Down-Regulation of miR-96 by Bone Morphogenetic Protein Signaling is Critical for Vascular Smooth Muscle Cell Phenotype Modulation

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ABSTRACT

The bone morphogenetic protein (BMP) signaling pathway is critical for the induction and maintenance of contractile phenotype in vascular smooth muscle cells (VSMCs). Inactivation of BMP signaling is common in abnormalities in vascular development and in vascular proliferative conditions, such as pulmonary artery hypertension. Herein, we identify microRNA-96 (miR-96) as a modulator of the VSMC phenotype in response to BMP4 signaling. We show that miR-96 is down-regulated by BMP4 treatment, which results in the derepression of a novel target, Tribbles-like protein 3 (Trb3). miR-96 targets a partially complementary sequence localized in the 3′ UTR of *Trb3*. Trb3 is an essential positive regulator of the BMP signaling pathway and promotes contractile phenotype in VSMCs. In conclusion, our study demonstrates a novel mechanism of regulation of SMC-specific gene expression and induction of a VSMC contractile phenotype by the BMP4 signaling pathway via suppression of the miR-96-Trb3 axis. J. Cell. Biochem. 115: 889–895, 2014.   © 2013 Wiley Periodicals, Inc.

KEY WORDS: BONE MORPHOGENETIC PROTEIN; VASCULAR SMOOTH MUSCLE CELLS; microRNA-96; TRIBBLES-LIKE PROTEIN 3

The transforming growth factor β (TGFβ) family of growth factors plays an essential role during vascular development and maintenance of homeostasis [Monzen et al., 2002; Owens et al., 2004]. TGFβ and BMP have been shown to modulate the phenotypes of VSMCs. VSMCs retain the ability to switch between differentiated "contractile" and dedifferentiated "synthetic" phenotypes in response to environmental signals [Lagna et al., 2007]. TGFβ and BMP inhibit VSMC proliferation and migration and increase contractile VSMC gene expression, which in turn promote the contractile phenotype and inhibit switching to the synthetic phenotype. Aberrant signaling pathways of TGFβ and BMP have been linked to vascular disorders, such as pulmonary artery hypertension (PAH) and hereditary hemorrhagic telangiectasia [ten Dijke and Arthur, 2007].

In VSMCs, TGFβ and BMP signaling is antagonized when phenotype switching is induced by platelet-derived growth factor (PDGF) [Chan et al., 2010]. PDGF signaling decreases Trb3 expression and consequently leads to the down-regulation of Smad signal transducers. Trb3 is a BMPRII-interacting protein and is released from BMPRII upon BMP stimulation, interacts with Smurf1, and leads to the degradation of Smurf1 through the ubiquitin-proteasome pathway [Chan et al., 2007]. The decrease in Smurf1 stabilizes molecules of the BMP pathway, such as Smad proteins, and potentiates the BMP response. The down-regulation of Trb3 by PDGF leads to the reduced expression of Smad proteins and decreases in BMP and TGFβ signaling, thereby promoting the synthetic phenotype in VSMCs.

MicroRNAs (miRNAs) have been proposed as important mediators for the modulation of VSMC phenotype by repressing the targets implicated in regulation of VSMC phenotype [Hata, 2013]. Multiple miRNAs have been found to be responsible for VSMC differentiation and proliferation under physiological or pathological conditions [Kato and Du, 2007]. Moreover, the expression of various miRNAs is modulated in response to extracellular signals, such as TGFβ, BMP,

PDGF, retinoic acid, and insulin [Kang and Hata, 2013]. For example, on BMP-induced signaling, Smad proteins translocate to the nucleus and modulate miRNA expression transcriptionally by directly associating with the promoter region of miR-302-367 gene cluster [Kang et al., 2013a]. TGFβ and BMP also regulate the posttranscriptional processing of miRNAs, such as miR-21, through the association of Smads, primary miRNA (pri-miRNA), and a Drosha Microprocessor [Davis et al., 2008; Kang et al., 2013b].

