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Differentiation of Bone Marrow Mesenchymal Stem Cells into the Smooth Muscle Lineage by Blocking ERK/MAPK Signaling Pathway

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Smooth muscle cells (SMCs) are major components of blood vessels and other hollow visceral organs required for tissue engineering of these organs. This study aims to evaluate whether adult bone marrow-derived mesenchymal stem cells (BMMSCs), multipotent cells, can be converted into SMCs. We examined the ERK/MAPK pathway as it exerts anti-myogenic signals in SMCs. Undifferentiated BMMSCs express most SMC marker genes, albeit mainly at low levels, except smooth muscle myosin heavy chain (SMMHC), the most definitive marker of differentiated SMC. The treatment of BMMSC with MEK inhibitor up-regulated the expression of alpha-smooth muscle actin (ASMA), *h*-caldesmon, and SMMHC in BMMSC in low serum condition. MEK inhibitor-treated BMMSC also contracted a collagen gel in response to endothelin. Interestingly, inhibition of MEK induced myocardin expression in BMMSC. In conclusion, BMMSCs treated MEK inhibitor gain a SMC-like phenotype with ligand-induced cell contractility to endothelin in vitro. This approach has obvious implications for cell therapeutics and tissue engineering of hollow visceral organs such as blood vessels.

Introduction

Vascular support is critical to the survival and functioning of all tissues. Thus, providing such vascular access is essential to recovery from tissue injury. Capillaries are readily regenerated from existing vessels, whereas large caliber conduits have been successfully engineered from biocompatible polymers (1). The missing links are the small-diameter (<6 mm) arteries such as coronary arteries which neither regenerate readily nor are amenable to the currently available materials. Thus, construction of these vessels is a target of regenerative medicine.

These vessels consist of three cell types, endothelial cells in the intima, smooth muscle cells (SMCs) in the media, and fibroblasts in the adventitia. While endothelial cells and fibroblasts are readily expandable from mature tissues, human adult SMCs have limited proliferative capacity, making ex vivo expansion of these cells very unpractical without immortalization of these cells (2, 3). Recently human tissue engineered blood vessels (TEBVs) with adequate mechanical strength were fashioned out of adult human endothelial cells and fibroblasts (4). However, lack of SMC layers did not allow these TEBVs to vasoconstrict and vasodilate in response to vasoactive mediators, thus limiting their utility. To create truly physiological and functional TEBVs, the presence of SMC layers would be indispensable. Because of these reasons, an alternative source of autologous adult SMCs is needed for regeneration and bioengineering of blood vessels and other visceral organs, and we speculate that bone marrow-derived mesenchymal stem cells (BMMSCs) might be an ideal cell source for adult SMCs.

BMMSCs, originally isolated as single-cell suspensions of bone marrow colonies of fibroblast-like cells that adhere to plastic (5), are pluripotent cells capable of differentiating into multiple cell lineages in vitro and in vivo after transplantation (6–13). BMMSCs are relatively easy to obtain and to expand in vitro. Encouragingly, there are many similarities noted between cultured BMMSC and SMC, such as expression of alpha-smooth muscle actin (ASMA)(14). Further, differentiation potential of transplanted BMMSCs into vascular smooth muscle cells (SMCs) has been shown in vivo (15–17). However, no protocol of driving BMMSC into SMC lineage in vitro has been established.

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The study of adult SMC differentiation and dedifferentiation has provided clues to key intracellular signaling pathways. Among them, the P44/42 extracellular signal-regulated protein kinase/mitogen activated protein kinase (ERK/MAPK) pathway has been shown as an anti-myogenic signaling pathway by several independent groups (18–23). The ERK/MAPK pathway is under the control of extracellular ligands. For example, basic fibroblast growth factor (bFGF) inhibited the transforming growth factor-beta (TGF-β)-induced up-regulation of SMC marker genes in 10T1/2 embryonic mesenchymal progenitor cells in ERK/MAPK pathway dependent manner (22).

Recent progress in vascular biology has revealed that the differentiation of vascular SMC is regulated by combinatorial interactions among several transcriptional regulators (24, 25). In particular, serum responsive factor (SRF) is a critical regulator for the differentiation of vascular SMC (26), and the activity of SRF is regulated by the interactions with myocardin or Elk-1. As a transcriptional coactivator of SRF, myocardin functions as a very potent myogenic factor for vascular SMC (27–29). Almost all of SMC marker genes are contingent on *cis*-regulatory CArG elements, and myocardin has been shown to induce all CArG-dependent SMC marker expression (25). Forced myocardin expression in BMMSCs induced SMC marker gene expression including smooth muscle myosin heavy chain (SMMHC), the late and most specific marker of differentiated SMC (30). However, genetic engineering of SMC is not currently considered a preferred option for tissue engineering due to either transient expression or chromosomal integration issues. Elk-1, another transcriptional cofactor of SRF, competes for the SRF binding site with myocardin and antagonizes myocardin (23). ERK/MAPK directly activates Elk-1 through phosphorylation (31).

These findings led us to hypothesize that the blockade of anti-differentiation signals and/or enhancement of pro-differentiation signals should drive BMMSCs into SMC lineage in vitro. In the present paper, we demonstrate that blocking of the ERK/MAPK pathway up-regulates SMC marker gene expression in BMMSCs, likely through induction of myocardin expression rather than inhibition of Elk-1 signaling, and these SMC-like BMMSCs contract collagen gel in response to endothelin, the most potent vasoconstrictive mediator (32). Thus, the establishment of SMC functionality provides a step forward in the tissue engineering of blood vessels and other hollow visceral organs.

Materials and Methods

Materials

Platelet derived growth factor (PDGF)-BB was obtained from Pepro Tech (Rocky Hill, NJ). PD98059, U0126, anti-alpha smooth muscle actin antibody, and alpha-tubulin antibody were from EMD Biosciences (San Diego, CA). Elk-1 antibody, phospho-Elk-1 antibody (Ser383), and phospho-P44/42 extracellular signal-regulated protein kinase/mitogen activated protein kinase (ERK/MAPK) (Thr202/Tyr204) antibody were purchased from Cell Signaling Technology (Beverly, MA). Anti connexin 43 antibody was from Chemicon International Inc. (Temecula, CA). Anti elastin antibody and anti β-actin antibody were from Sigma-Aldrich (Saint Louis, MO). Anti SMMHC antibody was from Biomedical Technologies (Stoughton, MA). Anti myocardin antibody and anti SRF antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti Kruppel-like zinc finger factor type 4 (KLF4) antibody and anti SM22α antibody were from Abcam (Cambridge, MA). HRP-conjugated secondary antibodies for enhanced chemiluminescence (ECL) were from Biosource International (Camarillo, CA). MEMα and DMEM culture media were from Cellgro (Kansas City, MO). FBS was from Atlanta Biologicals (Lawrenceville, GA). All of the remaining cell culture media and supplements were from Cellgro (Kansas City, MO) unless otherwise stated. All siRNAs were from Ambion (Austin, TX). Human myometrial tissue was obtained from Tissue Banking Department in the University of Pittsburgh to provide positive controls of SMC marker proteins and mRNA transcripts after approved as exempted (4e) by the University of Pittsburgh IRB.

