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MicroRNA in Cutaneous Wound Healing: A New Paradigm

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ABSTRACT

Repair of a defect in the human skin is a highly orchestrated physiological process involving numerous factors that act in a temporally resolved synergistic manner to re-establish barrier function by regenerating new skin. The inducible expression and repression of genes represents a key component of this regenerative process. MicroRNAs (miRNAs) are ~22-nucleotide-long endogenously expressed non-coding RNAs that regulate the expression of gene products by inhibition of translation and/or transcription in animals. miRNAs play a key role in skin morphogenesis and in regulating angiogenesis. The vascular endothelial growth factor signaling path seems to be under repressor control by miRNAs. Mature miRNA-dependent mechanisms impair angiogenesis *in vivo*. It is critically important to recognize that the understanding of cutaneous wound healing is incomplete without appreciating the functional significance of wound-induced miRNA. Ongoing work in our laboratory has led to the observation that the cutaneous wound healing process involves changes in the expression of specific miRNA at specific phases of wound healing. We hypothesize that dysregulation of specific miRNA is critical in derailing the healing sequence in chronic problem wounds. If tested positive, this hypothesis is likely to lead to completely novel diagnostic and therapeutic strategies for the treatment of problem wounds.

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INTRODUCTION

Repair of a defect in the human skin is a highly orchestrated physiological process involving numerous factors that act in a temporally resolved synergistic manner to re-establish barrier function by regenerating new skin. The inducible expression and repression of genes represents a key component of this regenerative process (Sen, 2003; Broughton *et al.*, 2006a; Branski *et al.*, 2007). The central dogma in molecular biology has been that DNA replicates its information and transcribes to RNA where it codes for the production of mRNA. mRNA is processed essentially by splicing and translocates from the nucleus to the cytoplasm. mRNA carries coded information to the ribosomes. Ribosomes translate the code for protein synthesis. The synthesis of specific proteins and their proper functionality at the correct temporal phase of healing is central to wound healing. Do all RNAs carry the code to synthesize protein? No. However, almost all means of gene identification assume that genes encode proteins. An important aspect of the central dogma remained under veils for a long time. Non-coding RNA (ncRNA) genes produce functional RNA molecules rather than encoding proteins. Several different systematic screens have identified a surprisingly large number of ncRNA genes. NcRNAs seem to be particularly abundant in roles that require highly specific nucleic acid recognition without complex catalysis, such as in directing post-transcriptional regulation of gene expression or in guiding RNA modifications. Although it has been generally assumed that most genetic information is transacted by proteins, recent evidence suggests that the majority of the genomes of mammals and other complex organisms are in fact transcribed into ncRNA, many of which are alternatively spliced and/or processed into smaller products (Mattick and Makunin, 2006). These RNAs (including those derived from introns) appear to comprise a hidden layer of internal signals that control various levels of gene expression in physiology and development, including chromatin architecture/epigenetic memory, transcription, RNA splicing, editing, translation, and turnover. This hidden layer of internal signals is now emerging to be of such critical significance that lack of consideration of that layer poses the serious risk of clouding our ability to understand the molecular basis of health and disease (Goodrich and Kugel, 2006; Mattick and Makunin, 2006; Racz and Hamar, 2006; Tomaru and Hayashizaki, 2006). In all forms of life, ncRNA includes ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA),

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interference RNA (RNAi), short interfering RNA (siRNA), and micro RNA (miRNA). The objective of this review article is to focus on the potential role of miRNA in cutaneous wound healing with the goal of developing the extraordinary significance of this new field.

FROM RNAi TO miRNA

Post-transcriptional gene silencing (PTGS), which was initially viewed as an isolated regulatory mechanism in some plant species, now represents a major frontier in molecular medicine (Filipowicz *et al.*, 2005; Racz and Hamar, 2006). RNAi was first observed inadvertently in an experiment to increase the purple pigment in petunias. However, the experiment backfired when the gene that was introduced caused PTGS of the pigment-producing gene. Subsequent studies on *C. elegans* and the fruit fly *Drosophila* revealed that PTGS could be triggered by dsDNA. A similar phenomenon in fungus was termed "quelling" in 1992. Andrew Fire and Craig Mello (Nobel Prize winners in Physiology or Medicine, 2006) are credited with the 1998 discovery of RNAi (Fire *et al.*, 1998). Earlier works had identified that both antisense RNA (Izant and Weintraub, 1984) as well as sense RNA (Guo and Kemphues, 1995) could silence genes although the results were inconsistent and the effects usually modest. In light of the observation that both sense and antisense RNA could cause silencing, Mello argued that the mechanism could not just be a pairing of antisense RNA to mRNA, and he coined the term RNAi for the unknown mechanism (Rocheleau *et al.*, 1997). The discovery that short RNA is the effector of RNAi was rapidly followed by the identification of a class of endogenous RNA molecules of the same size in worms, flies, mice, and humans. This small RNA was called miRNA (Reinhart *et al.*, 2000; Lagos-Quintana *et al.*, 2001; Lee and Ambros, 2001). miRNA can regulate gene expression by base pairing to mRNA, which results in either degradation of the mRNA or suppression of translation. There are 640 miRNA in human cells, which regulate about 30% of all genes.