miRNA-96 is an oncogenic miRNA as evidenced by the up-regulation of miR-96 in various types of cancer, such as bladder cancer, lung cancer, and hepatocellular carcinoma [Navon et al., 2009; Han et al., 2011; Ma et al., 2011; Chen et al., 2012]. This observation suggests that miR-96 may play a role in tumorigenesis or cell proliferation. Furthermore, miR-96 represses neural induction from human embryonic stem cells, suggesting that miR-96 may play a role in the maintenance of pluripotency or the self-renewal of embryonic stem cells [Du et al., 2013]. In this study, we demonstrate that BMP signaling down-regulates transcription of miR-96, which in turn leads to the up-regulation of Trb3. We show that Trb3 is a novel target of miR-96. BMP-miR-96-mediated up-regulation of Trb3 in VSMCs results in increased contractile gene expression.

MATERIALS AND METHODS

CELL CULTURE
Human primary pulmonary artery smooth muscle cells (PASMCs) were purchased from Lonza (CC-2581) and were maintained in Sm-GM2 medium (Lonza) containing 5% fetal bovine serum (FBS). Recombinant human BMP4 and PDGF-BB were purchased from R&D Systems. The cells were treated with 3 nM BMP4 or 20 ng/ml PDGF-BB under starvation conditions (0.2% FBS), as described previously [Lagna et al., 2007].

QUANTITATIVE REVERSE TRANSCRIPTASE–PCR (qRT–PCR)
Quantitative analysis of the change in expression levels was calculated by a real-time PCR machine (iQ5; Bio-Rad) as described previously [Chan et al., 2010]. mRNA level was normalized to GAPDH. The real-time PCR primer sequences for pri-miR-96 were 5′-CTGCCTTGAGTGCTCCTA-3′ and 5′-CCTGACACAAGGATGCAG-3′. Primer sets for ASMA, CNN, SM22α, Id3, Smad4, and Trb3 have been published previously [Chan et al., 2010]. For detection of mature miRNA-96, a TaqMan MicroRNA assay kit (Applied Biosystems) was used according to the manufacturer's instructions. Data analysis was performed using a comparative C_T method in Bio-Rad software. miRNA levels were normalized to U6 small nuclear RNA. The average of three experiments, each performed in triplicate, is presented with standard errors.

miRNA MIMIC AND ANTI–miRNA OLIGONUCLEOTIDES
Chemically modified double-stranded RNAs designed to mimic the endogenous mature miR-96 and negative control miRNA were purchased from Ambion. 2′-O-methyl-modified RNA oligonucleotides complementary to miR-96 or green fluorescent protein (GFP; control) sequence were purchased from IDT. The sequences were: anti-miR-96, 5′ AGCAAAAATGTGCTAGTGCCAAA 3′; anti-GFP,

5′-AAGGCAAGCUGACCCUGAAGU-3′. miRNA mimics and anti-miRNA oligonucleotides were transfected using RNAi Max (Invitrogen) according to the manufacturer's protocol at 5 and 40 nM, respectively.

RNA INTERFERENCE
Synthetic siRNA targeting human Smad4 (5′-CCUGAGUAUUGGU-GUUCCAUUGCUU-3′) was purchased from Invitrogen. Non-targeting scrambled siRNA (#1027280, Qiagen) was used as a control. The siRNAs were transfected at 40 nM using RNAi Max (Invitrogen), according to the manufacturer's protocol.

LUCIFERASE REPORTER CONSTRUCTS
The full-length 3′UTR sequence of Trb3 and the predicted MRE sequence were cloned into the pIS0 vector (Addgene) containing the luciferase gene. The full-length 3′UTR sequence of Trb3 was amplified by RT-PCR from mRNAs isolated from PASMCs using 5′-ATG-GAGCTCTAGGACCACCCTACTACACG-3′ and 5′-ATCGGCCGGC-CCCTTTATTAGGCACAGGTAAA-3′. 5′-AAGGGAGGTATCCCTGTG-CCAAA-3′ and 5′-TTTGGCACAGGGATACCTCCCTT-3′ were used for MRE and 5′-AAGGGAGGTATCCCTGTCGGTAA-3′ and 5′-TTACCGA-CAGGGATACCTCCCTT-3′ were used for the MRE mutant (MRE-mut).