Cell culture

Cultured primary human BMMSCs were provided by Dr. Darwin J. Prockop (33) (Tulane University, New Orleans, LA) and were cultured in MEMα supplemented with 15% FBS and 2 mM L-glutamine, 1 mM pyruvate, 100 μM nonessential amino acid. Human telomerase reverse transcriptase (hTERT)-immortalized human BMMSCs (hTERT-BMMSCs) were the kind gift from Dr. Junya Toguchida (Kyoto University, Kyoto, Japan)(34), and were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM pyruvate, 100 μM nonessential amino acid. The multipotency of these cells was confirmed previously (13). For the induction of SMC differentiation, BMMSCs were cultured in the FBS-reduced medium (3% FBS or "Low serum condition") supplemented with 10 μM of PD98059 for 5–7 days unless otherwise stated.

HS68 human foreskin fibroblasts were from American Type Culture Collection (ATCC, Rockville, MD) and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM pyruvate, and 100 μM nonessential amino acids. Immortalized human microvascular endothelial cells (HMECs) were obtained from the Center for Disease Control (Atlanta, GA) and cultured in MDCB 131 medium (Gibco, Gaithersburg, MD) supplemented with 10% FBS, 10 mM L-Glutamate, 1 ng/mL EGF (BD Biosciences, Bedford, MA), and 1 μg/mL hydrocortisone (Sigma, St. Louis, MO).

These studies were approved as exempted (4e) by the University of Pittsburgh IRB.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. In brief, cDNA systhesis, PCR amplification and detection of the template were carried out in the same tube with SuperScript III One-Step RT-PCR (Invitrogen, Carlsbad, CA) using gene-specific primer sets for humans. Primer sequences used in this study were given in Table (30, 35–37). All PCR primers were designed to span intron(s) to discriminate cDNA

Table 1. Primer Sequences for Reverse Transcriptase-Polymerase Chain Reaction

Primer	*Forward sequence*	*Reverse sequence*	*Reference*
SMMHC (SM1/2)	5′-GGAGGATGAGATCCTGGTCA-3′	5′-TTAGCCGCACTTCCAGTTCT-3′	
SMemb	5′-ATGGAGATAGACCTGAAGG-3′	5′-CCATCTGCTCTTTATACT G-3′	37
Calponin-1	5-AGAAGTATGACCACCAGC-3′	5-CAGCCCAATGATGTTCCG-3′	36
SM22	5-GCAGTCCAAAATCGAGAAGA-3	5-CTGTTGCTGCCCATCTGAAG-3′	36
ASMA	5′-TTCAATGTCCCAGCCATGTA-3′	5′-GAAGGAATAGCCACGCTCAG-3	
Smoothelin	5′-GCTGAGGAGCTGATGACTAT-3′	5′-CTCCTTCTCCAGCTTCTCAA-3′	35
SRF	5′-CTACCAGGTGTCGGAGTCTGA-3′	5′-CCAGATGATGCTGTCAGGAACA-3′	
Myocardin	5′-GGACTGCTCTGGCAACCCAGTGC-3′	5′-CATCTGCTGACTCCGGGTCATTTGC-3′	30
Elk-1	5′-CCACCTTCACCATCCAGTCT-3′	5′-TCTTCCGATTTCAGGTTTGG-3′	
GAPDH	5′-CCACCCATGGCAAATTCCATGGCA-3′	5′-TCTAGACGGCAGGTCAGGTCCACC-3′	Stratagene (La Jolla, CA)

SMMHC = smooth muscle myosin heavy chain, SMemb = smooth muscle myosin heavy chain embryonic form/ nonmuscle myosin heavy chain B, ASMA = alpha smooth muscle actin, SRF = serum response factor, GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

amplicons from genomic amplicons. The primer set for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Strategene, La Jolla, CA) was used as an endogenous invariant control (housekeeping gene).

RT-PCR was performed using an Eppendorf Mastercycler personal (Eppendorf North America, Westbury, NY). The RT-PCR reaction was performed according to the manufacturer's instruction under the following cycling conditions: 45°C for 30 minutes for RT and then, 25 or 40 cycles of three-temperature PCR at 94°C for 1 minute for denaturing, 48°C or 58°C for 1 minute for annealing, and 68°C for 1 minute for extension. Upon completion of the cycles, a final extension was achieved at 68°C for 5 minutes. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

Immunoblotting

After the indicated treatments, cells were lysed with sodium dodecyl sulfate (SDS)-sample buffer containing 0.1 M Tris-HCl, 4 % SDS, 0.2% Bromophenol Blue, and 5% β-mercapto-ethanol. Cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Blots were probed by primary antibodies before visualizing with HRP-conjugated secondary antibodies followed by development with an ECL kit (Amersham Biosciences, Piscataway, NJ) or SuperSignal West Femto (Pierce Biotechnology, Rockford, IL).

Immunofluorescent staining

Cells cultured on 8-well Lab-Tek™ II Chamber Slide™ (Nalge Nunc International, Rochester, NY) were fixed with 4% paraformaldehyde in PBS for 30 min followed by membrane permeabilization with 0.2 % Triton X in PBS for 10 min. After blocking with Power Block Universal Blocking Reagent (BioGene X, San Ramon, CA), cells were incubated with primary antibody solution for 60 min at room temperature. After washing unbound primary antibody, cells were incubated with Alexa Fluor 488 secondary antibody (Invitrogen, Carlsbad, CA) and Hoechst 33342 (Lonza, Basel, Switzerland) to visualize target molecules and nuclei. After washing out unbound secondary antibody and Hoechst dye, coverslips were mounted on slides and cells were examined with Olympus BX40 microscope (Olympus America, Melville, NY) and digital images were captured with CCD digital camera (Diagnostic Instruments, Sterling Heights, MI).

Scrape loading/dye transfer assay

The presence of functional gap junctions in Elk-1-repressed BMMSC was evaluated by scrape loading/dye transfer assay (38). In brief, the cultured cell monolayer will be scraped with a scalpel blade and incubated for 1 min with 1 mg/mL Lucifer yellow or rhodamine-dextran (Invitrogen, Carlsbad, CA). Then, the subsequent distribution of these dyes uptaken by scrape-wounded cells was observed under fluorescent microscope. Lucifer yellow diffuses intercellularly through functional gap junctions and spreads into adjacent unwounded cells due to its low molecular weight (522 Da), whereas rhodamine-dextran (10 KDa) is too large to diffuse intercellularly through gap junctions and therefore stay only within the wounded cells. After rinsing and fixation, the intercellular dye transfer was observed under fluorescent microscope.