miRNA: AN INTRODUCTION

miRNAs are ~22-nucleotide (nt) endogenously expressed RNAs that belong to the family of short ncRNA (Bartel, 2004). Transcribed in the nucleus by conventional mechanisms, miRNAs are exported to the cytoplasm (Yi *et al.*, 2003), where they form the mature miRNAs that can interact with matching mRNA causing degradation of specific mRNAs. In addition, this binding may cause translational or transcriptional inhibition (Fig. 1). This mechanism of action is termed as post-transcriptional gene regulation. In contrary to plants, in animals 100% nt match between miRNA and its target mRNA is not typically seen. Such binding leads to mRNA translational inhibition and not mRNA degradation (Carrington and Ambros, 2003). The interaction between the miRNA and its matching mRNA occurs between the 5′ untranslated region (UTR) of the miRNA to the 3′ UTR of the mRNA by a matching seed element in the miRNA. Computational algorithms estimate that miRNA can target 30% of the human genome (Lewis *et al.*, 2005; Kruger and Rehmsmeier, 2006; Smalheiser and Torvik, 2006). Furthermore, one miRNA can regulate more than one gene, and one gene can be regulated by a number of miRNAs. An important consideration in this context is that there is tissue specificity for miRNA expression. Thus, specific miRNA regulates specific sets of mRNA in a given tissue. As a result, miRNAs play a significant role in developmental biology and in cell and tissue phenotyping (Monticelli *et al.*, 2005; Song and Tuan, 2006; Sood *et al.*, 2006). A total of 640 miRNAs have been discovered in humans and more in other species, as recorded in the miRNA registry. It is not surprising that these RNA members are highly conserved among species and such conservation serves as one of the tools for identifying new miRNAs throughout the genome (Altuvia *et al.*, 2005; Berezikov *et al.*, 2005; Weber, 2005; Yousef *et al.*, 2006).

In the genome, miRNAs are distributed in non-coding DNA regions. They can be found in introns of protein-coding genes (Ying *et al.*, 2006), or introns and exons of ncRNA genes (Rodriguez *et al.*, 2004). Thus, miRNAs are under transcriptional regulation of the host genes. In addition, the miRNA genes are either genomically isolated or found in clusters (Onishi and Ueda, 2005; Yu *et al.*, 2006). There is experimental evidence connecting miRNA cluster expression to different types of cancers, such as lung cancer, lymphoma, and leukemia (Tanzer and Stadler, 2004; Hayashita *et al.*, 2005; Legendre *et al.*, 2005; Onishi and Ueda, 2005; Tagawa and Seto, 2005). Moreover, miRNAs have been implicated in tissue morphogenesis, cellular processes like apoptosis, and major signaling pathways linking its possible role in health and disease (Jin *et al.*, 2004; Mendell, 2005). There are four possible mechanisms by which miRNA can lead to disease (Plasterk, 2006): (i) a miRNA may acquire a mutation resulting in loss of function; (ii) a miRNA may acquire a mutation resulting in gain of function; (iii) a programmed target site may acquire a mutation and no longer be able to bind to the miRNA; and (iv) a gene may acquire a new and undesired miRNA target sequence that will result in silencing. These proposed mechanisms are hypothetical and remain to be fully validated in biological experiments. For example, gain and loss of miRNA target sites appears to be causal to some genetic disorders (Kloosterman and Plasterk, 2006). Furthermore, proteins participating in the biogenesis of miRNA can be candidates for disease cause. One of such protein, DGCR8 (discussed later), is commonly missing in DiGeorge syndrome. This syndrome involves heterogeneous defects, including cardiac deficiencies, immunodeficiency, schizophrenia, obsessive-compulsive disorder, and more (Alvarez-Garcia and Miska, 2005). In addition to host miRNA, there are studies connecting viral pathogenesis to miRNA produced by viruses that can influence either the pathogen itself or the infected host (Omoto *et al.*, 2004; Burnside *et al.*, 2006).

BIOGENESIS OF miRNA

Gene expression starts with transcription. Initially it was believed that the transcription of miRNA is mediated by RNA polymerase III, because it transcribes most of the small RNAs. However, primary microRNAs (pri-miRNAs) are sometimes several kilobases long and contain stretches of more than four uracils, which would have terminated transcription by polymerase III. Lee *et al.* (2004) have concluded that miRNA transcription is accomplished by RNA polymerase II. The miRNA is first transcribed as hundreds- to thousands-nt-long miRNA precursor

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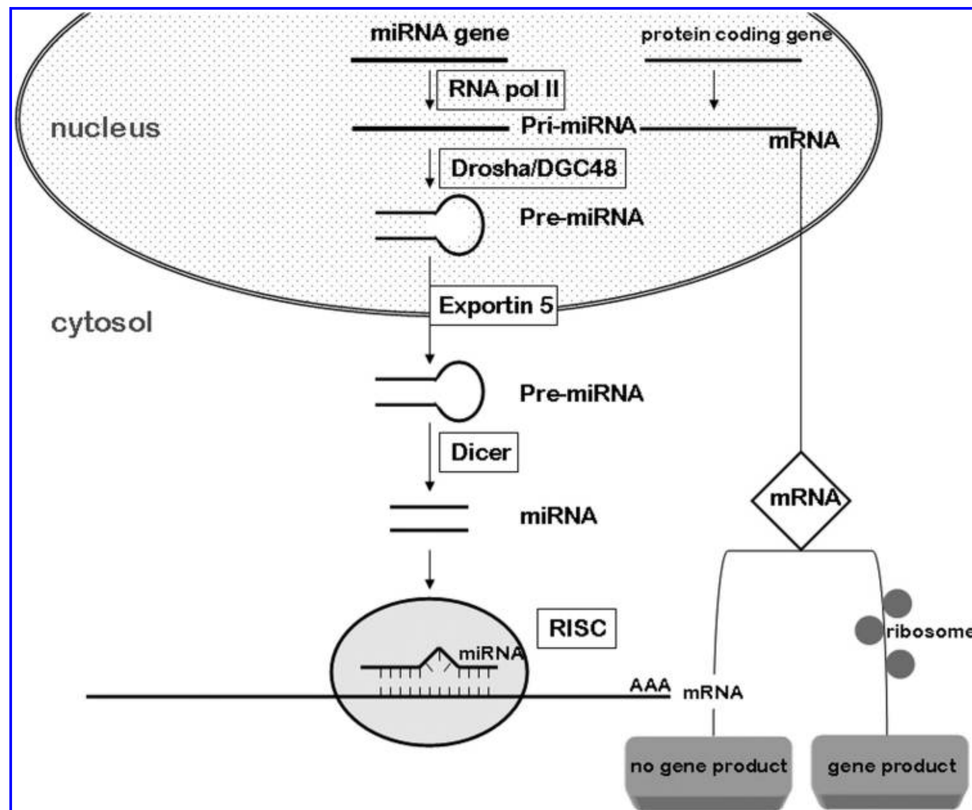