LUCIFERASE ASSAY
Cos7 cells were transfected with luciferase reporter constructs using Fugene HD (Roche) and a β-galactosidase (β-gal) expression plasmid as an internal transfection control. Twenty-four hours later, cells were transfected with 5 nM miR-96 mimic or control miRNA mimic using RNAi Max (Invitrogen). Luciferase assays were carried out and luciferase activities were presented after normalization to β-galactosidase activities.

IMMUNOBLOTTING
Cells were lysed in TNE buffer and total cell lysates were separated by SDS-PAGE, transferred to PVDF membranes (Millipore), immunoblotted with antibodies, and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences). Antibodies used for immunoblotting were an anti-GAPDH antibody (2E3-2E10, Abnova) and an anti-human Trb3 antibody (AP8157a, Abgent).

STATISTICAL ANALYSIS
The results presented were an average of at least three experiments that were each performed in triplicate with standard errors. Statistical analyses were performed by an analysis of variance followed by Student's t-test, using Prism 4 software (GraphPAD Software, Inc.). P values of <0.05 were considered significant and are indicated with asterisks.

RESULTS

BMP4 DOWN–REGULATES THE EXPRESSION OF miR–96
A miRNA expression profiling analysis in PASMCs indicated that miR-96 is repressed upon BMP4 stimulation (3 nM) for 24 h (data not shown). qRT-PCR analysis of PASMCs confirmed the miRNA expression profiling result and showed a decrease in miR-96 to 50% of the basal level upon BMP4 stimulation for 24 h (Fig. 1A). To
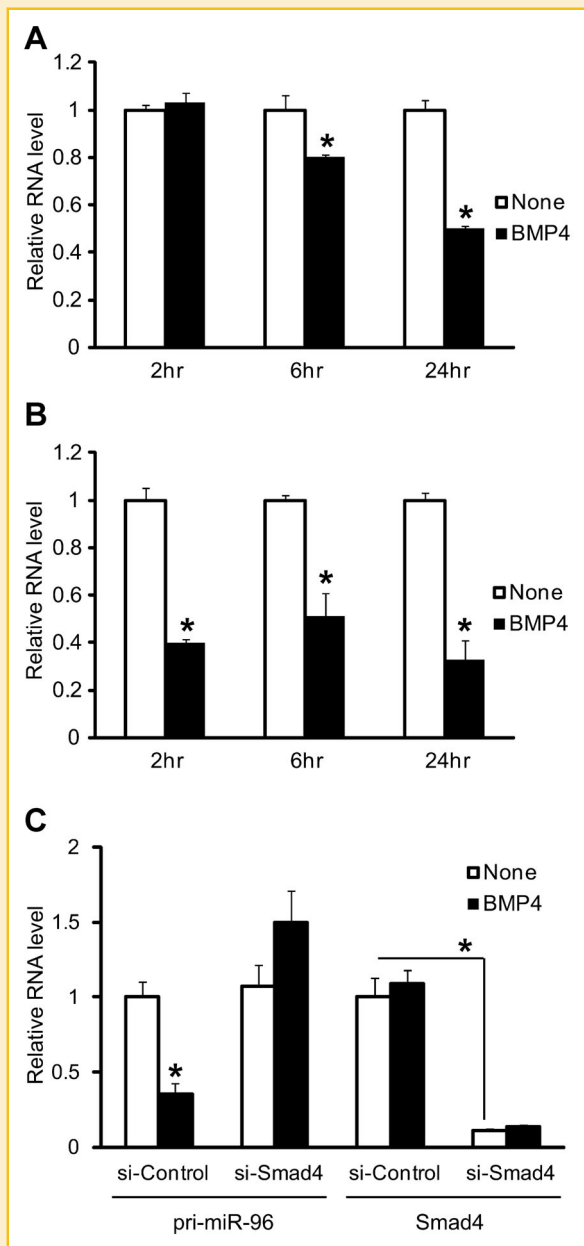
Fig. 1. miR–96 is regulated by BMP4 signaling. A: Expression levels of miR–96 normalized to U6 snRNA were examined by qRT–PCR in PASMCs stimulated with 3 nM BMP4 for a period of time as indicated. Data represent the means ± S.E. of triplicates. *$P < 0.001$. B: Expression levels of pri–miR–96 normalized to GAPDH were examined by qRT–PCR in PASMCs stimulated with 3 nM BMP4 for a period of time as indicated. Data represent the means ± S.E. of triplicates. *$P < 0.01$. C: PASMCs were transfected with non–targeting control siRNA (si–Control) and siRNA against Smad4 (si–Smad4), followed by 3 nM BMP stimulation for 24 h. Levels of pri–miR–96 and Smad4 mRNA relative to GAPDH were measured by qRT–PCR. Data represent the means ± S.E. of triplicates. *$P < 0.001$.