Gel contraction

Ligand-induced cell contractility was assessed by gel contraction (39). In brief, 8 volumes of type I collagen solution (3 mg/mL)(Inamed Biomaterials, Fremont, CA) was mixed with 1 volume of 10× MEMα and 1 volume of 0.1 N NaOH on ice to yield 2 mg/mL of collagen solution with pH 7.4 in final. Then, cell suspension was made in the collagen solution on ice (5×10^5 cells/mL), subsequently incubated at 37°C for 2 h for gelling, and added serum-starved medium over the gel. After allowing the cells to spread within the gel overnight, the gels were gently detached and lifted off the bottom of the well, and 50 nM of endothelin (ET) or 2.5 mM of carbachol (Cbchl) were administered. The contraction of

the gel was assessed by measuring the area of the gel in 24 h after addition of the indicated agents.

Elk-1 knockdown with siRNA

The Elk-1 siRNAs (Silencer® Validated; sense: 5′-GGU GAGCGGCCAGAAGUUCTT-3′, antisense: 5′-GAACUUCU GGCCGCUCACCTT-3′) and scrambled siRNA (sense: 5′-GGGCGUCGGUAGCAAUAGCTT-3′, antisense: 5′-GCUA UUGCUACCGACGCCCTT-3′) were purchased from Ambion (Austin, TX). Cells plated on 6-well plate at 70% confluence were transfected with siRNA using siPORT™ Amine (Ambion, Austin, TX) according to manufacture's instructions. Anti-Elk-1 or control siRNAs were added at the final concentration of 100 nM twice in 48 h apart, and the cell lysate was harvested after 72 h for immunoblot.

Statistical analysis

Data were analyzed using paired *t*-tests. Significance was set at $P < 0.05$ or more stringent as noted in the text and figure legends.

Results

Undifferentiated BMMSCs present transcripts for most SMC markers except SMMHC

A key question in attempting to direct stem cell differentiation is the level of cell type transcripts prior to lineage training. Interestingly, the primary BMMSCs cultured in the presence of 15% of FBS expressed CArG-dependent SMC markers (ASMA, SM22, Calponin) as well as CArG-independent SMC marker (Smoothelin), in addition to SMMHC embryonic form/Nonmuscle myosin heavy chain-B (SMemb) as detected using RT-PCR (Fig. 1A). However, expression of SMMHC, the most definite marker gene of differentiated SMC, was not detected even at the mRNA levels by RT-PCR. The findings are in accord with prior report of BMMSCs simultaneously expressing differentiation markers characteristic of various mesenchymal cell lineages including SMC (14, 40). These RT-PCR findings of SMC marker expression were validated with the transcripts of adult human uterine SMCs (Fig. 1B), which express these SMC markers including SMMHC, but not SMemb. Based on these findings, we focused our efforts on induction of SMMHC expression while maintaining of up-regulation levels of the other SMC markers.

A major issue in stem cell training is the possible inclusion in these isolates of multiple cell populations that are already differentiated along a specific lineage. To address, we also employed the clonal hTERT-BMMSC line, which can be differentiated into at least three lineages (13, 34). These clonal hTERT-BMMSCs expressed the same SMC markers as the primary human BMMSCs (Suppl Fig. 1A). Again, SMMHC was not detectable.

Inhibition of the MEK/ERK signaling axis induces SMC marker genes expression in BMMSCs

Undifferentiated BMMSCs express most of SMC marker genes including SMemb, relatively specific to de-differentiated/synthetic SMCs (25), but not SMMHC. Since the canonical ERK/MAPK pathway exerts anti-myogenic effects (18–23, 25), we asked whether the MEK inhibitor PD98059 would up-regulate the expression of SMC marker genes in BMMSCs. PD98059 up-regulated SMMHC transcription in a dose-dependent manner in both primary BMMSCs and immortalized BMMSCs (Fig. 2A and Suppl Fig 1B). This was specific to the ERK/MAPK pathway as the p38 MAPK inhibitor SB203580 failed to induce SMMHC expression (Fig. 2A).

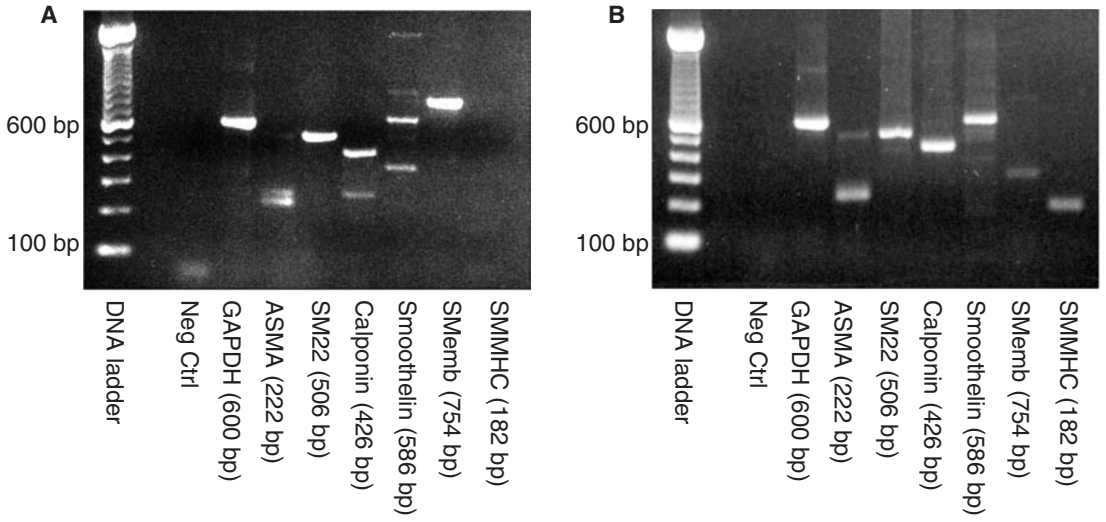

FIG. 1. Baseline mRNA expression of SMC marker genes in undifferentiated primary BMMSCs (**A**) and human uterine SMCs (**B**) evaluated by RT-PCR. The predicted size of amplified products was given in the parentheses. Negative control (Neg Ctrl) did not contain any specific primer pairs during RT-PCR reaction. GAPDH was a loading control. Transcripts from human uterine SMCs were included as positive controls of SMC marker gene transcripts. Note there was no amplified product corresponding to SMemb (754 bp) in (**B**). Shown are representative gels of 2 replicates.

