The target protein of miR-143, ERK5, suggests that miR-143 may be valuable in therapeutic applications as well. The ERK5 protein targets various cancer-related transcription factors such as MEF3, c-Myc, Sap-1, and c-Fos. Increasing miR-143 expression not only restricts cancerous cell growth and metastasis, but also mediates other abnormally expressed transcription factors into normative states (40).

2.5 miR-145

In many various cancer cell lines, miR-145 is under-expressed (41). Experiments by Wang et al. have shown that normal or over-expressed levels of miR-145 induce apoptosis and inhibit cell growth in various cell lines, such as breast cancer, cervical cancer, and colon cancer. In breast cancer, RTKN has been shown to be one of the targets of miR-145 (42, 43). RTKN codes for the Rho

factor in cells and decreases in expression as miR-145 is expressed. In normal cells, RTKN is typically very low, and most cancer cell lines have excessive RTKN expression, which protects the cells from scheduled apoptosis (44). In addition, miR-145 also seems to target the MUCI gene, which plays a role in cell invasion and cancer metastasis (45). miR-145 was shown to suppress the levels of MUCI gene, and in cancerous cells where miR-145 is under expressed, tend to promote the invasiveness of the cells. It has been suggested that the mechanism of MUCI tumorigenesis is through various growth factor signaling pathways (45).

2.6. miR-146

MiRNA-146 has been shown to be down-regulated in PC, with significant down-regulation in androgen-independent PC when compared to androgen-dependent PC (46). The expression of miR-146 is relatively normal in hyperplasia and decreases as the tissue turns malignant and the cancer advances. When the PC has progressed to hormone-refractory PC, the amount of miR-146 is barely detectable in the sample tissue. Previous studies have shown that the ROCK1 gene is targeted and knocked down by miR-146. When miR-146 was transfected into PC3 cells, the PC3 cells showed slowed growth, unaffected by Hyaluronal (HA) stimulation, which under normal conditions, should stimulate HRPC cell growth and metastasis. HA acts through the ROCK1 signaling pathway; thus, miR-146 works by silencing the ROCK1 gene pathway to affect cellular response to HA (47). HA-affected ROCK1 gene in PC can increase cell migration and support cancer cell proliferation and slow down apoptosis. This is expected, as miR-146 levels drop significantly as PCs advance to either become androgen-independent or metastasize to other parts of the body (47).

Because miR-146 seems to be deeply involved with the advancement of PC cells beyond its original domain, its mechanisms require further research to discover if there are opportunities to mediate or prevent the advancement of HRPC. The inverse correlation between levels of miR-146 and the progress of the cancer could potentially be utilized as a determining factor of the appropriate treatments.

2.7. miR-302

MiR-302 is up-regulated in PC cell lines, and over-expression of miR-302 has been associated with cell cycle inhibition, as it does in reprogramming cells (48). It targets E-CDK2 and D-CDK4/6 pathways which lead to cell cycle arrest and promote various tumor-suppressor genes to prevent cancer cell proliferation and metastasis (49). When human skin cells are transfected with miR-302, it is shown that the cells become reprogrammed into a pluripotent stem cell state, with expression of various genes associated with embryonic stem cells such as Oct4 and Sox2. Such genes are also expressed in various tumors (50).

The exact targets and mechanisms of miR-302 of PC have not been investigated and are still being speculated upon, but it has been shown in mice models injected with miR-302 reprogramed cancer cells they developed teratoma like cysts that self-terminated after a period of time (49). It suggests that miR-302 also reprograms the cells to have some self-regulating mechanisms to stop random growth. Further research may provide to be useful in determining the therapeutic effects of miR-302, not only in cancer treatment, but in other diseases as well, with its reprogramming capabilities.

2.8. miR-205

MiR-205 has been shown to be under-expressed in various PC cell lines (PC-3, LNCaP, DU145, VcaP) as well as cancerous and normal prostate tissue from PC patients. With the spread and metastasis of the cancer to other regions of the body, the miR-205 expression was further repressed. This shows that the expression of miR-205 is inversely proportional to the developmental progress of the cancer (51).