repression of pri-miR-96 by BMP4 was mediated by the signal transducer of the BMP signaling pathway, Smad4 was down-regulated by small interfering RNA (siRNA) and the levels of pri-miR-96 were examined (Fig. 1C). The repression of pri-miR-96 by BMP4 was inhibited when Smad4 was down-regulated, suggesting that down-regulation of miR-96 by BMP4 is dependent on the signal transducer of the BMP signaling pathway, Smad4.

miR–96 REGULATES SMC–SPECIFIC GENE EXPRESSION

We investigated a role for miR-96 in VSMCs. VSMCs were transfected with anti-miR-96 and the levels of SMC-specific genes, such as ASMA, CNN, and SM22α, and a transcription target of BMP signaling, Id3, were examined (Fig. 2A). The expression of SMC-specific genes and Id3 was enhanced in anti-miR-96-transfected cells in comparison with control cells, suggesting that endogenous miR-96 inhibits the induction of the contractile phenotype of VSMC characterized by an increased expression of the SMC-specific genes. Furthermore, the induction of SMC-specific genes upon BMP4 stimulation was significantly blocked in miR-96 mimic-transfected cells in comparison with control cells; however, miR-96 itself does not
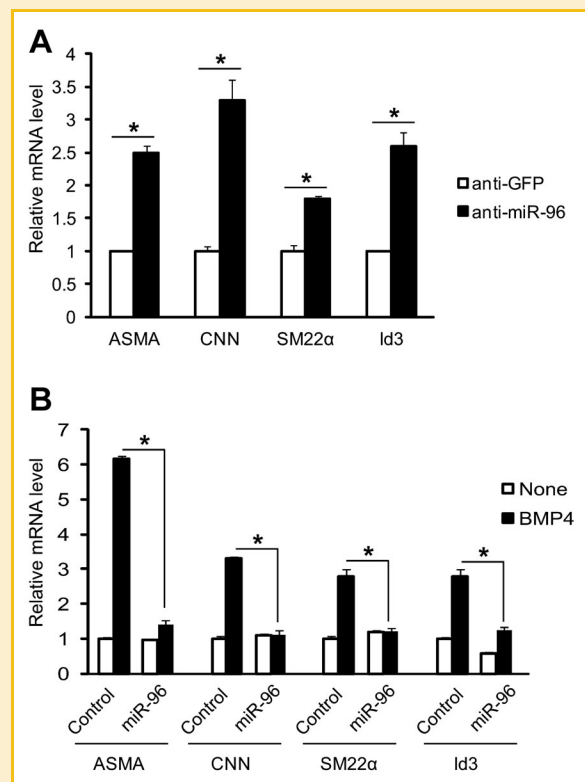
Fig. 2. miR–96 is critical for the BMP4–dependent induction of SMC–specific genes. A: The expression levels of SMC–specific genes (ASMA, CNN, SM22α) and Id3 normalized to GAPDH were examined by qRT–PCR in PASMCs transfected with 50 nM antisense oligonucleotides to GFP (anti–GFP) or miR–96 (anti–miR–96). Data represent the means ± S.E. of triplicates. *$P < 0.01$. B: PASMCs were transfected with negative control mimic or miR–96 mimic (5 nM). Cells were then treated with BMP4 for 24 h and subjected to qRT–PCR analysis of SMC–specific genes and Id3. Data represent the means ± S.E. of triplicates. *$P < 0.001$.

determine whether transcription of miR-96 is affected by BMP4, the pri-miR-96 expression level was examined. A time-course analysis of pri-miR-96 showed a rapid reduction of pri-miR-96 within 2 h after BMP4 stimulation, indicating that the down-regulation of miR-96 may be controlled transcriptionally (Fig. 1B). To examine whether the

seem to be sufficient to induce the expression of contractile genes (Fig. 2B). These results demonstrate that the down-regulation of miR-96 is essential for the induction of SMC-specific genes, leading to the contractile phenotype of VSMCs.

