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miR-142-3p Is a Regulator of the TGFβ-Mediated Vascular Smooth Muscle Cell Phenotype

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ABSTRACT

The transforming growth factor β (TGFβ) signaling pathway is critical for the promotion and maintenance of the contractile phenotype of vascular smooth muscle cells (VSMCs). Though multiple microRNAs (miRNAs) implicated in the regulation of the VSMC phenotype have been identified, the modulation of miRNAs in the VSMCs by TGFβ signaling has not been fully described. In this study, we identified microRNA-142-3p (miR-142-3p) as a modulator of the VSMC phenotype in response to TGFβ signaling. We show that miR-142-3p is induced upon TGFβ signaling, leading to the repression of a novel target, dedicator of cytokinesis 6 (DOCK6). The downregulation of DOCK6 by miR-142-3p is critical for cell migration. Thus, this study demonstrates that miR-142-3p is a key regulator of the TGFβ-mediated contractile phenotype of VSMCs that acts through inhibiting cell migration through targeting DOCK6. J. Cell. Biochem. 116: 2325–2333, 2015.

KEY WORDS: TGFβ; VASCULAR SMOOTH MUSCLE CELLS; MICRORNA-142-3p; DOCK6

miRNAs are short regulatory non-coding RNAs that control gene expression through the degradation of target mRNAs and/or inhibition of translation, thereby playing critical roles in determining cell fates by regulating subsets of genes during the differentiation of various cell types [Gammell 2007; Bartel 2009; Kim et al., 2009]. miR-142-3p was first identified as being preferentially expressed in the hematopoietic system and has recently been proposed to play regulatory roles in cell differentiation during erythropoiesis, granulocytopoiesis, chondrogenesis and hematopoiesis [Careccia et al., 2009; Kim et al., 2011; Jin et al., 2012; Nishiyama et al., 2012; Nimmo et al., 2013]. For example, miR-142-3p regulates TGFβ-mediated region-dependent chondrogenesis [Kim et al., 2011].

The TGFβ signaling pathway is critical for many cellular processes, including vascular development and homeostasis. TGFβ and bone morphogenetic protein (BMP) promote VSMC differentiation and maintain the contractile phenotype [Lagna et al., 2007; ten Dijke and Arthur, 2007]. In response to TGFβ and BMP, SMC-specific genes such as α-smooth muscle actin (ASMA) and SM22α are induced in the VSMCs, whereas cell migration and proliferation are inhibited. BMP signaling regulates the VSMC phenotype by modulating the levels of miRNA expression [Kang et al., 2011; Kang et al., 2012; Kim et al., 2014]. However, little is known about TGFβ-mediated miRNA modulation during VSMC differentiation and regulation of the VSMC phenotype. We hypothesized that miR-142-3p might regulate TGFβ-mediated VSMC phenotype modulation.

BMP signaling induces miR-21 expression and represses the transcription and translation of DOCK family members, which are guanine nucleotide exchange factors (GEFs) for Rac1[Kang, et al., 2011]. Rac1 is a GTPase that controls cell migration and cytoskeletal reorganization and BMP-induced miR-21 inhibits cell migration by reducing Rac1 activity via the repression of DOCK4, 5 and 7, leading to the contractile phenotype of VSMCs. The DOCK family consists of 11 genes, designated as Dock1 through Dock11, that are structurally divided into four classes: Dock-A, -B, -C and -D [Laurin and Cote, 2014]. Dock-A and -B subfamilies are typically GEFs specific for Rac1, while the Dock-D subfamily is specific for Cdc42. Though the Dock-A, -B and -D subfamilies have been characterized, the function

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of the members of the Dock-C subfamily remains elusive. Recently, Dock6, a member of the Dock-C subfamily, has been shown to exchange GDP for GTP in both Rac1 and Cdc42, inducing actin-based morphological changes during neurite outgrowth [Miyamoto et al., 2007].

In this study, we sought to investigate the role of miR-142-3p in VSMC phenotype modulation. Using pulmonary artery smooth muscle cells (PASMCs), we observed that TGFβ signaling upregulated miR-142-3p expression. We also examined the effects of overexpression or knockdown of miR-142-3p on the VSMC phenotype. Moreover, we identified DOCK6 as a novel target of miR-142-3p and examined the direct effects of the target gene on VSMC migration. Based on these examinations, we demonstrated that miR-142-3p is a critical regulator of the TGFβ-mediated contractile phenotype of VSMCs that acts by targeting DOCK6 to inhibit cell migration.

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 TGFβ and PDGF-BB were purchased from R&D Systems. The cells were treated with 400 pM TGFβ or 20 ng/ml PDGF-BB under starvation conditions (0.2% FBS). For starvation conditions, cells were maintained in Dulbecco's modified egles medium (DMEM) containing 0.2% FBS for 16 h.