FIG. 1. Outline of miRNA biogenesis. Primary microRNA (pri-miRNA) is synthesized in the nucleus by RNA polymerase II. The RNA endonuclease Drosha and its cofactor DGC48 cleave pri-miRNA to produce precursor miRNA that is about 70 nucleotides (nt) long. This product is exported to the cytosol by Exportin 5 where it is cleaved again by the second RNA endonuclease Dicer to form the approximately 20–22-nt-long mature miRNA. One of the double-stranded miRNA is incorporated to the RNA-induced silencing complex, where by base matching with the 3′ untranslated region end of mRNA, it captures the target mRNA in the complex. This causes inhibition of translation by the ribosome.

called primary miRNA. Analysis of several pri-miRNA precursors has shown that they all contain a 5′ 7-methyl guanosine cap and a 3′ poly-A tail. Therefore, this data indicates that pri-miRNAs are structurally analogous to mRNA (Cullen, 2004).

Following transcription, the miRNA goes through the first step of cleavage. It is initiated by the nuclear RNase III Drosha, a double-stranded RNA (dsRNA) specific endonuclease that introduces staggered cuts on each strand of the RNA helix (Lee and Kim, 2005). It is responsible for the nuclear processing of the pri-miRNA into stem-loop (hairpin shaped) precursors of ~70-nt named precursor miRNA (pre-miRNA). RNA interference of Drosha results in the strong accumulation of pri-miRNA, and the reduction of pre-miRNA and mature miRNA *in vivo* (Lee *et al.*, 2003). RNA stem-loops with a large, unstructured terminal loop (above 10 nt) are the preferred substrates for the cleavage of Drosha (Zeng *et al.*, 2005). In the nucleus, Drosha functions as a large complex where it interacts with DGCR8 (an essential cofactor for Drosha), which contains two dsRNA-binding domains (Han *et al.*, 2004; Yeom *et al.*, 2006). Recombinant human Drosha alone shows non-specific RNase activity, but the addition of DGCR8 renders it specific for pri-miRNA processing (Tomari and Zamore, 2005). The primary and secondary structures of miRNA precursors are conserved as internal loops and bulges that commonly appear in specific positions in the miRNA stem. This enables correct enzymatic processing leading to the maturation of the miRNA (Saetrom *et al.*, 2006).

Export of the pre-miRNA from the nucleus to the cytoplasm is mediated by Exportin 5 (Yi *et al*., 2003). It is a nuclear export receptor for certain classes of dsRNA, including pre-miRNA, viral hairpin RNA, and some tRNA (Chen *et al.*, 2004). The depletion of nuclear guanosine triphosphate (GTP)–bound Ran (RanGTP) impairs the export of pre-miRNA. It is therefore thought that the function of Exportin 5 is dependent on nuclear RanGTP (Bohnsack *et al.*, 2004). Once in the cytoplasm, the exported complex is disassembled by GTP hydrolysis (Matsuura and Stewart, 2004). In addition to supporting nuclear export of pre-miRNA, Exportin 5 likely prevents nuclear pre-miRNA degradation (Zeng and Cullen, 2004).

The second step of miRNA processing is confined to the cytoplasm (Lee *et al.*, 2002). The pre-miRNA goes through another cleavage step executed by Dicer. Dicer is a multi-domain ribonuclease that processes the hairpin precursor into a ~22-nt small dsRNA mature miRNA (Kolb *et al.*, 2005). Dicer functions through intra-molecular dimerization of its two RNase III domains, assisted by the flanking RNA binding domains, PAZ

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and dsRNA-binding domains (dsRBD), that generate products with 2-nt 3′ overhangs (Zhang *et al.*, 2004). PAZ domains are highly conserved domains of 130 amino acids that bind to RNA found only in Dicer and Argonaut proteins (discussed later) (Carmell and Hannon, 2004).

Following cleavage of the pre-miRNA by Dicer, the mature miRNA is incorporated into an RNA-induced silencing complex (RISC) whose diverse functions can include mRNA cleavage, translation suppression, transcriptional silencing, and heterochromatin formation (Andl *et al.*, 2006) (Fig. 1). This complex functions in RNAi as well. RISC is a multiple-turnover enzyme complex, meaning that miRNA can direct multiple rounds of target cleavage, once incorporated. One strand of the double-stranded miRNA is preferentially incorporated into RISC depending upon the thermodynamics of the duplex. It has been proposed that a ~500 kDa trimeric protein complex made up of Dicer, human immunodeficiency virus transactivating response RNA-binding protein (TRBP), and Argonaute2 (Ago2) is required for the biogenesis of miRNA (Gregory *et al.*, 2005). There is evidence that the complex forms prior to miRNA loading (Maniataki and Mourelatos, 2005). TRBP is a protein with three dsRBDs that are essential for the processing of miRNA (Haase *et al.*, 2005). Ago2 is a member of the Argonaute protein family and the only member in humans that is associated with both siRNA and miRNA silencing. It serves as the catalytic engine of RISC by virtue of a PIWI domain that contains an RNase H-like structure for its endonucleolytic-slicer activity (Sontheimer and Carthew, 2004; Miyoshi *et al.*, 2005). Ago2 is essential for mouse development, and cells lacking Ago2 fail to respond to siRNA. Moreover, mutations within the RNase H domain of Ago2 inactivate RISC supporting its fundamental role in miRNA-induced mRNA silencing (Rand *et al.*, 2005).