When PC cells are transfected with miR-205, they displayed lower rates of migration and better cell-adhesion properties. Further analysis has shown that there are increases of E-cadherin and beta-catenin after transfection; such pathways are down-regulated in cancerous cells, as they are products of contact inhibition of normal cell growth and adhesion. In addition, with an increase of miR-205, there was also a decrease in IL(interleukin)-6, EZH2, and caveolin-1, which are usually high in the presence of PCa, suggesting that there may be other genes mediated by miR-205 that requires further study (51).

Pathologically, miR-205 forces PC cells to undergo the mesenchymal to epithelial transition, reverse of the EMT, which is an indication in cancer metastasis and malignancy. Another target of miR-205 is protein Kknase Ce, which aids in the motility and migration of cancerous cells. Because its targets inhibit cancerous cells from immigrating into another part of the body, it is considered to have tumor-suppression functions and loss of miR-205 promote metastasis and malignancy. With this research, it is suggested that miR-205 may become useful as a possible drug, to reduce the malignancy and aggressiveness of various forms of PC and stop it from metastasizing (51).

2.9. miR-221 + 222

The miR-221 and miR-222 expression of PC cell lines depend on the nature of the cells: in studies by Galardi et al., it has been shown that miR-221 and 222 expressions are over-expressed in PC-3 cells, but are significantly reduced in LnCAP cells (52). This shows that miR-221 and 222 are under-expressed in hormone-dependent PC, while being over-expressed in more advanced, hormone-independent PC cells, like PC-3 (53, 54). This is indication that miR-221 and 222 may have an oncogenic

role in PCa metastasis and advancement. One of the targets of miR-221 and 222 has been shown to be the p27 mRNA, which regulates cell cycle arrest, as indicated by over-expression of miR-221 and 222 resulting in reduced expression of p27 in PC-3, LnCAP, and 22Rv1 cell lines (52). miR-221 and 222-induced reductions in p27 results in drastic cell growth in the cell lines and promotes the transition from the G1 phase to the S phase in most cells, as well as increasing the anchorage-independent characteristics of the cells (52, 54). PC-3's increased expression suggests that miR-221 and 222 are indicators of more advanced and aggressive PC, in contrast to the slow progressing, hormone-dependent likes of LnCAP or 22Rv1 cells (55).

Because of miR-221 and 222's variability within different types of PC cells, they could be accurate diagnostic tools. There are indications that the amount of miR-221 and 222 in PC tumors correspond with the Gleason scoring of the samples: with higher grade tumors showing significantly higher over-expression of the miRNAs and lower grade tumors with near normal levels (53). It would be more specific than most other miRNA markers in detecting the advancement of PC, as it seems to mitigate the transitional phase between hormone-dependent and hormone-independent PCs (55). In addition, miR-221 and 222 could become useful therapeutic tools, as blocking its effects on p27 can induce cell cycle arrest and reduce the tumorigenicity in PC (52, 55).

3. Conclusion

As most of the literature indicates, the ultimate goal of miRNA research in cancer seeks to find applications for miRNAs as diagnostic or therapeutic tools. For diagnostics, noting aberrant miRNA expression levels can potentially give a hazy overview of cancer progression (56), while additional knowledge on the mechanisms and targets of the various miRNAs is necessary for more accurate, targeted purposes (56).

With regards to miRNAs as therapeutic tools, there have been many developments, but such applications are still in the early stages of development (57), especially for PC, where many miRNAs target multiple genes, and each pathway needs to be more thoroughly explored. More research is necessary to determine the targets and mechanisms of action of the various abnormally expressed miRNAs and their target genes (57). In addition, successful therapy hinges on the efficiency in the method of delivery, and while there have been favorable results in targeting specific tumors with miRNA treatments, such localized delivery methods are powerless when faced with late staged, metastasized cancers (57).

Effective treatment for PC with miRNA therapy would require much more information regarding the targets and mechanisms of the various aberrantly expressed miRNAs in various types of PC to determine which sets of miRNA should be targeted for maximum efficiency in suppressing tumor growth and metastasis while diminishing the existent cancerous cell populations (58). Due to the nature of cancer cells, such treatment would require both localized therapies to target the origin of the cancer, as well as systemic delivery to minimize the chances of the cancer breaking out in another part of the body (58).