QUANTITATIVE REVERSE TRANSCRIPTASE-PCR (QRT-PCR)

Quantitative analysis of the change in expression levels was performed using real-time PCR. The mRNA levels were normalized to GAPDH. The primers used were as follows: DOCK5, 5′-AACTCACAGAGCAGCTGAAG-3′ and 5′-TGACTGAGGTGATGGACAAC-3′; DOCK6, 5′-TCTGCAAGAAATGTGAGGAT-3′ and 5′-AAACTTGCTCTGTTCAAGGA-3′; DOCK8, 5′-GGTAATGGTGGACTAATTGC-3′ and 5′-TATCGGAGAATTGTTTCCAT-3′. A TaqMan MicroRNA assay kit (Applied Biosystems Inc., Foster City, CA) was used according to the manufacturer's instructions to detect mature miR-142-3p. Data analysis was performed using a comparative C_T method in the Bio-Rad software. miRNA levels were normalized to U6 small nuclear RNA. Three experiments were performed in triplicate, and the average results with standard errors are presented.

miRNA MIMICS AND ANTI-miRNA OLIGONUCLEOTIDES

Chemically modified double-stranded RNAs designed to mimic the endogenous mature miR-142-3p and negative control miRNA were purchased from Ambion. 2′-*O*-methyl-modified RNA oligonucleotides complementary to the sequences of miR-142-3p or green fluorescent protein (GFP; control) were purchased from IDT. The sequences were as follows: anti-miR-142-3p, 5′-UCCAUAAAGUAGGAAACACUACA-3′; anti-GFP, 5′-AAGGCAAGCUGACCCUGAAGU-3′. The miRNA mimics and anti-miRNA oligonucleotides were transfected at 5 nM and 50 nM, respectively, using RNAi Max (Invitrogen) according to the manufacturer's protocol.

RNA INTERFERENCE

Synthetic siRNA targeting human DOCK6 (5′-AAGUGUCUGUCGCUCAAGUUC-3′) was purchased from Genolution Pharmaceuticals. Non-targeting scrambled siRNA (#1027280, Qiagen) was used as a control. The siRNAs were transfected at 50 nM using RNAi Max (Invitrogen) according to the manufacturer's protocol.

LUCIFERASE REPORTER CONSTRUCTS

The full-length 3′UTR sequence of DOCK6 was cloned into the pIS0 vector (Addgene) containing the luciferase gene. RT-PCR was used to amplify full-length 3′UTR sequence of DOCK6 from mRNAs isolated from PASMCs using 5′-ACTACGCGTGCCCACAAGGACCAA-3′ and 5′-ACTTCTAGAGCTTATAAAAACCATTTTAAATT-3′.

LUCIFERASE ASSAY

Cos7 cells were transfected with luciferase reporter constructs and a β-galactosidase expression plasmid as an internal transfection control using Fugene HD (Roche). Twenty four hours (hr) later, cells were transfected with 5 nM miR-142-3p 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 (50 mM Tris–HCl (pH 7.4). 100 mM NaCl. 0.1 mM EDTA) and total cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, immunoblotted with antibodies and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences). Antibodies used for immunoblotting were an anti-β-actin antibody (sc47778, Santa Cruz) and an anti-DOCK6 antibody (PD016, MBL Life Science).

IN VITRO SCRATCH WOUND ASSAY

PASMCs were plated in a six-well plate at 2×10^5 cells/well and transfected with the miR-142-3p mimic, anti-miR-142-3p or siRNAs for 24 h. Then, three scratch wounds were generated with a 200 μl disposable pipette tip. Cells were maintained in DMEM containing 0.2% FBS and scratch wounds were photographed over an 8 or 24 h period with a Nikon inverted microscope equipped with an attached digital camera. The widths of the scratch wounds were quantitated using ImageJ software.

COLLAGEN MATRIX CONTRACTION ASSAY

PASMCs were transfected with the miR-142-3p mimic, the control miRNA mimic or anti-miR-142-3p for 24 h. After tripsinization, cells were suspended at 1.5×10^5 cells/ml in DMEM containing 0.2% FBS. Collagen solution (BD biosciences) and 1 M NaOH were added to the cell suspension and 500 μl of the mixture was plated into a 24 well plate. After adding DMEM containing 0.2% FBS or media containing 400 pM TGFβ, the PASMC embedded collagen matrices were dissociated from the mold by a pipet tip. After 24 h, the gel matrixes were photographed.