In mammals, imperfect match between miRNAs and their target mRNA is commonly noted. As a result, in mammals miRNAs are primarily responsible for translational inhibition of mRNA (Fig. 1). RISC containing miRNA may directly interfere with translation initiation or elongation, and perhaps target the mRNA to centers of degradation. These centers, which contain untranslated mRNA, are sites of mRNA degradation. They have been previously observed in yeast and animal cells and are called processing (P) bodies (Jabri, 2005). Supporting this notion is the evidence of the presence of Argonaute family proteins in these P-bodies. However, it is not clear whether P-bodies are a cause or a consequence of inhibiting protein synthesis. RCK/p54 is the effector molecule in miRNA-RISC that represses translation (Chu and Rana, 2006). RCK/p54, the human homolog of yeast Dhh1p, is a P-body protein and a member of the ATP-dependent DEAD box helicase family. In human cells, RCK/p54 interacts in P-bodies with the translation initiation factor, eIF4E. The overall result of the binding of mRNAs in the RISC complex by their matching miRNA is inhibition of translation of the mRNA. This, in turn, leads to decreased levels of the protein encoded by the target mRNA for any given miRNA (Fig. 1).

miRNA IN SKIN MORPHOGENESIS

The skin is the largest organ of the body, accounting for about 15% of the total body weight in adult humans (Kanitakis, 2002; Healy, 2005). In brief, the skin is made up of three distinct layers of tissue: epidermis, dermis, and hypodermis. The mammalian epidermis is a stratified epithelium layer that retains the ability to self-renew under both homeostatic and injury conditions by maintaining a population of mitotically active cells in the hair follicles and innermost basal layer (Segre, 2006). It is populated by keratinocytes (80%) and other cell types, such as dendritic cells, melanocytes, Langerhans, and Merkel cells. The dermis consists of collagenous and elastic fibers embedded in an amorphous ground substance. It is populated by fibroblasts, macrophages, mast cells, and lymphocytes. The hypodermis is composed of adipocyte lobules defined by fibrous connective tissue septa. In addition, the skin contains hair follicles. Developmentally, hair follicles represent an outgrowth of the primitive epidermis (Stenn, 2003). It has a very complex structure and consists of over 20 different cell types distributed into six main compartments, namely the connective tissue sheath, the dermal papilla, the outer root sheath, the inner root sheath, the shaft, and the sebaceous gland. These compartments lie within the dermis and the epidermis (Bernard, 2005). Moreover, the hair follicle has a reservoir of pluripotent stem cells that can also regenerate the epidermis (Lavker *et al.*, 2003; Ma *et al.*, 2004). The skin is responsible for many functions, such as epidermal barrier and defense, immune surveillance, UV protection, thermoregulation, sweating, lubrication, pigmentation, the sensations of pain and touch, and the protection of various cutaneous stem cell niches (Ross and Christiano, 2006). Nevertheless, the most crucial function of the skin is to defend the body as a barrier interface between the internal organs and the environment. This barrier function of the skin is critical in newborn animals, as shown by transgenic animal models with barrier defects that die shortly after birth from transepidermal water loss (Segre, 2003).

It is fortunate that in this early phase of miRNA research, one of the organs about which we know more than most others is skin. Recent works on the significance of miRNA in skin morphogenesis and development provide important insight that lays the foundation for wound healing research. Our laboratory has initiated a project specifically directed to address the significance of miRNA in cutaneous wound healing. Recent works by the Fuchs laboratory have addressed the role of miRNA in mouse skin epidermis and hair follicle (Yi *et al.*, 2006). First, after isolating RNA they cloned and sequenced small RNA and found that most of them correspond to known mouse miRNA. They characterized the relative miRNA levels in these tissues and discovered that many skin miRNAs are differentially expressed by epidermal and hair follicle lineages. There were distinctive expression patterns of miRNAs in these two tissues. In both, the most abundant miRNA was mmu-miR-16. This miRNA is abundant in most tissues of the body (Krutzfeldt *et al.*, 2005). In addition, many skin miRNAs could be classified into discrete groups on the basis of similar 5′ seed sequences, although they were transcribed from distinct genomic loci. These data support the notion that target mRNAs in the skin are efficiently regulated by miRNAs.

Testing of the significance of miRNAs in skin development has led to very interesting findings. The Dicer-deficient mouse model has been informative (Yi *et al.*, 2006). Dicer was conditionally knocked out in skin epithelial progenitor cells. Because Dicer is one of the key enzymes in the processing of

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miRNA to functional mature miRNA, ablation of Dicer arrests mechanisms triggered by mature miRNA. The conditional knockout animals began to lose weight within 1–2 days after birth, and neonatal conditional knockout mice appeared dehydrated and did not survive past postnatal day 4–6. The most striking histological finding in the skin was that instead of invaginating downward into the dermis, hair germs appeared to evaginate into the epidermis. With age, hair germ–like cysts became prevalent markedly distorting the overlying epidermis. In addition, skin of the conditional knockout showed signs of apoptosis although there were larger numbers of cells in the follicles of conditional knockout mice. This continual upward proliferation of follicle cells grossly perturbed the integrity of the skin of the mutant mice. Cyst-induced epidermal perturbations likely accounted for the loss of weight, dehydration, and eventual death of the Dicer1 conditional knockout animals. It is clear that miRNAs play a critical role in skin morphogenesis. Furthermore, the essential role of skin in life and death was evident (Yi *et al.*, 2006).