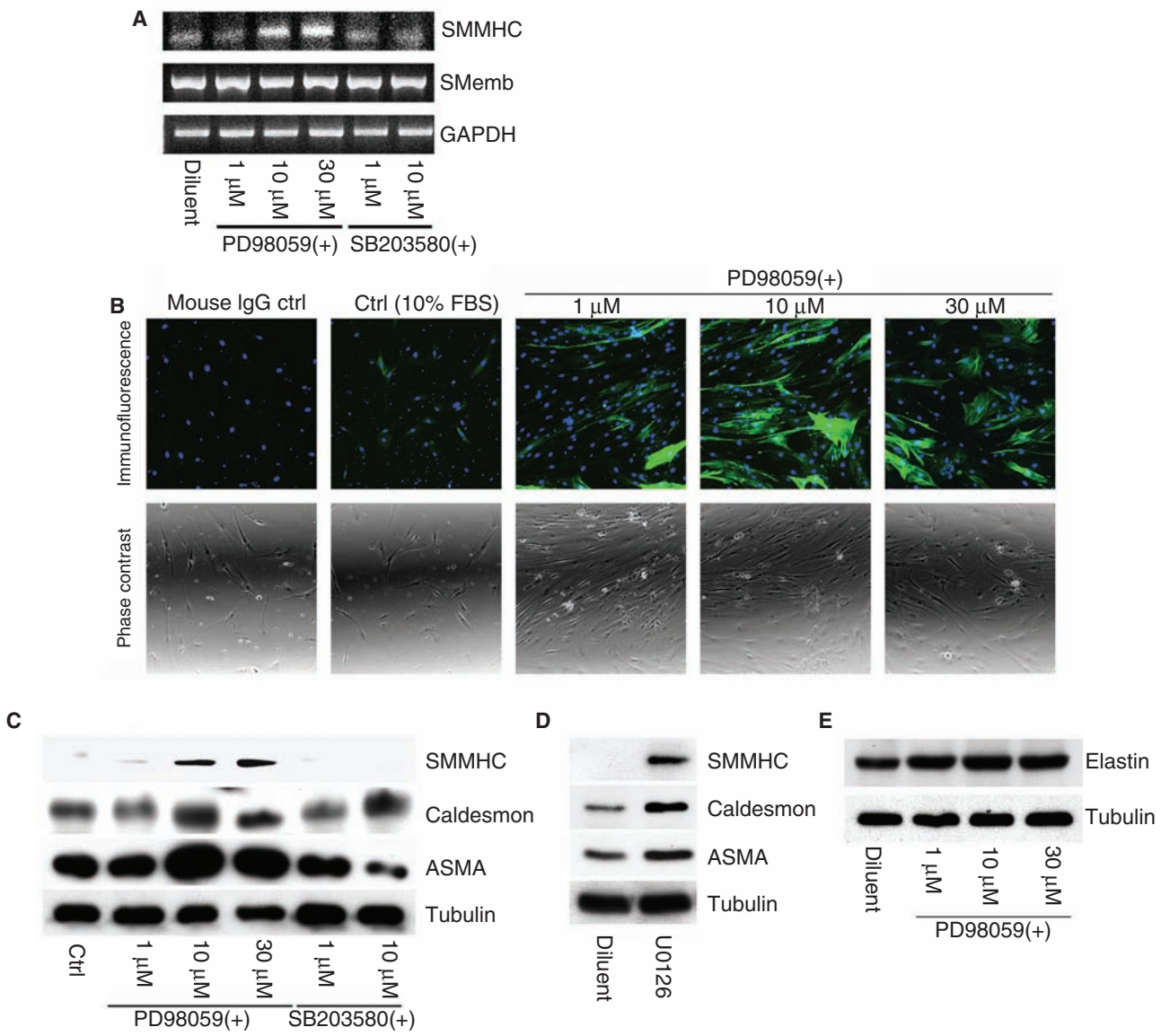

FIG. 2. Effects of MEK inhibitors PD98059 and U0126 and p38 MAPK inhibitor SB203580 on SMC marker gene expression. (**A**) RT-PCR was utilized to evaluate the effects of PD98059 treatment on mRNA expression of SMMHC and SMemb in primary BMMSCs. The effect of SB203580 was also evaluated in primary BMMSCs. GAPDH was used as a loading control. (**B**) ASMA expression was evaluated by immunofluorescent staining of primary BMMSCs in green. Nuclei were counterstained by Hoechst 33342 in blue. Lower rows show the corresponding phase-contrast images. Each photograph is 900 micrometer square. (**C**) Effects of PD98059 and SB203580 on the expression of ASMA, SMMHC and *h*-caldesmon in primary BMMSCs were evaluated by immunoblot. (**D**) Effects of U0126 (10 μM) on the expression of ASMA, SMMHC, and *h*-caldesmon in primary BMMSCs by immunoblot. (**E**) Effects of PD98059 on elastin expression in primary BMMSCs were evaluated by immunoblot. Tubulin blots were used as loading controls (**C, D,** and **E**). Shown are representative gels, images, and blots of at least 2 replicates per assessment.

Curiously, SMemb expression was not altered by these treatments (Fig. 2A and Suppl Fig. 1B) suggesting that these cells were not fully differentiated. The effects on differentiation markers were translated to the protein level (Fig. 2B – 2D), as SMMHC, ASMA and *h*-caldesmon were up-regulated. These effects were also reproduced with U0126, another MEK inhibitor (Fig. 2D). Low serum (3% FBS) condition alone weakly induced SMC marker gene expressions including SMMHC, presumably due to weak stimulation of ERK/MAPK pathway by growth factors and cytokines in FBS. Specificity for ERK/MAPK was shown again by SB203580 reducing ASMA and not altering *h*-caldesmon and SMMHC expression by immunoblot (Fig. 2C). This increase in protein expression was widespread as immunofluorescent staining showed that roughly 50% of BMMSCs strongly expressed ASMA after PD98059 (10 μM) treatment for 5 days; in contrast only 15% of the undifferentiated BMMSCs expressed ASMA and that only at modest levels (Fig. 2B). The up-regulation of ASMA and *h*-caldesmon

by PD98059 in hTERT-BMMSCs was less than that noted in the primary BMMSCs (data not shown).

One further marker of SMC-containing vessels is elastin, which plays a pivotal role with providing mechanical compliance and avoiding aneurysm formation (41). Elastin protein was expressed in the BMMSCs, independently of PD98059 treatment (Fig. 2E). Expression of elastin provides additional impetus for employing BMMSC as a source of vascular SMCs for production of tissue engineered blood vessels.