There are many distinctively abnormally expressed miRNA markers that are indicative of PC. These miRNAs mitigate the growth and expansion of the cancer and has the potential to deter growth and inhibit the toxicity of the disease through its tumor-suppressor ways. However, key information about such miRNAs is still missing from the picture. More in-depth information regarding the targeted genes of many of the miRNAs, as well as the mechanisms by which they function, are necessary to paint a more well-rounded picture of their potential as diagnostic or therapeutic tools in the detection and treatment of PC. In addition, due to the invasive nature of cancer, more research in the realm of miRNA therapy delivery for cancer is necessary to facilitate the potential of miRNA therapies in targeting both the site of origin of the disease, as well as other locales after possible metastasis for higher grade cancers.

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INDEX

A

Adipocytes 181–193
Adipogenesis 181
Adipose tissue 181–193
AGO (argonaute) 8, 9, 11, 22, 23, 92, 352
Amplification 84–88, 152, 154, 163, 164, 169, 170, 190, 225, 226, 263, 291, 301, 302, 306, 308, 331, 339
Angiogenesis 48, 347, 348
Antisense 2, 7, 9, 37, 119, 121, 130, 131, 150, 199, 200, 212, 215, 219, 221–223, 225, 227, 273, 274, 280, 288, 290, 292, 303–305, 307, 330, 333, 352
Apoptosis 48, 78, 97, 100, 216, 281, 299, 311, 314, 350, 359–363
Arabidopsis 9, 210, 280
Artificial introns 213, 214, 216, 223, 272, 281, 282
Asymmetric assembly 273–275
ATP 24, 26–29, 84, 106, 109, 115, 161, 221–223, 289, 290, 305, 306, 330, 348, 361
Aubergine 8

B

Bacteriophage 4
β-actin 218, 273, 283
β-catenin 283–286, 292, 349, 364
Biogenesis 6, 10–12, 21, 22, 35, 78, 131, 173, 210–212, 215, 220, 231–233, 281–284, 298, 303, 314, 326, 343–344, 346–348
Bioinformatics 35, 36, 38, 50, 77–81, 92, 98, 100, 118, 212, 236, 271, 300
Biomarkers 313–316
BLAST,
Blastn,
BMI-1 ..... 299, 300, 302
BMP4 286

C

Caenorhabditis elegans 4, 126, 129, 140, 143, 158, 210, 280, 314
Cancer 13, 14, 16, 50, 78–79, 92, 93, 99, 118, 157–171, 210, 213, 216, 280, 281, 296, 299, 301, 309, 313–322, 326, 349–350, 353, 357–366
CDK2 ..... 296, 298–300, 360, 363
CDK4/6cell cycle 296, 299, 360, 363
CDKN1A 295
Cell culture 85, 108, 145, 161, 196–197, 202, 223, 226, 234–239, 249, 258–259, 290, 292, 306, 309–311, 334–336
Central nervous system (CNS) 38, 44
Chicken 14, 17, 96, 132, 135, 210, 283–286, 290–292
Chromosome 3, 11, 71, 72, 94–96, 98
Chromosome X (Hs X-17cl) 8, 11
Cloning 12, 13, 22, 83–87, 89, 119, 121–123, 126, 131, 134, 146–148, 150, 152, 155, 157–171, 182, 190, 196, 200–202, 205, 222, 225, 226, 233, 234, 289–291, 305, 308
cMyc 79, 247, 254, 296, 362
CNS. *See* Central nervous system (CNS)
Computational prediction 92, 94–98, 100
Cosuppression 4
Cyclin D 296, 299, 300, 360
Cytomegalovirus 225, 273, 281
Cytoplasm 3, 5, 11, 12, 22, 60–63, 78, 92, 158, 173, 181, 182, 197, 198, 210, 211, 213, 216, 257, 272, 282, 286, 288, 303, 327, 328, 344
Cytotoxicity 281, 285, 288

D

Database 31, 37, 69–75, 80, 81, 94, 95, 98, 99, 101
Depigmentation 326
DGCR8. *See* DiGeorge syndrome critical region gene 8 (DGCR8)
DH5α 147, 159, 164, 202, 222, 225, 226, 289, 291, 305, 308
Diagnostics 79, 196, 222, 259, 289, 306, 314, 331, 359, 362, 365, 366
DIANA LAB microT 38, 42
Dicer 4–6, 9–13, 21–33, 78, 92, 158, 181, 198, 205, 211–213, 218, 220, 232, 233, 257, 272, 273, 275, 285, 298, 327, 328, 344, 345, 347