In a study that utilized the skin as a model system to investigate the functions of *Dicer* in mammalian organogenesis, it was first discovered by *in situ* hybridization of mouse embryos and mouse literates that Dicer is present in both epidermis and hair-follicle outer root sheath (Andl *et al.*, 2006). To determine whether *Dicer* is required for the development of hair follicles or epidermis, epidermal-specific deletion of the *Dicer* gene was performed in mice. This was achieved by crossing *Dicer*^{flox} mice with a transgenic mouse line in which Cre recombinase was expressed under the control of a keratin 14 promoter. In agreement with the former study, here, newborn *Dicer* mutant mice were initially grossly indistinguishable from control littermates. However, by postnatal day 7, mice were stunted and lacked external hair growth with poor viability of the mutant mice. Evagination of the epidermis by hair follicles was noted. In addition, hair follicles were also replaced by cyst-like structures or disorganized clumps of epithelial cells within the dermis. Examination of the molecular details revealed that expression of the progenitor cell marker Keratin 15 was absent in the skin of newborn *Dicer* mutant (Andl *et al.*, 2006). Keratin 15 is a specific marker for hair-follicle stem cells, although its significance is not yet known. In contrast to the findings in the mutant hair follicles, epidermis of the *Dicer* mutant displayed marked elevation in the numbers of both basal and supra-basal cell-layers compared with the epidermis of control littermates. Interestingly, the expression of *Notch1* [a trans-membrane receptor that once signaled activates transcription (Wilson and Radtke, 2006)] was reduced in the epidermis as well as in the hair follicles of *Dicer* mutant mice. Deletion of *Notch1* in the epidermis causes hyperproliferation and tumor development, suggesting that the observed decrease in *Notch1* expression in the *Dicer* mutant could contribute to the epidermal phenotype (Proweller *et al.*, 2006). Furthermore, it has been noted that embryonic as well as postnatal inactivation of *Notch1* shortly after birth or in adult mice results in almost complete hair loss followed by cyst formation (Vauclair *et al.*, 2005). This may lead to the hypothesis that *Notch1* is the key protein that is affected in the Dicer knockouts leading to abnormalities in hair follicle that will sequel in skin layer impaired morphogenesis and eventually end in transdermal water loss and death. Another phenomenon in the mutant skin was the appearance of clusters of dermal cells, apparently in the process of being surrounded by epidermal cells. Because *K14-Cre* does not cause recombination of the *Dicer*^{flox} allele in dermal cells, this phenotype must be because of Dicer deficiency in the epidermis or hair follicle epithelium. These studies by the groups of Elaine Fuchs and Sarah Millar provide first evidence describing the fundamental role of miRNA in skin tissue morphogenesis. The stage is now set for testing the significance of miRNA in skin-related diseases, including wound healing. miRNA-based therapies may be expected in the near future.

WOUND HEALING AND ANGIOGENESIS

Wound healing may be broadly split into three overlapping basic phases: inflammation, proliferation, and maturation (Broughton *et al.*, 2006b). First in sequel, the inflammatory phase is characterized by hemostasis and inflammation. The next phase consists mainly of epithelialization, angiogenesis, granulation tissue formation, and collagen deposition. The final phase includes maturation and remodeling. This phase is characterized by an organized deposition of collagen (Broughton *et al.*, 2006a). The complexity of wound healing is augmented by the influence of local factors (such as ischemia, edema, and infection) and systemic factors (such as diabetes, age, hypothyroidism, malnutrition, obesity, and more) (Harvey, 2005). Angiogenesis is often identified as the rate-limiting step of wound healing (Lingen, 2001). Wound angiogenesis is marked by endothelial cell migration and capillary formation where the sprouting of capillaries into the wound bed is critical to support the regenerating tissue. The granulation phase and tissue deposition require nutrients supplied by the capillaries. Impairments in wound angiogenesis therefore may lead to chronic problem wounds (da Costa Pinto and Malucelli, 2002; Galeano *et al.*, 2003; Chbinou and Frenette, 2004).

Expression of the angiogenic phenotype is a complex process that requires a number of cellular and molecular events to occur in sequential steps. Some of these activities include endothelial cell proliferation, degradation of surrounding basement membrane, migration of endothelial cells through the connective tissue stroma, formation of tube-like structures, and maturation of endothelial-lined tubes into new blood vessels. Angiogenesis is controlled by positive and negative regulators (Li *et al.*, 2005). In addition to endothelial cells, cells associated with tissue repair, such as platelets, monocytes, and macrophages, release angiogenic growth factors into injured sites that initiate angiogenesis. Vascular endothelial growth factor (VEGF) is believed to be the most prevalent angiogenic factor in the skin repair process during wound healing (Sayan *et al.*, 2006). The significance of the VEGF family in wound angiogenesis has been recently described elsewhere (Roy *et al.*, 2007).

miRNA AND ANGIOGENESIS

At present, the significance of miRNA in cutaneous wound healing remains unpublished. In this section, literature that directly addresses the role of miRNA in angiogenesis has been reviewed with the objective to highlight the potential significance of studying miRNA in the context of wound angiogenesis.

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The Dicer gene is significantly expressed in embryos from day 11 and remains constant through day 17, evenly expressed throughout the embryonic tissues (Yang *et al.*, 2005). To further determine the *in vivo* function of Dicer during development, the *dicer*$^{ex1/2}$ mutant mice model has been developed. These mutant mice lack the first two exons of *dicer* that are essential for the function of the protein, that is, maturation of miRNA. Homozygous mutant mice were not viable; therefore, the embryos were examined. Starting from embryonic day 11.5, virtually all *dicer*$^{ex1/2}$ embryos were growth- and developmentally-retarded as compared with their wild-type or heterozygous litter mates (Yang *et al.*, 2005). The embryos that were still viable at this stage had thin and sub-optimally developed blood vessels, providing first evidence for the involvement of miRNA in angiogenesis. Moreover, microscopic examination of the yolk sac from mutant embryo revealed that there were fewer blood vessels in the *dicer*$^{ex1/2}$ yolk sacs and that these vessels were thin, small, and less organized than those of control yolk sacs. Together, these observations lead to the hypothesis that Dicer is required for the development of blood vessels during embryogenesis. When yolk sacs from 11.5-day embryos were stained with anti-PECAM antibodies specific to endothelial cells, it was noted that the blood vessels in *dicer*$^{ex1/2}$ yolk sacs were thin and disorganized compared to their healthy controls. The vascular defects found in the Dicer mutant embryo led to question the levels of key angiogenic genes in the mutant mice. Interestingly, mRNA levels of VEGF and the genes of its receptors, *Flt1* and *Kdr*, were significantly higher than those in wild-type embryos. Although this finding seems to predict favorable angiogenic environment in the mutant mice, the actual observation was in direct contrast. Results of the experiment suggest that up-regulation of the VEGF signaling pathway alone may not lead to functional angiogenesis. Furthermore, it seemed likely that the VEGF signaling path is under repressor control by miRNA-dependent mechanisms. Other explanations of the observation include a compensatory up-regulation of the VEGF system in the face of impaired angiogenesis or induction of other pro-VEGF signaling pathways such as that driven by hypoxia (Gerber *et al.*, 1997). In *dicer*$^{ex1/2}$ mutant mice, the mRNA level of *tie-1*, a receptor tyrosine kinase gene, was lower than in corresponding wild-type mice. *Tie-1* is a member of the tie receptor family that is required for the angiogenic remodeling of vessels during embryonic development and for the stabilization of blood vessel in quiescent adult vasculature (Jones *et al.*, 2001). Taken together, studies with the *dicer*$^{ex1/2}$ mutant mice present compelling evidence that arrest of mature miRNA-dependent mechanisms impair angiogenesis *in vivo* (Yang *et al.*, 2005).