PDGF-BB inhibits SMC marker gene expression in BMMSCs

Several pro-myogenic reagents have been reported to drive the differentiation of progenitor cells towards the SMC lineage (25). Among them, the role of PDGF-BB is quite controversial; although PDGF-BB promoted SMC differentiation of embryonic stem cells (42) and bone marrow derived mononuclear SMC progenitor cells (43), PDGF-BB also has been identified as a strong negative regulator of SMC differentiation (25). Therefore, we evaluated whether PDGF-BB induce SMC marker gene expression alone or in combination with PD98059. PDGF-BB did reduce ASMA and *h*-caldesmon protein levels (Fig. 3A) and SMMHC transcript induced by PD98059 (Fig. 3C). It did not enhance SMMHC transcript alone (Fig. 3B). PDGF-BB activates ERK/MAPK very strongly (13), which should be the mechanism of PDGF-BB-induced inhibition of SMC marker gene expression.

Functional gap junctions are well maintained in BMMSCs treated with MEK inhibitor

Coordinated responses among vascular wall cells are critical to the local modulation of vasomotor tone and to the maintenance of circulatory homeostasis. Vascular SMC are the final effectors of vascular tone, and syncytial activation of vascular SMC is a key phenomenon for these modulations (44). Intercellular communication through gap junctions utilizing connexin 40 and 43 provides the mechanistic basis for strong electrical and metabolic coupling, and the lack of connexin 43 expression in vascular SMCs enhanced neointimal formation in response to vascular injury in mice (45). Thus the presence of functional gap junctions is indispensable for the functionality of differentiated vascular SMC (44). BMMSCs expressed connexin 43 in the presence and absence of PD98059 (Fig. 4A). The presence of functional gap junctions among BMMSCs was further confirmed by scrape loading/dye transfer assay (38). BMMSCs remain coupled independently of PD98059 treatment, as testified by the presence of Lucifer yellow dye in the cells located at several cells' distance from the scrape line, whereas Rhodamine dextran dye, gap junction-impermeant due to its large size (MW: 10 kDa), stayed only in the wounded cells at the scrape line (Fig. 4B). The presence of functional gap junctions among BMMSCs is another desirable characteristic for employing BMMSC as a SMC source. While scrape loading/dye transfer is essentially a qualitative assay, it is interesting to note that PD98059 treatment increased the ratio of gap junction connected cells (green) to loaded cells (red) (diluent is 4.83 ± 0.50 compared to PD98059 at 6.04 ± 0.59, $P < 0.05$).

MEK inhibitor-treated BMMSCs contract a collagen gel in response to endothelin

The ultimate goal of cellular therapeutics and tissue engineering is to revive the lost or compromised tissue/organ with transplanted cells or bioengineered tissue to regain the lost function. For vascular smooth muscle cells, this implies the ability to contract or relax in response to stimuli. After treatment with PD98059, BMMSCs contracted a collagen gel in response to endothelin, the most potent vasoconstrictive mediator (32), whereas BMMSCs untreated with PD98059 barely contracted a collagen gel in the presence of endothelin. BMMSCs did not contract a collagen gel in response to acetylcholine analog carbachol, even after PD98059 treatment (Fig. 5A and 5B). To demonstrate lineage specificity, neither HMECs nor human HS68 foreskin fibroblasts demonstrated ligand-induced cell contractility in response to endothelin or carbachol (Fig. 5C and 5D).

BMMSCs express both myogenic and antimyogenic transcription factors

Undifferentiated BMMSCs express most of SMC marker genes, even at low levels (Fig. 1A and Suppl Fig. 1A), and

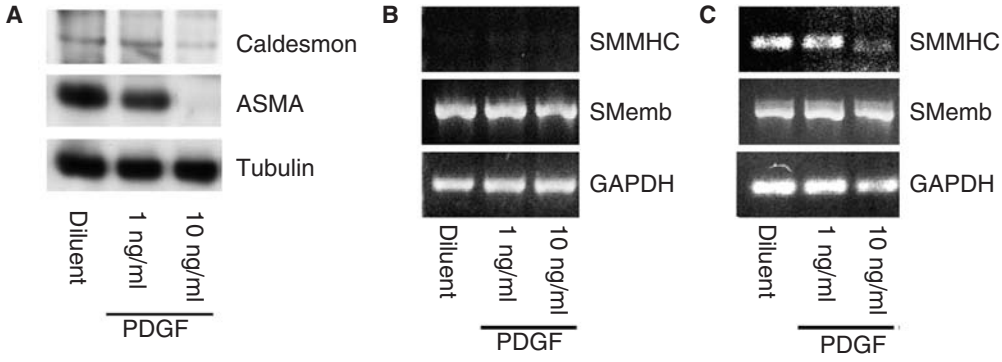

FIG. 3. Effects of PDGF-BB on the expression of ASMA and *h*-caldesmon by immunoblot (**A**) and of SMMHC and SMemb in the absence (**B**) and presence (**C**) of PD98059 by RT-PCR. Tubulin blot (**A**) and RT-PCR of GAPDH (**B, C**) were loading controls. Shown are representative immunoblots and gels of at least 2 replicates per assessment.

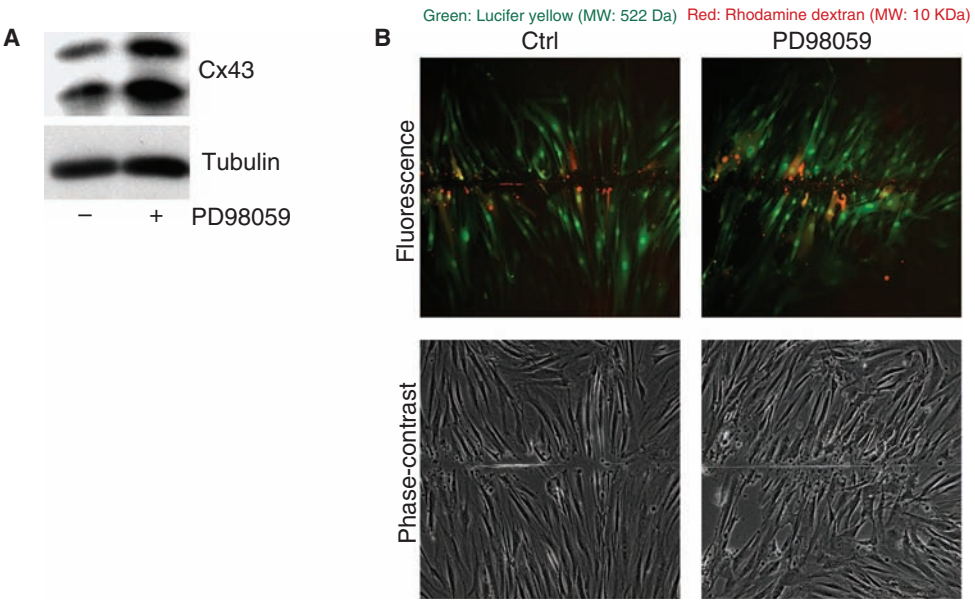

FIG. 4. Presence of functional gap junctions in BMMSCs after 5 days treatment with and without PD98059 (10 μM). (**A**) Expression of connexin 43 by immunoblot. Tubulin blots were loading controls. (**B**) Scrape loading/dye transfer assay for evaluation of functional gap junctions. After scrape wounds were given on the cultured cell monolayer, intercellular diffusion of the uptaken dyes (Lucifer yellow and Rhodamine-dextran) by scrape-wounded cells was evaluated under fluorescent microscope. Lower rows show the corresponding phase-contrast images. Scrape wound was given horizontally in these images. Each photograph is 900 micrometer square. Shown are representative immunoblots and images of at least two replicates per assessment.