STATISTICAL ANALYSIS

For each of the assays, three experiments were performed in triplicate, and the results were presented as the average 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

TGFβ INDUCES THE EXPRESSION OF miR-142-3p

It was recently discovered that the differential induction of miR-142-3p is critical during TGFβ-induced chondrogenic differentiation [Kim et al., 2011]. We hypothesized that TGFβ signaling might modulate miR-142-3p expression in VSMCs to regulate VSMC phenotypes. To test this hypothesis, PASMCs were treated with TGFβ, and the expression of miR-142-3p was examined over a time course of 24 h. miR-142-3p expression was induced by three-fold at 24 h, suggesting that TGFβ signaling differentially modulates the expression level of miR-142-3p in VSMCs (Fig. 1A).

miR-142-3p IS ESSENTIAL FOR TGFβ-MEDIATED SMC-SPECIFIC GENE REGULATION

To examine the effect of increased expression of miR-142-3p on the VSMC phenotype, we transfected PASMCs with miR-142-3p mimic or control mimic and examined the mRNA level of SMC-specific genes such as ASMA and SM22α by qRT-PCR. Exogenous miR-142-3p increased the expression of ASMA and SM22α and potentiated TGFβ-mediated induction of SMC-specific genes (Fig. 1B).

To examine the role of endogenous miR-142-3p in the regulation of the SMC-specific gene expression, PASMCs were transfected with 2′-O-methyl-modified RNA oligonucleotides complementary to the miR-142-3p sequence (anti-miR-142-3p) and the levels of ASMA and SM22α were examined by qRT-PCR in the absence or presence of TGFβ. The expression of ASMA was reduced in anti-miR-142-3p transfected cells in comparison with control cells. Moreover, the induction of ASMA and SM22α upon TGFβ was blocked when miR-142-3p function was inhibited by anti-miR-142-3p (Fig. 1C). These results suggest that miR-142-3p is essential for the induction of SMC-specific genes, leading to the contractile phenotype of VSMCs.

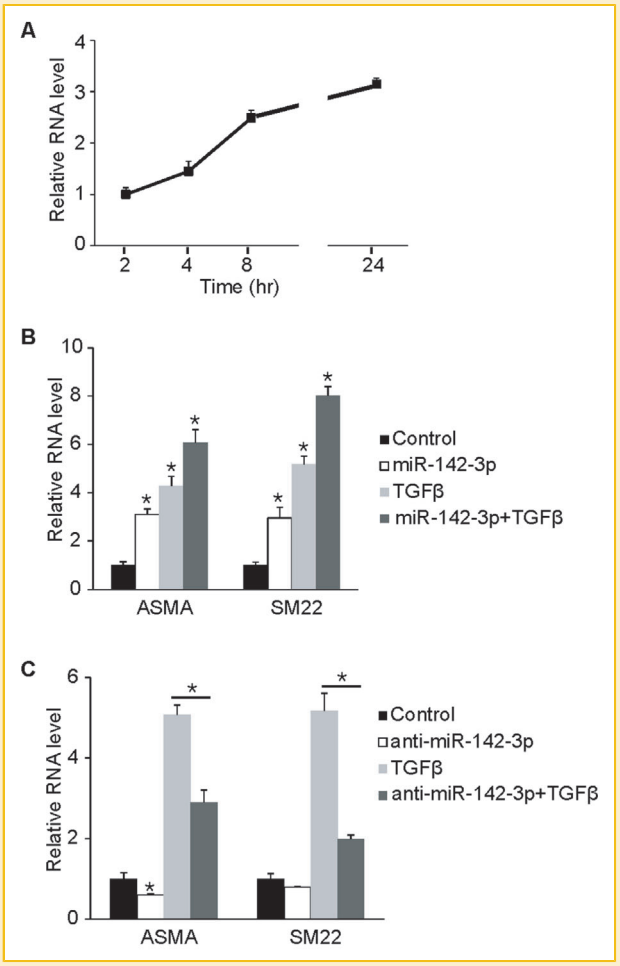

Fig. 1. miR-142-3p is induced by TGFβ signaling. A: Expression levels of miR-142-3p normalized to U6 snRNA were examined by qRT-PCR in PASMCs stimulated with 400 pM TGFβ for the indicated periods of time. B: Levels of ASMA and SM22α relative to GAPDH were measured by qRT-PCR in PASMCs transfected with miR-142-3p in the absence or presence of TGFβ. C: PASMCs were transfected with anti-miR-142-3p and levels of ASMA and SM22α relative to GAPDH were measured by qRT-PCR in the absence or presence of TGFβ. Data represent mean ± SEM. *$P < 0.01$.

miR-142-3p INHIBITS CELL MIGRATION

TGFβ signaling has been demonstrated to promote the contractile phenotype by inhibiting the migration and growth of VSMCs and by enhancing the expression of SMC-specific contractile genes during normal vascular development and homeostasis [ten Dijke and Arthur 2007].