The field of miRNA and cancer has developed rapidly (Calin and Croce, 2006; Cummins and Velculescu, 2006; Dalmay and Edwards, 2006; Mocellin *et al.*, 2006; Pfeffer and Voinnet, 2006; Silveri *et al.*, 2006; Tomaru and Hayashizaki, 2006). Unlike wound healing research where we practically know nothing about the significance of miRNA in wound angiogenesis *in vivo*, the role of miRNA in tumor angiogenesis has been directly addressed. c-myc is a leucin zipper transcription factor that has been found to have a role in neo-vascularization of neoplasms (Brandvold *et al.*, 2000). miRNAs are implicated in the regulation of the c-myc pathway (Dews *et al.*, 2006). c-myc does not seem to induce angiogenic pathways. Instead, c-myc seems to down-regulate anti-angiogenic factors, such as thrombospondin-1 (Tsp-1) and connective tissue growth factor (CTGF). This observation is consistent with previous observations that c-myc down-regulates Tsp1 not by blocking promoter activity, but by decreasing Tsp1 mRNA half-life (Janz *et al.*, 2000). Recently it has been established that c-myc directly activates the miRNA cluster miR-17-92 in human lymphocytes (O'Donnell *et al.*, 2005). We now know that levels of miRNAs miR-18 and miR-19, which are the cleavage products of the miR-17-92 cluster, are up-regulated by c-myc as well (Dews *et al.*, 2006). To further elucidate the direct effect of c-myc on these miRNA, transfection of antisense oligonucleotides to individual miRNA has been performed. miR-19 is primarily responsible for the down-regulation of Tsp1, and miR-18 for the down-regulation of CTGF in response to c-myc. Thus, a substantial role of miRNA in regulating c-myc-dependent tumor vascularization has been unveiled. This constitutes first evidence supporting the involvement of specific miRNA in angiogenesis.

Support for our call to study the significance of miRNA in wound angiogenesis has been provided by a recent work addressing the role of miRNA on the angiogenic properties of human umbilical vein endothelial cells (HUVEC) (Poliseno *et al.*, 2006). Twenty-seven abundant miRNAs have been identified in these endothelial cells. Prediction algorithms have been utilized to look for angiogenic receptors that may be target candidates of the miRNAs identified in HUVEC. miR-221 and miR-222 were predicted to target c-kit. c-kit is a receptor tyrosine kinase that binds stem cell factor (SCF). Inhibition of c-kit results in down-regulation of VEGF expression (Litz and Krystal, 2006). Moreover, it has been shown that c-kit is involved in neovascularization and tumor progression. Arresting c-kit results in tumor containment (Strumberg, 2005; Roboz *et al.*, 2006). In wounds, the expression of c-kit in mast cells is induced slowly when healing, while in chronic wounds as well as in psoriatic lesions, c-kit is intensely expressed (Huttunen *et al.*, 2002). Transfection of miR-221/222 mix decreased c-kit protein levels without changing the mRNA level. In addition, the transfection inhibited the ability of endothelial cells to promote tube formation in response to activation by SCF. Furthermore, introducing miR-221/222 mix to HUVEC diminished SCF-induced survival. Thus, miR-221 and miR-222 modulate the angiogenic activity of SCF by modulating the level of its receptor c-kit. This is valuable information although very preliminary and only demonstrated *in vitro* (Poliseno *et al.*, 2006).

It is clear from *in vivo* studies of experimental Dicer knockdown discussed earlier that miRNAs play a fundamental role in the biology of skin morphogenesis and angiogenesis (Fig. 2). Although many questions remain to be addressed, explaining how miRNAs are involved in wound healing and related angiogenesis, it is critically important to realize that the study of cutaneous wound healing is incomplete without appreciating the functional significance of miRNA. A mature understanding of the molecular processes and the role of miRNA in cutaneous wound healing biology will unveil new and modified therapeutic strategies.