the expression of SMC marker genes including SMMHC is inducible by blocking ERK/MAPK pathway (Fig. 2 and Suppl Fig. 1B). These data led us to speculate that BMMSCs might express myogenic and antimyogenic transcription factors, even before induction of differentiation. Myocardin is a very potent myogenic transcriptional cofactor for SRF (27, 29, 46, 47) whereas phosphorylated Elk-1 is a transcription factor which antagonizes myocardin through competing binding of myocardin to SRF (23). As postulated, both primary BMMSCs and hTERT-BMMSCs expressed all three players in this transcriptional regulation; myocardin, Elk-1, and SRF (Fig. 6A and 6B and Suppl Fig. 1C and D).

Elk-1 is activated by direct phosphorylation by ERK/MAPK (31). Therefore, we speculated, and then demonstrated that PD98059 would inhibit the phosphorylation of Elk-1, enabling endogenous myocardin to induce SMC marker gene expression (Fig. 6C). PD98059 blocked nearly completely the phosphorylation of ERK/MAPK and Elk-1 in a low serum environment. However, in the presence of higher levels of serum, PD98059 did not block the Elk-1 phosphorylation, presumably because serum contains various compounds, including PDGF-BB, which strongly activate ERK/MAPK pathway (Fig. 6C).

Elk-1 abrogation induces SM22α expression, but not SMMHC, ASMA, or h-caldesmon in BMMSCs

We initially speculated that inhibition of phospho-Elk1 signaling should be responsible for MEK inhibitor-mediated SMC marker gene up-regulation (Fig. 2 and Suppl Fig. 1B), despite the multitude of downstream targets of ERK/MAPK pathways besides Elk-1. Elk-1 siRNA specifically abrogated Elk-1 and up-regulated SM22α expression (Fig. 7). However, and contrary to our expectations, Elk-1 siRNA treatment decreased *h*-caldesmon expression and did not alter ASMA and SMMHC expression (Fig. 7), whereas MEK inhibitor increased the expression of ASMA, SMMHC, and *h*-caldesmon (Fig. 2). Elk-1 siRNA treatment did not reproduce the effects of MEK inhibitor on SMC marker gene expression, with this suggesting that inhibition of phospho-Elk-1 is not responsible for MEK inhibitor-induced SMC marker gene up-regulation.

MEK inhibitor treatment induces myocardin expression without inhibiting KLF4 expression

To further explore the mechanisms of MEK inhibitor-induced SMC marker gene expression in BMMSCs, we also evaluated the effects of MEK inhibitor on myocardin expression by immunoblot. Myocardin was expressed in BMMSCs, which was further enhanced by PD98059 treatment (Fig. 8A). This effect was not specific to PD98059, as another MEK inhibitor U0126 also enhanced myocardin expression in BMMSCs (Fig. 8B). Myocardin expression was shown to be suppressed by a transcription factor KLF4 in SMC (48). KLF4, in turn, was shown to be induced by ERK-MAPK pathway in colon cancer cell (49). Therefore, we hypothesized that MEK inhibitor would induce myocardin expression through down-regulating KLF4 in BMMSCs; however, it was not observed by PD98059 treatment (Fig. 8A). SRF expression

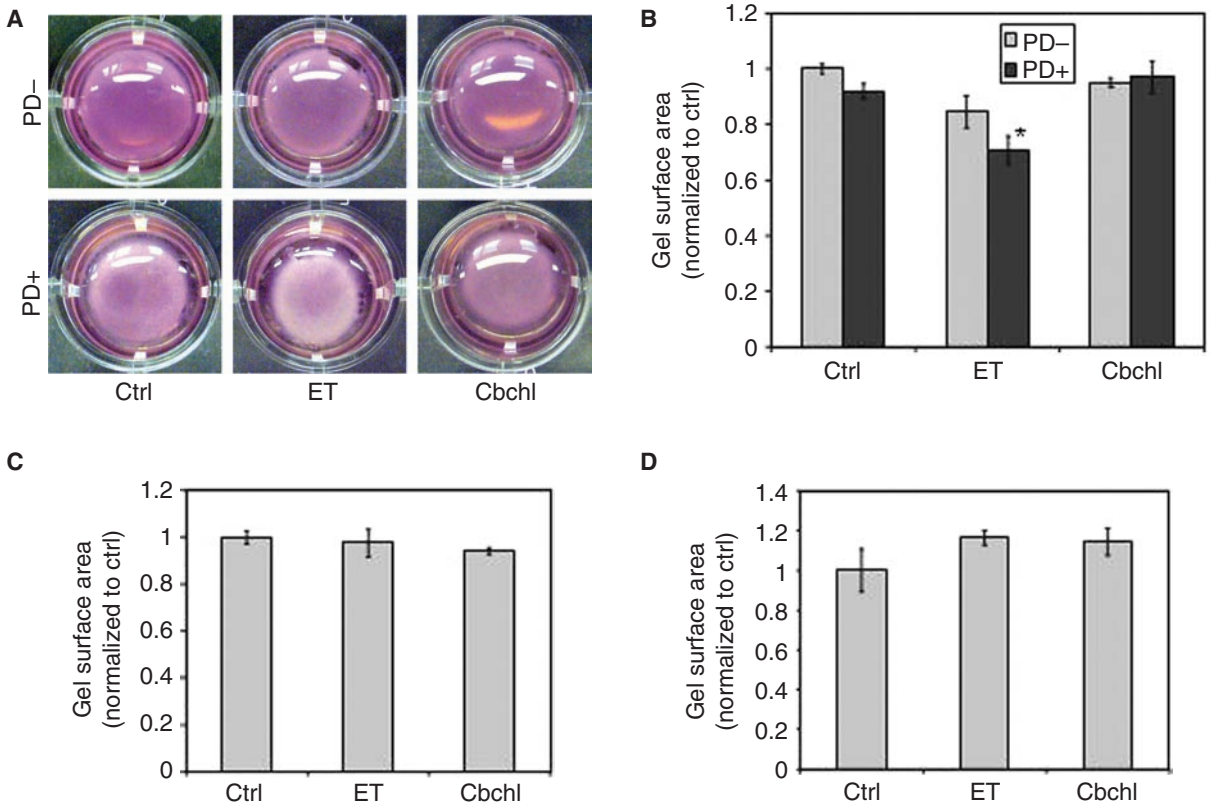

FIG. 5. Ligand-induced gel contraction of primary BMMSCs (**A** and **B**), HMECs (**C**) and HS68 fibroblasts (**D**). The endothelin (ET) or carbachol (Cbchl)-induced gel contraction of BMMSCs was evaluated after treatment with and without PD98059 (10 μM) in low serum condition for 5 days. Representative gel images of 3 experiments in 24-well plate were shown in (**A**). The difference of gel contraction by ET and diluent (control) in PD98059-treated BMMSCs was statistically significant (*$p < 0.05$, $N = 3$ each performed in triplicate).

was also unaltered by PD98059 treatment (Fig. 8C). Hence, it is likely that MEK inhibitor induces myocardin expression independent of KLF4 down-regulation in BMMSCs.