To determine whether induction of miR-142-3p by TGFβ plays a role in regulating cell migration, a scratch wound assay was performed. A wound was created in PASMCs transfected with the control mimic, the miR-142-3p mimic or anti-miR-142-3p. The wound closure was monitored using images captured at the beginning of the assay and at regular intervals during cell migration. Similarly to the TGFβ-treated cells, the migration distance of miR-142-3p-transfected cells was reduced to 65% of the migration distance of cells transfected with the control miRNA 8 h after the scratch wound was generated (Fig. 2A). In contrast, when miR-142-3p function was inhibited by anti-miR-142-3p, the rate of cell migration was enhanced by 55% at 8 h after a scratch wound was generated, indicating a differential level of miR-142-3p expression determines the migration of VSMCs (Fig. 2A). We also examined the effect of anti-miR-142-3p on the TGFβ-induced inhibition of VSMC migration (Supplemental Fig. S1). Anti-miR-142-3p blocked anti-migratory effect of VSMC by TGFβ.

PDGF-BB and TGFβ signaling play opposing roles in a mechanism for changing the VSMC phenotype [Chan et al., 2010; Kim and Kang 2013]. PDGF-BB promotes cell migration and the growth of PASMCs. To determine whether miR-142-3p affects PDGF-BB-mediated cell migration, the scratch wound closure was monitored in a time-course after PDGF-BB stimulation. Overexpression of miR-142-3p reduced the PDGF-BB-induced cell migration by 20%, suggesting that the regulation of miR-142-3p levels is critical for the