Trb3 IS A NOVEL TARGET OF miR-96

In a search for potential targets of miR-96 using the TargetScan target prediction algorithm, we observed an evolutionarily conserved miRNA recognition element (MRE) partially complementary to miR-96 in the 3′ UTR of *tribbles homolog 3* (*Trb3*) gene (Fig. 3A). Trb3 is a critical molecule for regulatory mechanism of the BMP signaling pathway that functions as a positive regulator. To assess whether endogenous miR-96 regulates the expression of *Trb3*, miR-96 was inhibited by the transfection of antisense oligonucleotides complementary to the miR-96 (anti-miR-96) in PASMCs, and the relative expression level of *Trb3* to *GAPDH* was examined
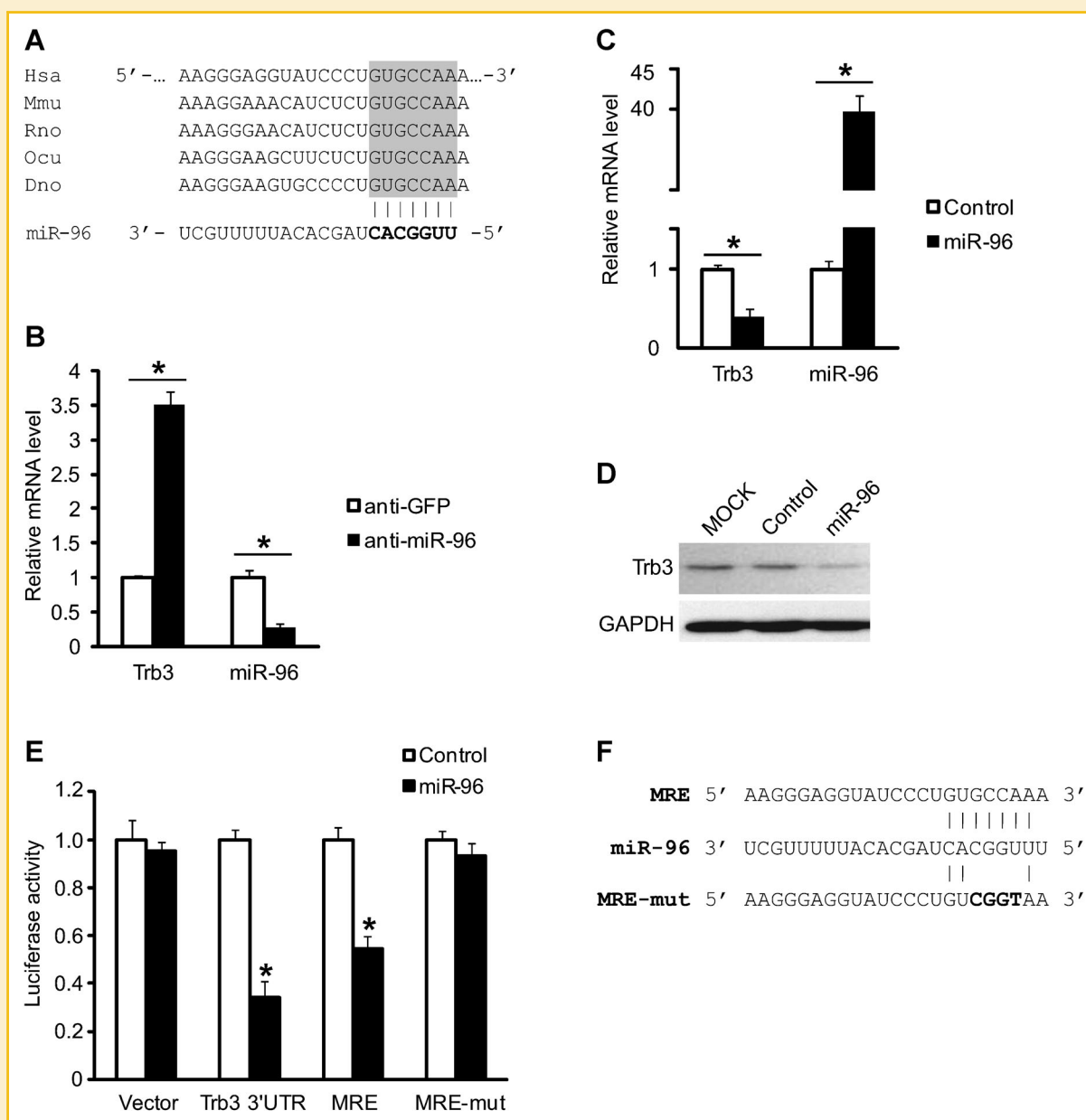