Discussion

In this study, we demonstrated that BMMSCs could be differentiated into smooth muscle like cells in vitro in low serum condition by the treatment with MEK inhibitor PD98059 or U0126. These cells gain ligand-induced cell contractility to endothelin, and this characteristic is regarded as a key attribute of SMC. Elastin expression and the presence of functional gap junctions with connexin 43 expression in BMMSCs are other favorable characteristics of BMMSCs as a source of SMCs. These data suggest that BMMSCs could be potentially used as a source of SMCs for cellular therapeutics and tissue engineering.

The current work relies on prior examination of intracellular signaling pathways that showed that the ERK/MAPK exerts an anti-myogenic effect in SMC differentiation (18–23). Since ERK/MAPK directly phosphorylates and activates Elk-1(31), which, in turn, antagonizes the pro-myogenic cofactor myocardin (23), we initially speculated that pro-myogenic effect of MEK inhibitor would be attributed to the inhibition of Elk-1 activation/signaling in BMMSCs. Indeed, PD98059 treatment inhibited Elk-1 activation/signaling (Fig. 6C); however, this is not the case as the specific knockdown by Elk-1-targeted siRNA did not replicate MEK inhibitor-induced SMC marker gene up-regulation in BMMSCs; SM22α expression was induced, but *h*-caldesmon expression was reduced and SMMHC and ASMA expression was unaltered. In agreement with our finding, Zhou and his colleagues showed that Elk-1 knockdown increased SM22α expression, but did not increase the expression of ASMA in rat A10 aortic SMCs (50).

Rather, MEK inhibitor was found to induce myocardin expression, which should further up-regulate SMC marker gene in BMMSCs (Fig. 8). To our knowledge, this is a first report showing the up-regulation of myocardin expression by MEK inhibitor. Since another transcription factor KLF4, which was shown to be induced by ERK-MAPK pathway in colon cancer cells, suppressed myocardin expression in SMC (48, 49), we hypothesized that MEK inhibitor would induce myocardin expression through down-regulating KLF4 in BMMSCs (21); however, it was not the case also (Fig. 8A). Additionally, the expression of SRF was not altered by MEK inhibitor treatment (Suppl Fig. 2), consistent with a previous report showing that ERK-MAPK pathway activation did not alter the SRF expression in mouse 10T1/2 embryonic multipotent mesenchymal cells (22). The intracellular

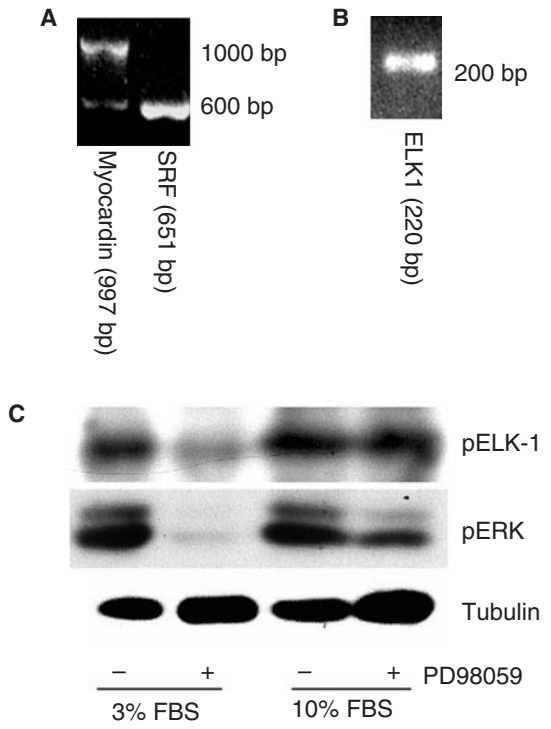

FIG. 6. RT-PCR analysis of (**A**) myocardin and SRF expression, and (**B**) Elk-1 expression in primary BMMSCs. (**C**) Effects of PD98059 and serum on the phosphorylation of ERK/MAPK and Elk-1 by immunoblot. Tubulin blots were loading controls (**C**). Shown are representative gels and immunoblots of at least 3 replicates.

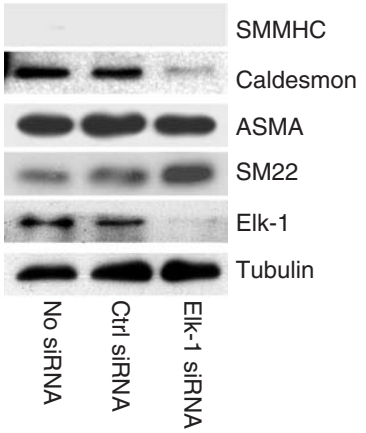

FIG. 7. Effects of anti-Elk-1 siRNA on SMC marker gene expression in BMMSCs. Effects of Elk-1 knockdown by siRNA on the expressions of ASMA, SMMHC, SM22α, and *h*-caldesmon were evaluated in primary BMMSCs by immunoblot. Tubulin blots were loading controls. Shown are representative gels and immunoblots of at least 3 replicates.

signaling pathway linking between ERK-MAPK pathway and myocardin is still uncertain. There are other translational factors and signaling pathways involved in the regulation of SMC differentiation (21, 24, 25), and this incomplete recapitulation suggests other transcriptional regulation is at work in these cells, which is the subject of further studies that lie beyond the scope of the present communication.

SMCs in the vascular walls respond to exogenous stimuli within seconds to adjust the vascular tone in a timely manner. It would be ideal to measure the cell contraction in a matter of seconds; however, a special instrument would be necessary for that experiment (51). Due to the inaccessibility to such instruments, we utilized a simple gel contraction assay, which just needs longer time (in a matter of hours) for gel contraction. An important finding is that MEK inhibitor-treated BMMSCs gained the gel-contractile response to endothelin, the most vasoconstrictive reagent, whereas PD98059-untreated BMMSCs or other types of cells (HMECs or HS68 fibroblasts) failed to respond. Although not definitive, that is suggestive of BMMSCs acquiring contractile phenotype by MEK inhibitor treatment.