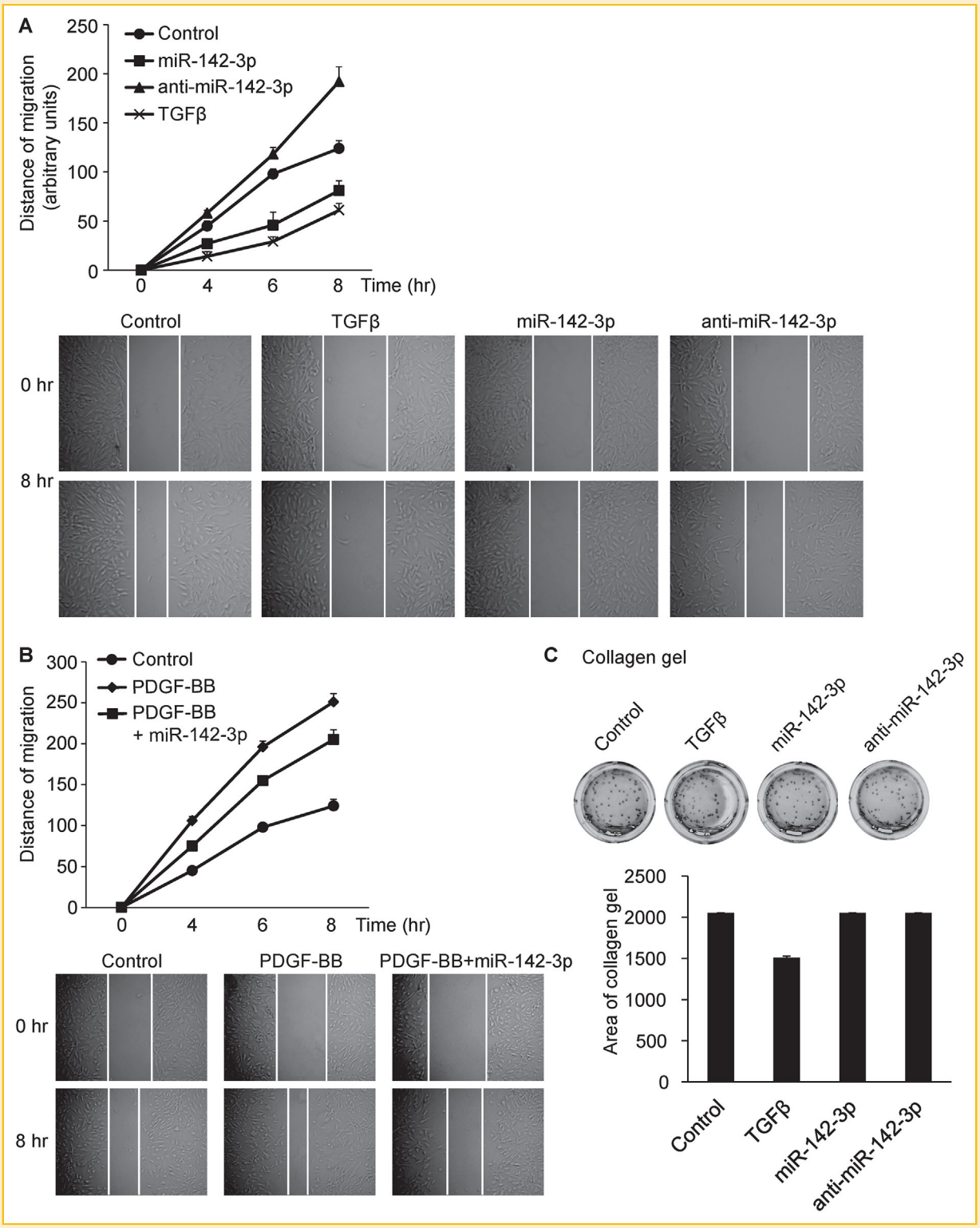

Fig. 2. miR-142-3p regulates VSMC migration. A: PASMCs were transfected with control mimic, miR-142-3p mimic or anti-miR-142-3p and were subjected to the scratch wound assay. As a control, PASMCs were treated with 400 pM TGFβ. The representative images of scratch wound assay were shown. B: PASMCs transfected with the control mimic or the miR-142-3p mimic were subjected to the scratch wound assay in the presence or absence of 20 ng/ml PDGF-BB. The results are the average ± S.E. of measurements taken in triplicate from three independent experiments. The representative images of scratch wound assay were shown. C: PASMCs were transfected with the control mimic, the miR-142-3p or anti-miR-142-3p. PASMCs transfected with the control mimic were stimulated with 400 pM TGFβ (TGFβ) or left unstimulated (Control). The PASMC-embedded collagen lattices were photographed using a digital camera to measure the gel contraction.

pro-migratory function of PDGF-BB in VSMCs (Fig. 2B). These data, therefore, further support that miR-142-3p induced by TGFβ mediates the inhibition of VSMC migration.

We examined the effect of miR-142-3p on the contractility of PASMCs using a collagen lattice contraction assay. TGFβ increases the contractility of PASMCs through actin remodeling, which can be visualized by a decrease in the collagen lattice. Unlike TGFβ stimulation, either the overexpression of the miR-142-3p mimic or the knock-down of miR-142-3p did not affect contraction in the collagen lattice. This result suggested that miR-142-3p has a specific role in the regulation of cell migration to promote the TGFβ-mediated contractile phenotype (Fig. 2C).

DOCK6 IS A NOVEL TARGET OF miR-142

Previously, we demonstrated that BMP induces an increase in miR-21 expression in VSMCs, inhibiting cell migration by targeting DOCK family members such as DOCK4, 5 and 7 [Kang et al., 2011]. To test whether TGFβ-induced miR-142-3p targets DOCK family members to regulate VSMC migration, we examined whether the 3′UTRs of DOCK family members contain miRNA recognition elements (MRE) that are partially complementary to miR-142-3p. Using RNA22 to search for the MRE of miR-142-3p, we observed MREs in the 3′UTRs of DOCK3, 5, 6, and 8 (Fig. 3A). To assess whether endogenous miR-142-3p regulates the expression of these DOCK family members, PASMCs were transfected with anti-miR-142-3p or the control and the relative expression levels of DOCK5, 6 and 8 to GAPDH were examined. DOCK6 expression increased by two-fold when endogenous miR-142-3p was inhibited, indicating that miR-142-3p represses DOCK6 (Fig. 3B). Conversely, exogenous miR-142-3p reduced the expression level of endogenous DOCK6 (Fig. 3C). The endogenous DOCK6 expression was also downregulated by TGFβ stimulation (Supplemental Figure S2). Neither DOCK5 nor 8 were affected by the modulation of miR-142-3p expression levels, indicating that DOCK5 and 8 may not be targets of miR-142-3p. The neuro-specific expression of DOCK3 has been reported [Kashiwa et al., 2000]. To confirm that miR-142-3p targets DOCK6, we measured the expression of the luciferase reporter containing the 3′UTR of DOCK6 downstream of the luciferase reporter gene in the presence of the miR-142-3p mimic. Consistent with the qRT-PCR results, the luciferase activity of the DOCK6 3′UTR construct was reduced by approximately 50% upon expression of the miR-142-3p mimic, demonstrating that DOCK6 is a target of miR-142-3p (Fig. 3D). The activity of the luciferase construct containing the DOCK6 3′UTR was also decreased by TGFβ (Supplemental Figure S3). Moreover, immunoblot analyses indicated that endogenous DOCK6 protein in PASMCs was downregulated upon miR-142-3p overexpression in comparison with the control miRNA mimic (Fig. 3E). Therefore, DOCK6 is a novel target of miR-142-3p.