Fig. 3. miR-96 targets Trb3. A: miR-96 MRE sequence found in the 3′ UTR of Trb3 mRNA is evolutionarily conserved. Has: human, Mmu: mouse, Rno: rat, Ocu: rabbit, Dno: armadillo. B: Levels of Trb3 mRNA relative to GAPDH and miR-96 relative to U6 snRNA were quantified by qRT–PCR analysis in PASMCs transfected with 50 nM anti-GFP or anti-miR-96. Data represent the means ± S.E. of triplicates. *P < 0.001. C: Levels of Trb3 mRNA relative to GAPDH and miR-96 relative to U6 snRNA were examined by qRT–PCR analysis in PASMCs transfected with 5 nM negative control mimic or miR-96 mimic. Data represent the means ± S.E. of triplicates. *P < 0.001. D: Total cell lysates of PASMCs transfected with 5 nM control mimic or miR-96 mimic for 48 h were subjected to immunoblot analysis of endogenous Trb3 and GAPDH. E: Luciferase activities of constructs with full-length 3′UTR of Trb3, wild type Trb3 MRE (MRE), or mutated Trb3 MRE (MRE-mut) were examined in Cos7 cells by transfecting control or miR-96 mimic. Data represent the means ± S.E. of triplicates. *P < 0.01. F: Sequence of wild type miR-96 MRE and mutated MRE (MRE-mut) cloned into the 3′UTR of the luciferase gene is shown.

(Fig. 3B). The antisense oligonucleotides complementary to *GFP* (anti-GFP) were used as controls. *Trb3* expression increases approximately 3.5-fold when endogenous miR-96 is inhibited, indicating that endogenous miR-96 represses *Trb3* expression. Conversely, exogenous miR-96 down-regulates endogenous *Trb3* expression levels (Fig. 3C). In VSMCs transfected with 5 nM miR-96 mimic, the relative mRNA level of *Trb3* to *GAPDH* is reduced to approximately 40% compared with the 5 nM control miRNA-transfected VSMCs. Moreover, immunoblot analyses indicated that the endogenous Trb3 protein in PASMCs was down-regulated upon miR-96 over-expression in comparison with control miRNA mimic (Fig. 3D). These results indicate that Trb3 is a novel target of miR-96.

To confirm that miR-96 targets Trb3, we measured the expression of the luciferase reporter construct containing the 3′UTR of *Trb3* at the downstream of the luciferase reporter gene in the presence of miR-96 mimic (Fig. 3E). Consistent with the qRT-PCR results, the luciferase activity of the *Trb3* 3′UTR construct was significantly reduced upon expression of the miR-96 mimic, demonstrating that Trb3 is a target of miR-96. To determine whether miR-96 targets Trb3 through the evolutionarily conserved MRE in the 3′ UTR of *Trb3* gene, luciferase reporter containing MRE was generated. miR-96 reduced the luciferase activity of MRE by approximately 50%. Mutations in MRE, which disrupted base pairing with miR-96 (Fig. 3F), abrogated the inhibition of luciferase activity by miR-96, suggesting that the conserved MRE is a critical target site for recognition of *Trb3* mRNA by miR-96.

miR-96 IS NOT AFFECTED BY PDGF SIGNALING

Trb3 is known to be an essential positive regulator of the BMP-mediated signaling pathway to modulate VSMC phenotype [Chan et al., 2007]. Moreover, it has been shown that Trb3 is down-regulated by miR-24 upon PDGF signaling which promotes the synthetic phenotype of VSMCs [Chan et al., 2010]. We confirmed that the *Trb3* mRNA level is down-regulated to approximately 40% of the basal level of the control after 24 h of PDGF-BB stimulation (Fig. 4A). To test the possibility that PDGF-BB induces miR-96 to down-regulate Trb3, mature miR-96 levels in VSMCs were examined by qRT-PCR following PDGF-BB stimulation. PDGF signaling had no effect on the expression of miR-96, suggesting that miR-96 is modulated only by BMP signaling, not PDGF-BB (Fig. 4B).