Shao-Yao Ying (ed.), *MicroRNA Protocols*, Methods in Molecular Biology, vol. 936,
DOI 10.1007/978-1-62703-083-0, © Springer Science+Business Media, LLC 2013

Dicer-like 9, 11, 212, 303
Differentiation....15, 21, 48, 78, 79, 92, 105–115, 130, 234, 249, 254, 257–268, 300, 311, 314, 345, 347, 358–360
DiGeorge syndrome critical region gene 8 (DGCR8)....15, 21, 22, 343, 345
DMEM. *See* Dulbecco's modified eagle medium (DMEM)
DNA chip....259, 263–265, 267
DNA constructs 84
DNA–miRNA....16
DNA–RNA hybrid 216
DNA/RNA methylation 3, 11, 63, 296
DNAse 153, 175, 178, 259, 263, 264, 316, 318, 322
Donor introns....7
Drosha....5, 10, 12, 21–23, 78, 91, 157, 181, 197, 198, 204, 205, 211, 232, 233, 257, 303, 327, 328, 343
Drosophila.... 131, 151
Drosophila melanogaster....15, 210, 280
Drug development....98
dsDNA....87, 334
Dulbecco's modified eagle medium (DMEM).... 85, 106, 148, 150, 161, 165, 166, 196, 202, 235, 236, 239, 243, 249, 258, 260, 306

E

EBV. *See* Epstein-Barr virus (EBV)
E-cadherin....284, 349, 350, 364
EGFP.... 148, 198, 204, 214, 215, 217–219, 225, 227, 273–275, 278, 282, 283, 290, 305
Embryonic development 17, 79, 105, 118, 210
Embryonic stem cells (ESC) 4, 50, 58, 79, 234, 235, 237, 240, 244, 247–255, 296, 297, 363
Enrichment analysis 91–101
Epstein-Barr virus (EBV) 143, 146, 153
ES cells (ESC). *See* Embryonic stem cells (ESC)
Evolution.... 3, 7, 9, 11, 46, 48, 50, 64, 96, 210, 212, 213, 220, 272, 303, 359
Exons....3, 6, 10–12, 69, 71, 72, 75, 78, 100, 138, 198, 210, 211, 214–217, 223, 224, 226, 227, 282, 283, 303, 326, 327
Exosomes....3, 315
Exportin-5....5, 10, 22, 78, 91, 158, 211, 303, 344

F

FASTA 36, 44, 70, 71, 74, 80, 81
Feather....284, 286
Fine tuning....6, 7, 11, 50, 64, 117, 210, 242, 298, 302
Fish....210, 218, 219, 274, 280, 282
Fragile X mental retardation protein 213
Functional annotation 73, 94, 95, 97–98

G

Galaxy....36, 38–40, 42, 43
GAPDH. *See* Glyceraldehydes phosphate dehydrogenase (GAPDH)
Gene
  cloning....291
  endogenous....4
  function 2, 4, 6, 14–17, 78, 97, 216, 281
  homogenous 69, 296, 297
  host....3, 69–73, 75, 131
  splicing 213
  therapy.... 14–16, 287, 351
  transfer....4
  tumor....14, 50, 63, 78, 79, 296, 297, 300, 302
Gene–gene interactions 38
Gene ontology (GO)....73–75, 95, 97, 98, 100
Gene silencing 2, 4–6, 10, 11, 14, 15, 78, 92, 118, 119, 209–227, 257, 272–275, 278, 280–284, 286–288, 297, 298, 327
  in vivo 209–227
Genome.... 2, 7, 8, 10–16, 36, 37, 39, 40, 43, 47–49, 71, 74, 75, 77, 80, 81, 91, 96, 99, 129, 133, 157, 162, 209, 210, 220, 234, 249, 300, 314, 343, 359
Glyceraldehydes phosphate dehydrogenase (GAPDH).... 153, 154, 263, 264, 285, 287
GO. *See* Gene ontology (GO)