DOCK6 IS CRITICAL FOR CELL MIGRATION

As downregulation of DOCK4, 5 and 7 by miR-21 inhibits VSMC migration and promotes the contractile phenotype [Kang, et al., 2011], we hypothesized that TGFβ-mediated inhibition of VSMC migration occurs through the downregulation of DOCK6 by miR-142-3p. To test for the role of DOCK6 in cell migration, PASMCs transfected with siRNA against DOCK6 (siDOCK6) were followed by a scratch wound assay (Fig. 4A). As a control, a siRNA with a non-targeting sequence (Control) was transfected. Knock-down efficiency of siDOCK6 was confirmed by immunoblotting (Fig. 4B). When DOCK6 was downregulated by siRNAs, cell migration was inhibited to levels that were comparable to those of cells over-expressing miR-142-3p or stimulated by TGFβ, suggesting that DOCK6 is critical for the regulation of VSMC migration (Fig. 4A and Supplemental Figure S4). Therefore, we can conclude that TGFβ signaling regulates the miR-142-3p-DOCK6 axis along with miR-21-DOCK4, 5 and 7 axis to inhibit cell migration, resulting in the contractile phenotype.

DISCUSSION

The TGFβ signaling pathway has been known to promote the contractile phenotype together with the BMP signaling pathway [ten Dijke and Arthur, 2007]. However, with the exception of miR-143/145 [Davis-Dusenbery et al., 2011], little is known about the regulation of miRNAs by the TGFβ signaling pathway in the modulation of the VSMC phenotype. In this study, we discovered a novel mechanism by which TGFβ signaling promotes the contractile phenotype of VSMCs (Fig. 4C). We demonstrated that miR-142-3p is induced by TGFβ signaling and we identified DOCK6 as a novel target of miR-142-3p. The downregulation of DOCK6 by miR-142-3p is critical for promoting the contractile VSMC phenotype through inhibiting cell migration.

Because a single miRNA can potentially target several hundred genes, and conversely, a single gene can be targeted by more than one miRNA, multiple miRNAs may regulate target genes cooperatively to modulate the VSMC phenotype [Kang and Hata 2012]. Thus, miRNAs hold the potential to modulate the complex physiological or disease phenotypes by regulating entire cellular networks.

miR-142-3p has been shown to play an important role in regulating immunity. In hematopoietic cells, miR-142-3p expression is regulated by the binding of transcription factors such as PU.1, C/EBPβ and Runx1 to the miR-142 gene promoter [Sun et al., 2013]. The expression level of miR-142-3p modulates the inflammatory cytokine interleukin-6 (IL-6). In dendritic cells, miR-142-3p regulates the expression of IL-6 in response to the endotoxin lipopolysaccharide (LPS), which activates a prototypical toll-like receptor-mediated inflammatory response [Sun et al., 2011]. Moreover, miR-142-3p negatively regulates the production of the pro-inflammatory mediators NF-κB, TNF-α and IL-6 in macrophages by targeting IL-1 receptor-associated kinases (IRAK-1) [Xu et al., 2013]. In response to injury, VSMCs begin to produce inflammation mediators including IL-6, which directly stimulates VSMC migration and proliferation, resulting in changing to the synthetic dedifferentiated phenotype [Ikeda et al., 1991; Morimoto et al., 1991]. Thus, it would be interesting to test whether miR-142-3p plays a role in regulating VSMC inflammation.

Vascular inflammation causes various cardiovascular disorders such as pulmonary arterial hypertension (PAH). BMP pathway defects and inflammation are hallmarks of PAH [Yang et al., 2005;