We have not confirmed the expression of endothelin receptors in MEK inhibitor-treated BMMSCs, but the contractile response of these cells to endothelin (Fig 5A and B) suggested that these cells express endothelin receptors (ETA and/or ETB2)(52). As we did not observe carbachol-induced contractile response in MEK inhibitor-treated BMMSCs, we speculate that the levels in M_2 and M_3 muscarinic receptor expression might be low, although the possibility of desensitization of muscarinic receptors by 24h carbachol exposure cannot be ruled out (53). MEK inhibitor-untreated BMMSCs failed to show the contractile response to endothelin, but it is unclear whether the lack of contractile response is due to lack of corresponding receptor expression and/or insufficient contractile protein expression such as ASMA in untreated BMMSCs. The possible up-regulation of the expression of these receptors by MEK inhibitor remains to be examined. Evaluation of these receptor expression profile and cell contraction assay in a matter of seconds (51) should address these issues, but these are beyond the scope of the current study. Thorough receptor expression profile of vasoactive agents along with comprehensive physiological and pharmacological studies of these cells should be conducted to minimize the unwanted contractile or relaxing response of these cells in vivo.

Although these MEK inhibitor-treated BMMSCs express the differentiation-specific SMMHC protein, these cells also express SMemb, which tends to be down-regulated in fully differentiated SMCs. We noted that though MEK-inhibitor-treated BMMSCs expressed SMMHC, the protein expression level was less than the one in fully-differentiated human uterine SMCs (data not shown). Thus, MEK inhibitor-treated BMMSCs do not reach full differentiated state of SMC and these cells might still need further differentiation in vitro before utilized as a source of SMCs for the production of TEBV. Nevertheless, the SMC marker expression including SMMHC, elastin expression, the presence of functional gap junctions with connexin 43 expression, and gel contractility in response to endothelin indicate that MEK inhibitor-treated BMMSCs are at least primed to differentiate towards SMCs, which should allow for further exploration of the potential use of BMMSCs as a source of SMCs for production of TEBVs and other SMC-rich organs.

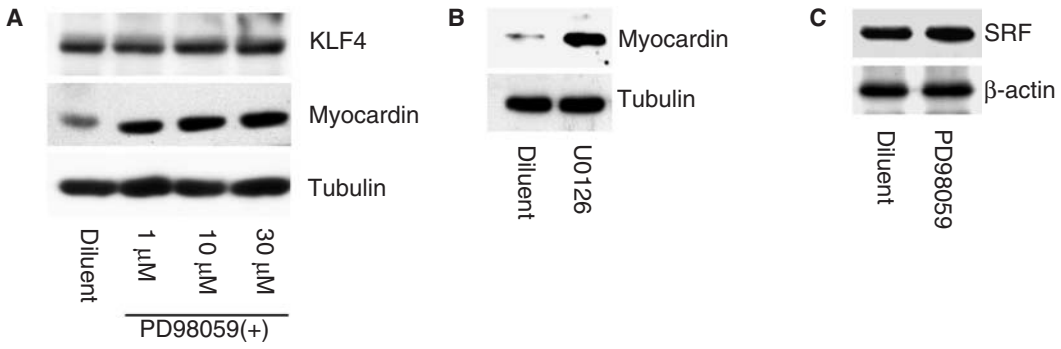

FIG. 8. Effects of MEK inhibitor PD98059 (**A**) or U0126 (**B**) on the expression of myocardin and KLF4. Tubulin blots were loading controls. Shown are representative gels and immunoblots of at least 3 replicates. (**C**) Effects of MEK inhibitor PD98059 on the expression of SRF. β-actin blots were loading controls. Shown are representative gels and immunoblots of at least 2 replicates.

We conducted the current study based on the previous findings that ERK-MAPK pathway exerts an anti-myogenic effect in SMC differentiation (18–23); however, there were other contradictory reports suggesting that ERK-MAPK pathway might exert a pro-myogenic effect or at least not prohibit SMC differentiation. For example, contrary to our findings (Fig. 3), PDGF-BB was also reported to promote the differentiation into SMC lineage in certain progenitor cells, even though it strongly activates ERK-MAPK pathway (42, 43). In another report, PDGF-BB did not inhibit TGF-β-induced differentiation of multipotent adult progenitor cells (MAPC) into contractile cells with positive SMMHC expression, phenotypically similar to neonatal SMCs in vitro (54). Moreover, Jeon et al. showed that an MEK inhibitor blocked TGF-β-induced differentiation of adipose tissue-derived mesenchymal stem cells (ADMSCs) into SMC lineage, suggesting that ERK-MAPK pathway exerts pro-myogenic effect in ADMSCs, though these groups did not demonstrate SMMHC expression and the functionality of these cells (55). In another report, an MEK inhibitor did not alter TGF-β-induced differentiation of mouse 10T1/2 mesenchymal cells into SMC lineage (56). The apparent reason why these cells respond differently to PDGF-BB or activation of ERK-MAPK pathway in SMC marker gene expression is unclear; but it might attribute to phenotypical differences between BMMSCs and these cells. For instance, MAPCs were reported to express embryonic stem cell-specific transcription factor Oct 3/4 with high telomerase activity (57) and are thought to be phenotypically similar to embryonic stem cells (58–61). These phenotypical differences in BMMSCs and MAPCs might be a reason why MAPCs respond differently to TGF-β and PDGF-BB in SMC marker gene expression.

Previous reports showed that TGF-β increased SRF expression, which in turn led to SMC differentiation of 10T1/2 mesenchymal cells or ADMSCs (22, 55, 62). Since MEK inhibitor treatment increased myocardin expression (Fig. 8) without increasing SRF expression (Suppl Fig. 2) in this study, it might be interesting to evaluate whether the combination of MEK inhibitor and TGF-β synergistically promote BMMSC differentiation to SMC lineage. This lies beyond the scope of the current study due to the possible pluriopotential effects of TGF-β. While TGF-β was shown to up-regulate ASMA in BMMSCs (63, 64), TGF-β is also known to induce BMMSC differentiation into the chondrogenic lineage (13, 65).

SMC generation for tissue engineering has been explored from other stem cell sources. While these studies represent promising starts to making TEBVs a reality, the optimal cell source for the SMC progenitors is yet to be determined. As such, it is valuable to continue investigating the SMC program in multiple stem cells. Herein, we find that BMMSCs treated with a small molecular inhibitor gained not only molecular but also functional attributes of SMC. This finding could potentially be used for cell therapeutics and tissue engineering of hollow visceral organs such as blood vessels.

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