H

Heat-shock proteins 84, 86, 123, 147, 165, 191, 193, 339
HEK 293 cells 85, 87, 309
HeLa cells 120, 123, 202
Herpes....16
High throughput sequencing....38, 182
Human immunodeficiency virus (HIV).... 16, 58, 59, 215, 282
Hybridization 3, 13, 27, 30–32, 106, 107, 111, 115, 130, 221, 223, 225, 227, 249–251, 254–255, 264, 265, 267, 275, 276, 278, 289, 290, 292, 304, 307, 315, 330, 333
Hyperpigmentation 339
Hypoxia 347, 348

I

Induced pluripotent stem cells (iPS/iPSC)....79, 247–255, 295–311
Ingenuity Pathway Analysis (IPA) 38, 41–42, 44
In situ hybridization 13, 130
Intronic miRNA-expression construct 89, 106, 108
Introns.... 3, 69–75, 77–81, 91, 130, 171, 197, 209–227, 271, 279–292, 295–311, 325–341
IPA. *See* Ingenuity Pathway Analysis (IPA)
iPS/iPSC. *See* Induced pluripotent stem cells (iPS/iPSC)

K

KEGG....73–75, 97, 98, 100
Klf4....79, 247, 254, 296, 299, 302

L

Lentiviral vector....148, 150
let-7....5, 6, 12–14, 130, 193, 211, 253, 267
lin-4....5, 6, 12–14, 130, 211
Loss-of-function....8, 14, 16–17, 280, 283
Luciferase assay....85, 88, 89, 120, 145, 151–153
Luciferase reporter gene assay....117–126

M

Melanin....286, 287, 326, 329, 332, 334–338, 345, 349
Metastasis....9, 350, 360–366
Microarray....13, 79, 93, 94, 98, 99, 105–107, 110–112, 114, 115, 182, 187, 248–251, 253, 296, 303, 315, 322
Microprocessor....5, 21, 198, 343
MicroRNA (miRNA)
biogenesis....10, 22, 35, 232, 233, 282–284, 298, 303, 343–344, 346, 347
expression patterns....130, 258
functional analysis....2, 130, 144, 157–171
inhibitors....232, 233, 238–241, 352
intergenic miRNAs....6, 7, 12, 69, 72, 210–212, 326–328
intronic miRNA....6–8, 11, 12, 14, 15, 17, 69–75, 77–81, 91, 130, 131, 138, 209–227, 271–273, 275, 279–292, 295–311, 325–341
mimics....232, 234, 236–244
miRanalyzer....37, 38, 40–41, 43, 44
pre-miRNAs....5, 10, 15, 23, 24, 80, 81, 89, 91, 92, 197, 215, 233, 241, 272–275, 278, 281, 303, 315, 327, 328, 343
pri-mRNAs....5, 10–12, 15, 21, 23, 78, 91, 96, 131, 157, 158, 197, 198, 204, 211–220, 222–223, 225–227, 232, 303, 315, 327, 328, 343, 344
profile....48, 50, 75, 94, 99, 100, 106, 130, 248–249, 254, 258, 263–265, 267, 314
regulatory element....118
targeting sequence....91, 92, 118, 119, 125, 126
target protectors....232, 240
targets....38, 41–42, 44, 73, 91–101, 170, 204, 236, 352, 357–366
miR-124a....265, 266
miR-125....265
miR-138....118–121, 123–125
miR-302....79, 249, 265, 295–311, 363–364
miR-302 cluster....252, 298, 303, 305–308
miR–Tyr....238–341
miR–Tyro....329, 331–340
Mouse embryonic carcinoma (EC) P19 cell....105–115, 257–268
mRNA target....6, 8, 11, 39, 41, 44, 50, 59, 91, 92, 129, 144, 153–154, 181, 199, 204, 231, 232, 236, 237, 242–244, 248, 280, 285, 326–328, 344, 358
Mutagenesis....14, 84–87, 89

N

Nanog....50, 79, 247, 248, 296, 297, 299, 302
NCBI Ref Seq data base....71
Neural differentiation....150–115
Neuronal differentiation....257–268
Noncoding RNA (ncRNA)....2–9, 11, 21, 36, 37, 48, 50, 63, 77, 91, 117, 257, 276, 314, 326, 328, 358, 359
Northern blotting....24–31, 96, 130, 182