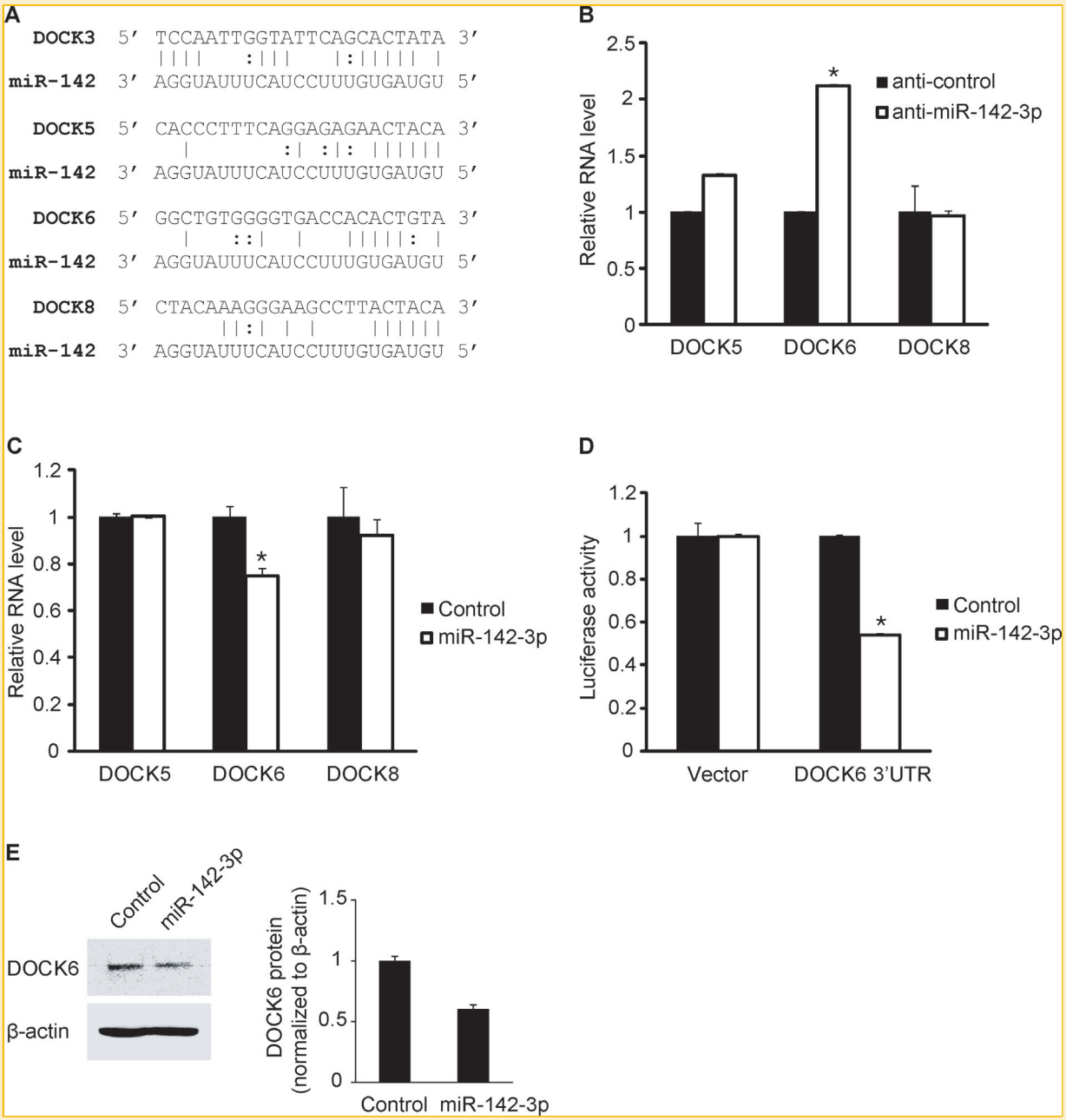

Fig. 3. miR-142-3p targets DOCK6. A: Schematic diagram of the predicted miR-142-3p MREs in the 3′UTR of DOCK5, 6 and 8 transcripts. B: Total RNA was harvested from PASMCs transfected with control or anti-miR-142-3p oligonucleotides and mRNA levels of the indicated DOCK genes relative to GAPDH were measured by qRT-PCR. The experiments were performed in triplicate and the data represent fold changes of the mRNA levels in anti-miR-142-3p transfected cells relative to the mRNA levels of cells transfected with control oligonucleotides ± SEM. *$P < 0.01$. C: PASMCs were transfected with the control mimic or the miR-142-3p mimic for 24 h. mRNA levels of the indicated DOCK genes relative to GAPDH were measured using qRT-PCR. The data represent the fold changes ± SEM. *$P < 0.01$. D: Cos7 cells were transfected with a luciferase reporter containing 3′UTR of DOCK6 gene transcripts and the control mimic or the miR-142-3p mimic. Relative luciferase activities were examined. An empty luciferase vector (Vector) was used as a control. The data represent the fold changes ± SEM. *$P < 0.01$. E: PASMCs were transfected with the control mimic or the miR-142-3p mimic. Total cell lysates were subjected to immunoblotting with anti-DOCK6 or β-actin antibodies.

Pullamsetti et al., 2011]. The expression levels of inflammatory cytokines and chemokines such as CCL2, CCL3, IL-1β, and IL-6 are increased in PAH patients. While PAH can be induced by the overexpression of IL-6 and TNFα, BMP signaling inhibits the TNFα-induced activation of NF-κB by promoting an MRTF-A/NF-κB inhibitory complex, thereby preventing inflammation in PASMCs [Wang et al., 2012]. Interestingly, mutations in members of the TGFβ superfamily of receptors, such as endoglin and the activin-like