DISCUSSION

In this study, we elucidated a novel mechanism by which BMP signaling promotes the contractile phenotype of VSMCs. BMP4-dependent down-regulation of miR-96 elevates the expression of Trb3, which in turn leads to potentiate the BMP signaling pathway (Fig. 4C). We identified Trb3 as a novel target of miR-96 and provided evidence that down-regulation of miR-96 is critical for promoting the contractile VSMC phenotype by inducing the SMC-specific contractile gene expression.

The aberrant regulation of the VSMC phenotype, in particular a switch from the contractile to the synthetic phenotype, is a critical phenomenon underlying the pathogenesis of a variety of vascular proliferative diseases, including PAH. Differential expression of
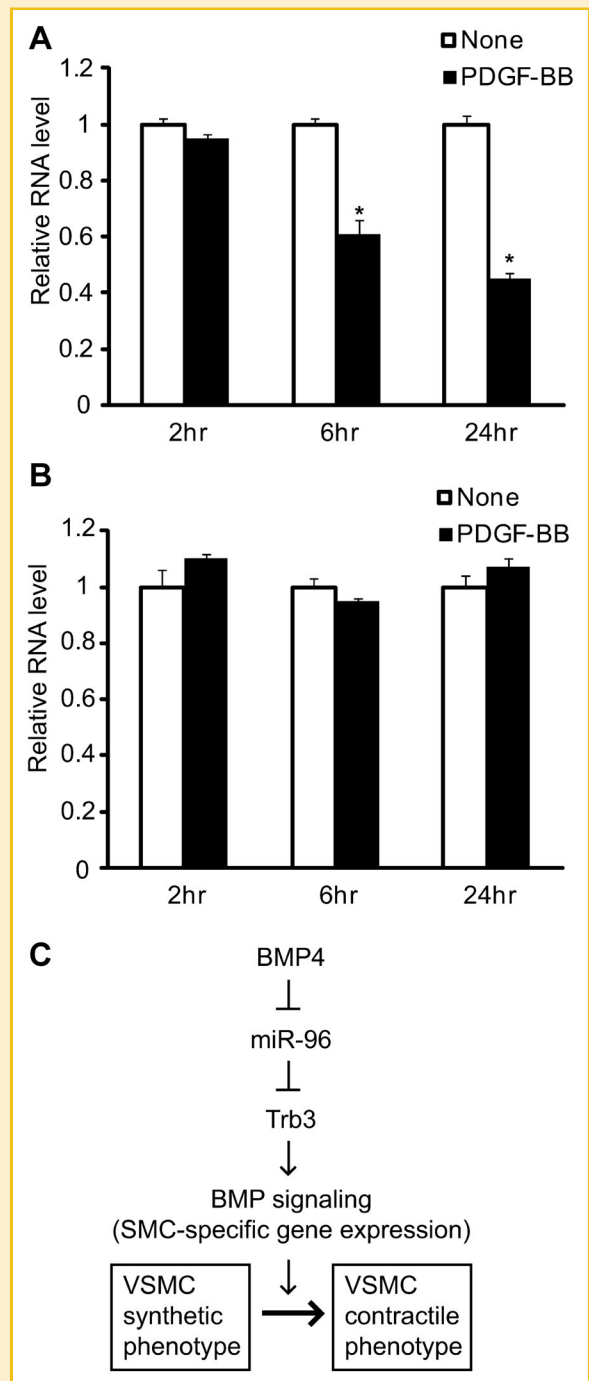