Interestingly, in the field of skin and wound healing, antisense-hypoxia inducible factor (aHIF) (Thrash-Bingham and Tartof, 1999; Rossignol *et al.*, 2002; Cayre *et al.*, 2003)

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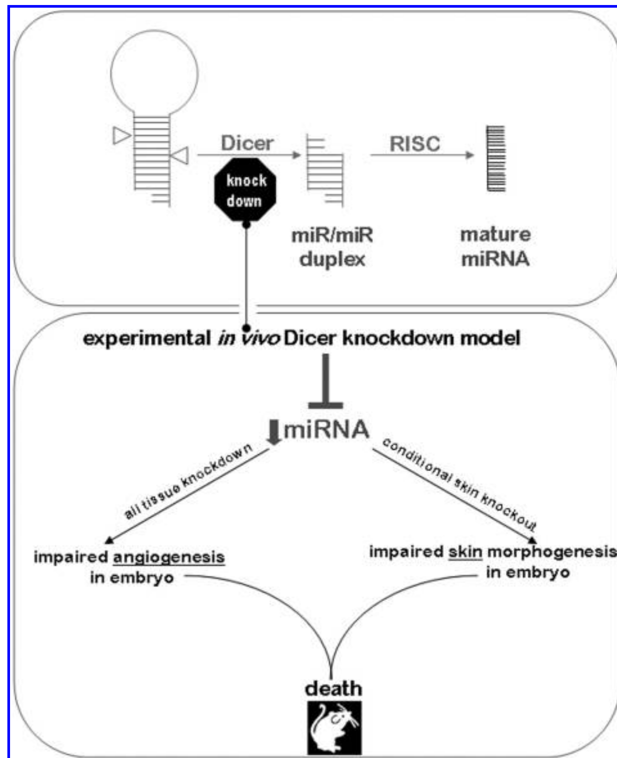

FIG. 2. Lessons from the Dicer knockdown mouse model. In Dicer knockdown mice, processing of precursor miRNA to mature miRNA is arrested. In the global knockout model, the most aberrant phenomenon was impaired embryonic angiogenesis that led to embryonic death. In the conditional skin Dicer knockout model, impaired skin morphogenesis was followed by early neonatal death of mice.

could well be an miRNA. HIF1 is one of the main genes regulating the molecular responses to hypoxia. HIF1 is composed of two subunits. HIF1β is constitutively expressed. However, HIF1α is controlled by oxygen tension and is degraded under normoxic conditions. Hypoxic conditions with partial pressure of oxygen lower than 40 torr lead to HIF1α accumulation, translocation to the nucleus, and, as a result, transcriptional activity through binding to specific hypoxia-responsive elements (Diaz-Gonzalez *et al.*, 2005). This leads to the expression of oxygen-dependent genes (Albina and Reichner, 2003). In experimental skin wound models, it is known that hypoxia upregulates HIF1α (Vihanto *et al.*, 2005). The expression of HIF1 in wounds may induce inducible nitric oxide synthase and VEGF, two HIF1-responsive genes intimately related to the process of repair (Albina *et al.*, 2001). In 1999, aHIF was reported as a natural antisense transcript of HIF. aHIF sequence is complementary to the sequence in the 3′ UTR of HIF1α mRNA (Thrash-Bingham and Tartof, 1999). aHIF is present in human adult as well as fetal tissue (Rossignol *et al.*, 2002). In breast cancer patients, aHIF, not HIF1α transcript, served as a reliable marker of poor prognosis (Cayre *et al.*, 2003). Unlike miRNA, aHIF is more than 1 kb long and complements HIF1α for almost all of its length. Thus, aHIF is not an miRNA. Of note, aHIF is non-coding and is functionally analogous to miRNA. These observations suggest that there may be another non-coding entity of longer RNA, which regulates mRNA transcription like miRNA.

MIRNA-BASED THERAPEUTICS: FUTURE POTENTIAL

RNAi-based therapeutics represents one of the hottest novel avenues in biomedical treatment (Boudreau and Davidson, 2006; Ke *et al.*, 2006; Ketzinel-Gilad *et al.*, 2006; Storvold *et al.*, 2006; Waseem, 2006). miRNA-based therapies represent a sub-discipline that holds significant promise (Weiler *et al.*, 2006; Ying *et al.*, 2006; Liu *et al.*, 2007). The ability to modulate miRNA activity *in vivo* is likely to have tremendous impact on disease therapy and on *in vivo* research opportunities. Initial efforts in this direction are in motion. There are two major options available: over-expression and silencing of the prospective miRNA. For the former, delivery of corrective synthetic miRNA in the form of (siRNA-like) dsRNA may be productive. For a disease phenotype caused by abnormal miRNA-dependent inhibition of a specific subset of mRNA, oligonucleotides complementary to either the mature miRNA or its precursors can be designed such that the miRNA will be functionally arrested and will not be able to bind the target mRNA subset. Successful design of such oligonucleotide should include considerations such as successful *in vivo* delivery, resistance to degradation in tissues, and specificity and high-binding affinity to the specific miRNA in question. This can be achieved by chemical modification of the nucleotides, especially the addition of chemical groups to the 2′-hydroxyl group. Three forms of chemically modified oligonucleotides that have been used as means of silencing miRNA include (a) 2′-*O*-methyl-group (OMe)–modified oligonucleotides; (b) 2′-*O*-Methoxyethyl–modified oligonucleotides that show to have higher affinity and specificity to RNA than their OMe-analogs; and (c) Locked nucleic acid (LNA)–modified oligonucleotides in which the 2′-*O*-oxygen is bridged to the 4′-position via a methylene linker to form a rigid bicycle, locked into a C3′-endo (RNA) sugar conformation (Weiler *et al.*, 2006). Most current data in this direction originate from *in vitro* studies. Results from *in vivo* studies involving manipulation of tissue miRNA are limited.