O

Oct3/4....79, 297
oligo (dT)....152, 259, 263, 276, 277
Oral cancer....118, 313–323

P

p21Cip1/Waf1....295
p16Ink4a, p14/p19Arf....299–302
PIWI....8–9, 258
Poliomyelitis....16
Post-transcriptional gene regulation....5
Post-transcriptional gene silencing (PTGS)....5, 11
Program....13, 14, 16, 36–40, 44, 51, 57, 63, 80, 81, 97–101, 110, 124, 151, 152, 154, 163, 168, 170, 196, 249, 288, 300
Promoters....6, 7, 9, 12, 17, 50, 69, 72, 78, 118, 130–134, 138–140, 144, 146, 171, 196, 204, 211, 212, 214, 215, 218, 225, 232, 233, 244, 273, 281, 282, 291, 315, 328
Prostate cancer....160, 213, 281, 309, 353, 357–366
pSM30....196, 198–204
pSM155....196, 198–204
PTGS. *See* Post-transcriptional gene silencing (PTGS)

R

RA. *See* Retinoic acid (RA)
Real time polymerase chain reaction (RT-PCR)....37, 81, 96, 107, 110, 143–145, 151–154, 160, 162, 182, 184–185, 190–191, 193, 248, 263, 264, 319–321
Real time quantitative polymerase chain reaction (RT-qPCR)....146, 155, 182, 185, 190, 191, 193, 259, 264–266
Red fluorescent membrane protein (rGFP)....213–219, 221–227, 274, 281–283, 285, 289–292, 298, 303, 305–310

Reprogram 79, 247, 248, 296, 297, 299–302, 309, 363, 364
Restriction enzymes 84–88, 119, 125, 126, 132, 134, 135, 138, 139, 144, 147, 158, 159, 162, 164, 196, 201, 202, 205, 216, 221–225, 289, 305, 307, 308, 330, 331, 333
Retinoic acid (RA) 105, 258, 260–262, 264–266
Retrotransposon 7, 50, 257, 258
Retroviral vector 148, 158, 160, 204, 291
Retroviruses 7, 64
rGFP. *See* Red fluorescent membrane protein (rGFP)
RhoC.....118, 119, 121, 123, 125
Ribonuclear protein (RNP) 3, 10, 212, 213, 216, 272
Ribonuclease
  RNAse III..... 3–6, 10, 12, 22, 78, 91, 92, 157, 181, 198, 211, 303, 343, 344
Ribosomes 3, 92, 344
RNA activation (RNAa).....9
RNA hybride..... 98, 250
RNAi. *See* RNA interference (RNAi)
RNAi-mediated methylation..... 213, 272
RNA-induced silencing complex (RISC)..... 6, 8, 10, 11, 15, 22, 78, 92, 212, 217, 219, 220, 231–233, 244, 257, 273–275, 327, 328, 344, 351
RNA interference (RNAi).....2, 4–6, 9, 11–15, 77, 81, 181, 195, 199, 204, 205, 210, 211, 215, 216, 218, 220, 257, 272, 280, 282, 283, 327, 328
RNA polymerase II (pol II).....2, 3, 6, 9, 12, 78, 91, 144, 181, 196, 198, 204, 210, 211, 215, 216, 232, 258, 280–284, 303, 320, 327, 328, 343
RNA polymerase III (pol III).....12, 196, 204, 281, 320, 327, 328
RNAs
  ds RNA..... 4–6, 9–11, 13, 22, 78, 198, 216, 281, 288, 344
  hairpin-like RNAs..... 14, 195, 213, 217, 272, 278
  non-coding RNAs (nc RNAs)..... 2–9, 11, 21, 36, 37, 48, 50, 63, 77, 91, 117, 257, 276, 314, 326, 328, 358, 359
  nuclear RNA 3–4, 6, 178, 303
  piRNAs 8–9, 258
  rasiRNA 9
  si RNAs 4–6, 8–12, 17, 50, 63, 78, 195, 197–200, 204, 210–212, 216–220, 233, 244, 258, 273–276, 278, 280, 281, 284, 286–288, 303
  small modulatory ds RNAs.....5
  small nuclear RNAs (snRNAs)..... 3, 4, 169, 276, 320
  small RNAs 2–9, 36, 37, 106–110, 112, 114, 130, 131, 157, 158, 162, 168, 169, 178, 185, 186, 218, 234, 244, 248, 250, 254, 258, 273, 275–277, 280, 283, 284, 314, 321, 322
  sno RNAs 2, 3, 6, 7, 145, 151, 171, 276
  splicing 3, 4, 6, 7, 12, 211, 212, 216, 217, 282, 298, 303, 327
  ss RNA 1, 4, 5, 12, 77, 117, 129, 210, 211, 216, 280, 281, 286, 326, 328
  tasiRNAs 9
  tRNA..... 2, 3, 186
  wave..... 49, 51, 63
RNP. *See* Ribonuclear protein (RNP)
RT-PCR. *See* Real time polymerase chain reaction (RT-PCR)
RT-qPCR. *See* Real time quantitative polymerase chain reaction (RT-qPCR)