Fig. 4. miR-96 is not affected by PDGF signaling. A: Expression levels of Trb3 mRNA normalized to GAPDH were examined by qRT-PCR in PASMCs stimulated with 20 ng/ml PDGF-BB for time as indicated. Data represent the means ± S.E. of triplicates. *P < 0.01. B: Expression levels of miR-96 normalized to U6 snRNA were examined by qRT-PCR in PASMCs stimulated with 20 ng/ml PDGF-BB for time as indicated. Data represent the means ± S.E. of triplicates. C: Schematic diagram of BMP signaling pathway mediated by down-regulation of miR-96 in VSMCs.

miRNAs responsible for the regulation of the VSMC phenotype has been demonstrated in the pulmonary vasculature of patients with PAH or other vascular proliferative conditions [McDonald et al., 2012]. Therefore, the modulation of the miRNA level in vivo by delivery of anti-miR oligonucleotides or adeno-associated virus is considered a novel therapy. For example, miR-145 and miR-21 are dysregulated in the mouse model of PAH [Caruso et al., 2010, 2012]. Down-regulation of miR-145 prevents the development of PAH, while down-regulation of miR-21 promotes the development of PAH. In addition, miR-424 and miR-503 are down-regulated in PAH and restoring the expression of miR-424 ameliorates PAH in experimental models [Kim et al., 2013].

We demonstrate that miR-96 level is modulated by BMP4 and affects the regulation of the VSMC phenotype. miR-96 inhibits the induction of contractile phenotype by BMP signaling. However, further investigation remains to determine whether miR-96 expression is altered in cardiovascular diseases. Differential expression and regulation of miR-96 have been observed during the differentiation of various cell types. For example, miR-96 is differentially expressed during osteogenesis, adipogenesis, and neurogenesis [Suomi et al., 2008; Laine et al., 2012; Du et al., 2013]. miR-96 expression was increased during osteogenic and adipogenic induction of human bone marrow-derived MSCs [Laine et al., 2012]. However, miR-96 was uniquely down-regulated in neuroectodermal precursor cells and induced neuronal differentiation through regulating its target, PAX6, a key transcription factor of neural induction [Du et al., 2013]. Furthermore, an increased expression of miR-96 has been observed in various tumors such as bladder cancer and colorectal cancer, suggesting that miR-96 may be involved in a relatively common mechanism during tumorigenesis [Xu et al., 2012; Yoshino et al., 2013]. Therefore, miR-96 may be critical for a primary regulatory mechanism governing basic cellular functions, cell differentiation, and tumorigenesis.

We identified Trb3 as a novel target of miR-96. Trb3 has been shown to play a critical role in the regulation of various signaling pathways. For example, Trb3 inhibits Akt activity and impairs insulin signaling in hepatic cells and in C2C12 muscle cells [Du et al., 2003; Kato and Du, 2007]. Trb3 also mediates ER stress-induced insulin resistance in skeletal muscle through regulation of programmed cell death [Ohoka et al., 2005]. In the BMP signaling pathway, Trb3 is known to mediate the degradation of Smurf1, which facilitates the degradation of Smads and RhoA. Smads and RhoA are downstream signal transducers critical for mediating the pro-contractile signal from the BMP signaling pathway in VSMCs [Chan et al., 2007]. Thus, we speculate that the induction of Trb3 by down-regulation of miR-96 leads to pro-contractile signaling via multiple mechanisms through the reduction of Smurf1.

In previous studies, Trb3 has been shown to mediate the BMP-dependent regulation of SMC-specific gene expression [Chan et al., 2007]. The PDGF signaling pathway induces miR-24, which targets Trb3 [Chan et al., 2010]. Repression of Trb3 results in a decrease in BMP signaling, promoting the synthetic phenotype in VSMCs. Therefore, we investigated a potential crosstalk between miR-96 and PDGF signaling by monitoring the effect of PDGF-BB on the level of miR-96. We did not observe significant changes in the expression of miR-96 upon PDGF-BB stimulation, suggesting that miR-96 is not regulated by PDGF at the level of transcription or processing in VSMCs. Therefore, the regulatory mechanism of miR-96 by BMP is specific and miR-96 does not play a role in PDGF-mediated regulation of the synthetic phenotype.

ACKNOWLEDGMENTS

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