Another approach to manipulate miRNA includes genetic or non-genetic mechanisms (Krutzfeldt *et al.*, 2006). The genetic approach includes (i) knockout of miRNA genes in mice, (ii) mutation of miRNA target sites in protein-encoding genes, and (iii) conditional alleles of the miRNA-processing gene *Dicer1* leading to deficiency of all mature miRNA. The non-genetic approaches may be broadly divided into two categories: antisense oligonucleotide (ASO) (Esau *et al.*, 2006) and antagomirs (Krutzfeldt *et al.*, 2005). 2′-*O*-Methoxyethyl phosphorothioate–modified ASO represents an effective tool to silence miRNA such as miR-122. Intraperitoneal injection of ASO was sufficient to achieve the desired results. Verification of miR-122 silencing was additionally proven by the increase of mRNA levels of four target genes of miR-122. No target mRNA changes were observed in mice treated with control ASO, demonstrating specific inhibition of miR-122. The ASO approach has been applied to a disease model of obesity in mice. C57Bl/6 mice that had been fed a high-fat diet for 19 weeks were treated with

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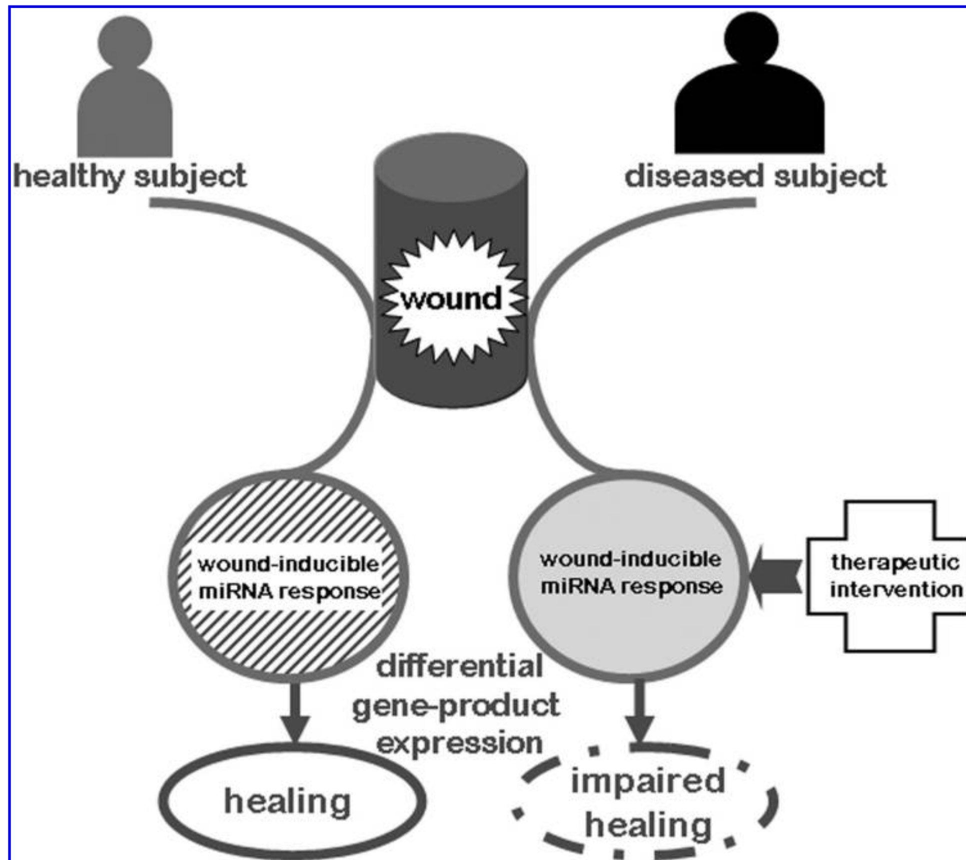

FIG. 3. Overall hypothesis to examine the significance of miRNA in cutaneous wound healing. In healthy subjects, wound induces a specific pattern of miRNA expression, which in turn regulates overall gene-product responses to healing. Such response favors healing responses, including wound angiogenesis, and finally leads to successful healing. In diseased subjects, wound induces the expression of a different pattern on miRNA expression. Such unfavorable response tilts the wound-induced expression of gene-products such that the healing process is stalled resulting in chronic problem wound. Prolonged lack of barrier function of the skin causes infection and further complicates healing.

miR-122 ASO. Blocking miR-122 resulted in 35% decrease of plasma cholesterol levels compared to control mice (Esau *et al.*, 2006). Antagomirs are chemically modified, cholesterol-conjugated, single-stranded RNA analogues designed to be complementary to specific miRNA. Antagomirs are synthesized starting from a hydroxyprolinol-linked cholesterol solid support and 2′-OMe phosphoramidites (Krutzfeldt *et al.*, 2005). Intravenous injection of antagomir-122 specifically decreased miR-122 levels. Antagomir-122 (240 mg per kg body weight) resulted in a complete loss of miR-122 signal, and levels of miR-122 were undetectable for as long as 23 days after injection. Antagomir-16 effectively silenced miR-16 in all body tissues, besides the brain. Therefore, antagomirs are useful in silencing miRNA *in vivo*. Silencing miR-122 resulted in down-regulation of 3-hydroxy-3-methylglutaryl-CoA-reductase (Hmgcr), a rate-limiting enzyme of endogenous cholesterol biosynthesis. In agreement with the last finding, plasma cholesterol levels were decreased more than 40% in antagomir-122–treated animals. Moreover, antagomir injection did not seem to have any toxic effect. This aspect of antagomir biology needs more rigorous testing, however. Thus, antagomirs represent useful tools to silence miRNA *in vivo* and offer a new platform for studying miRNA and related therapeutic opportunities (Krutzfeldt *et al.*, 2005).

In vivo over-expression of miRNA is another related area of outstanding importance. Pre-*miR-1* plus its flanking sequence has been sub-cloned into α-MHCclone26 or β-MHCclone32 vectors, and introduced into mice (Zhao *et al.*, 2005). Northern blots of the transgenic mice confirmed that they expressed miR-1. Western blot demonstrated a significant decrease in Hand2 protein levels compared with non-transgenic littermates, while mRNA levels of Hand2 remained unchanged. This observation confirmed that Hand2 is an miR-1 target *in vivo* and that up-regulation of a single miRNA using its precursor can elevate specific protein levels. Studies ongoing in our laboratory have led to the observation that cutaneous wound healing involves changes in the expression of specific miRNA at specific phases of wound healing. We hypothesize that dysregulation of specific miRNA is critical in derailing the healing sequence in chronic problem wounds (Fig. 3). If tested positive, the hypothesis is likely to lead to novel diagnostic and therapeutic strategies for the treatment of problem wounds.

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