S

Saliva..... 313–322
Skin..... 14, 283–288, 309, 325, 326, 332, 335–339, 343–354
Skin whitening 325, 326, 332, 335–340, 349
Small-hairpin RNA (shRNA).....195–206, 217, 303
Small RNA library 106–110, 130
snoRNP.....3
Somatic cell 79, 247, 249, 296–298, 300, 309
Sox2.....50, 79, 247, 248, 296, 297, 302, 363
Sponge.....143, 144, 146–155, 232, 233, 352, 353
Stem cell..... 4, 50, 60, 79, 105, 106, 112–114, 215, 234, 235, 237, 240, 244, 247–255, 281, 296–299, 302, 345, 358, 363
Subcellular fractionation..... 173, 179
Synaptoneurosomes..... 173–179
Synaptosomes..... 173, 174

T

TaqMan microRNA assays.....145, 151, 162, 167–169, 171, 259, 266, 315–320
Target prediction 38, 41–42, 73, 75, 92, 94, 96–98, 100, 300
Targetrons 7
Target validation.....83–90
TAR-RNA-binding protein (TRBP).....22
Telomerase..... 3, 301, 302
Therapeutics.....203, 216, 281, 351
Transfection..... 15, 23, 79, 84, 85, 87–89, 119, 120, 123–124, 152, 158–160, 166, 170, 202, 205, 213–215, 217–219, 223, 226, 233, 234, 236–244, 273–275, 278, 286–288, 290, 292, 298, 306, 309–311, 327, 331–332, 334–336, 340, 364
Transgenes..... 3, 5, 11

Transgenic animal.....14, 16–17, 196, 279–292
Transposons.....7–9, 11
TRBP. *See* TAR-RNA-binding protein (TRBP)
Tumor suppressor.....78, 92, 297, 314, 359, 362, 363, 366
Tyrosinase (Tyr).....287, 326–329, 332, 334–336, 349

U

Untranslated regions (UTR).....5, 6, 10, 11, 50, 73, 78, 83, 85, 86, 89, 91, 92, 96, 118, 119, 121, 125, 126, 132, 134, 138, 143, 144, 149, 152, 153, 155, 210, 213, 237, 242, 243, 272, 288, 305, 307, 308, 314, 343, 358

V

Vaccine (antiviral).....14, 16
Vertebrates.....3, 10, 11, 14, 96, 101, 210, 212, 283
Virus.....5, 7, 11, 143, 148–150, 154, 166, 170, 215, 289–292

W

WALDI. *See* Weighted average length of diced RNA (WALDI)
Web resources.....98, 100
Weighted average length of diced RNA (WALDI).....24, 30, 33
Western blotting.....143–146, 153, 169, 171, 178, 197, 202, 205, 215, 296, 332, 334, 335
Workflow.....35–44, 235, 236
Wound healing.....343–354

X

Xenopus, oocytes.....4, 23
Xist.....11

Z

Zebrafish.....10, 13, 14, 16, 17, 50, 210, 212, 217, 218, 220, 272–273, 281, 283, 286
Zinc finger protein-9 gene.....8, 212, 272
Zygote.....7, 296, 299