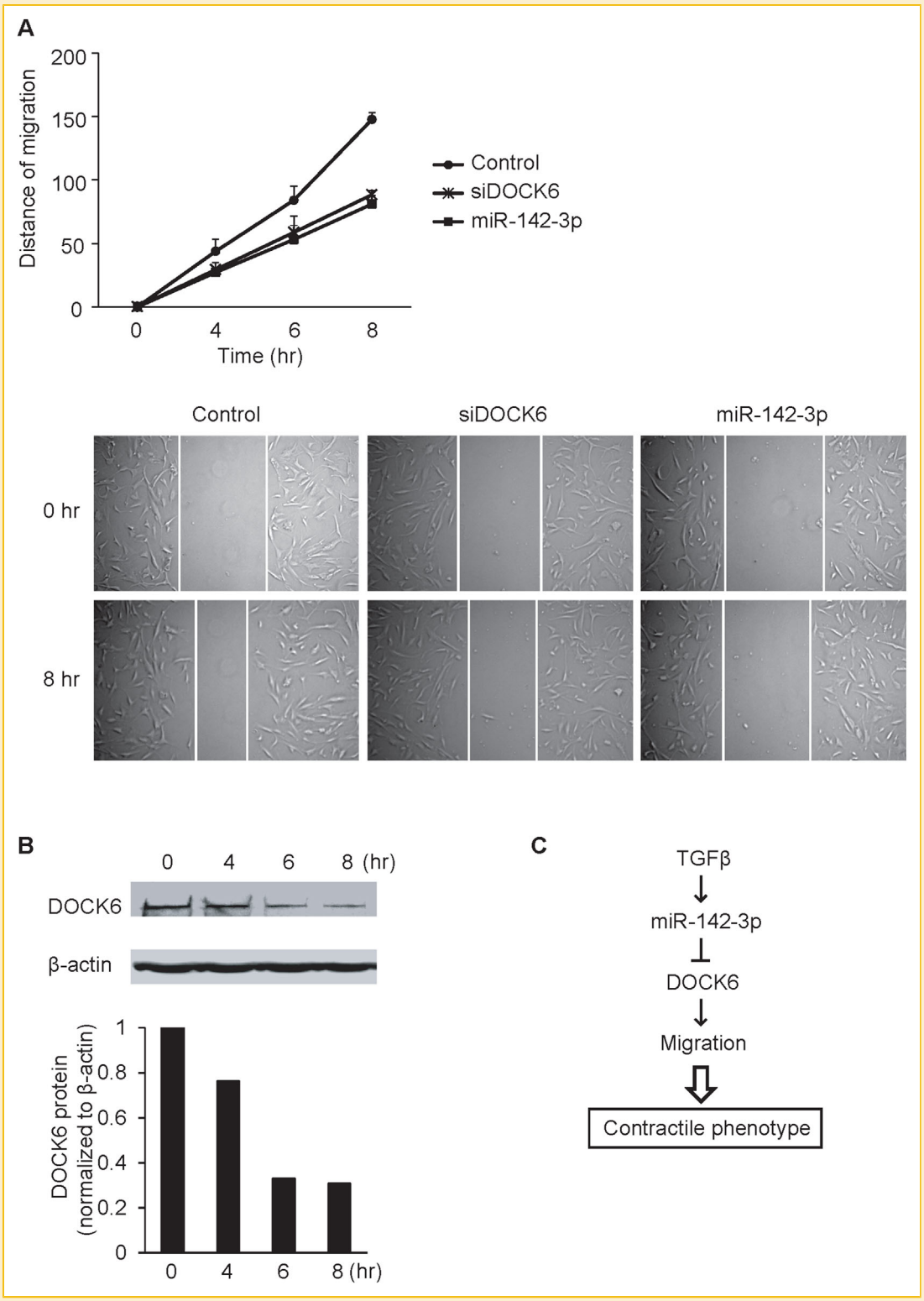

Fig. 4. Downregulation of DOCK6 inhibits VSMC migration. A: PASMCs transfected with a non-targeting control siRNA (Control), siRNA against DOCK6 or miR-142-3p mimic were subjected to the scratch wound assay. The data are the means ± S.E. of triplicate measurements from three independent experiments. The representative images of scratch wound assay were shown. B: PASMCs were transfected with siDOCK6 for 4, 6, 8 h. Total cell lysates were subjected to immunoblotting with anti-DOCK6 or β-actin antibodies. C: Schematic representation of the cellular effects of the miR-142-3p-mediated downregulation of DOCK6 in vascular smooth muscle cells.

receptor 1 (ALK-1), have also been observed in PAH [Gore et al., 2014]. Therefore, further investigation remains necessary to determine whether TGFβ-induced miR-142-3p plays an anti-inflammatory role in VSMCs by targeting pro-inflammatory cytokines such as IL-6.

miR-142-3p might be implicated in various cardiovascular diseases in addition to PAH, such as cardiac hypertrophy, heart failure and cardiac arrhythmias. miRNA expression profiling studies using a cardiac hypertrophy-regression surgical model have shown that miR-142-3p negatively regulates cardiac hypertrophy, although the mechanism underlying the anti-hypertrophic effects has not yet been elucidated [Jeong et al., 2012]. miR-142-3p might target genes controlling cell survival and differentiation as well as DOCK6, as cardiac hypertrophy is promoted by inhibiting the expression of genes involved in cell survival and differentiation.

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