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**Hypoxia-induced miR-1260b regulates vascular smooth muscle cell
proliferation by targeting GDF11**

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Abstract

Vascular smooth muscle cells (VSMCs) are a unique cell type that has unusual plasticity controlled by environmental stimuli. As an abnormal increase of VSMC proliferation is associated with various vascular diseases, tight regulation of VSMC phenotypes is essential for maintaining vascular homeostasis. Hypoxia is one environmental stress that stimulates VSMC proliferation. Emerging evidence has indicated that microRNAs (miRNAs) are critical regulators in the hypoxic responses of VSMCs. Therefore, we previously investigated miRNAs modulated by hypoxia in VSMCs and found that miR-1260b is one of the most upregulated miRNAs under hypoxia. However, the mechanism that underlies the regulation of VSMCs via miR-1260b in response to hypoxia has not been explored. Here we demonstrated that hypoxia-induced miR-1260b promotes VSMC proliferation. We also identified growth differentiation factor 11 (GDF11), a member of the TGF- β superfamily, as a novel target of miR-1260b. miR-1260b directly targets the 3'UTR of *GDF11*. Downregulation of GDF11 inhibited Smad signaling and consequently enhanced the proliferation of VSMCs. Our findings suggest that miR-1260b-mediated GDF11-Smad-dependent signaling is an essential regulatory mechanism in the proliferation of VSMCs, and this axis is modulated by hypoxia to promote abnormal VSMC proliferation. Therefore, our study unveils a novel function of miR-1260b in the pathological proliferation of VSMCs under hypoxia.

Keywords

microRNA, miR-1260b, GDF11, Smad signaling, hypoxia, vascular smooth muscle

cell

Introduction

Vascular smooth muscle cells (VSMCs) have a unique ability to turn into a contractile or synthetic phenotype in response to microenvironmental stimuli (1). Contractile VSMCs are characterized by a very low rate of proliferation, migration and expression of SMC contractile-associated genes, such as smooth muscle α -actin (α -SMA), SM22 α and calponin (2). In contrast, synthetic VSMCs demonstrate increased proliferation and migration. The transforming growth factor-beta (TGF- β) superfamily of growth factors and the platelet-derived growth factor (PDGF) are involved primarily in the regulation of VSMC phenotypes (3, 4). The TGF- β signaling pathway has been demonstrated to promote the contractile phenotype by inhibiting proliferation and migration of VSMCs (4). In contrast, the PDGF signaling pathway leads the synthetic phenotype by stimulating VSMC proliferation and migration (3). As dysregulation of VSMC phenotypes is associated with vascular diseases, VSMC proliferation and differentiation are dynamically regulated under physiological or pathological conditions.

Hypoxia is a unique environmental stress that affects cellular functions, such as proliferation, apoptosis and DNA repair (5). In VSMCs, hypoxia stimulates proliferation and can lead to vascular diseases including pulmonary artery hypertension (6). Under hypoxia, gene expression is regulated by master transcription factors, hypoxia-inducible factors (HIF) (7, 8). In addition, specific microRNAs (miRNAs), termed hypoxamirs, regulate gene expression through

posttranscriptional mechanisms in response to hypoxia (9). Several miRNAs have emerged as crucial regulators of the VSMC phenotype by repressing their target mRNAs (10). We previously investigated miRNAs modulated by hypoxia in VSMCs and found that miR-1260b is one of the most upregulated miRNAs under hypoxia (11). miR-1260b was reported to be upregulated and promoted cell proliferation and invasion in non-small cell lung cancer cells, renal cancer cells, and HCC tissues (12-14). Thus, miR-1260b is considered a putative target for the treatment or diagnosis of such cancers. In this study, we attempted to explore the role of hypoxia-induced miR-1260b in VSMC functions.

Growth differentiation factor 11 (GDF11), a TGF- β superfamily member, is known to regulate development and differentiation (15-17). GDF11 controls anterior-posterior patterning of the axial skeleton in mouse embryos (18). Activin type II receptors, ActRIIA and ActRIIB, mediate GDF11 signaling in axial vertebral patterning. GDF11 binds to both receptors and induces the phosphorylation of Smad2/3 (19, 20). GDF11 is also known to suppress neural stem cell proliferation by modulating the expression of multiple genes implicated in the process (17). However, the function of GDF11 in VSMCs has not been illustrated in detail.

As enhanced proliferation of VSMCs under hypoxia can lead to vascular disorders, understanding the functions of hypoxia-induced miRNAs in the modulation of VSMC proliferation is important. In this study, we demonstrate that hypoxia-induced miR-1260b expression promotes cell proliferation. We also report that GDF11 is targeted by miR-1260b, which results in enhanced VSMC proliferation. Furthermore, we show that Smad signaling is inhibited by the modulation of the miR-1260b-GDF11 axis under hypoxia. Taken together, these results suggest that the upregulation of miR-

1260b by hypoxia leads to the repression of GDF11 expression, which, in turn, results in the inhibition of Smad signaling and promotion of VSMC proliferation.

Results

miR-1260b is upregulated under hypoxia and promotes VSMC proliferation

Our previous study has shown that miR-1260b is one of the most upregulated hypoxamirs in VSMCs (11). We confirmed that the expression of mature miR-1260b was significantly increased in pulmonary artery smooth muscle cells (PASMCs) after exposure to hypoxia for 24 h (Figure 1A). As it is well known that hypoxia stimulates VSMC proliferation (6), we determined whether hypoxia-induced miR-1260b expression has effects on VSMC proliferation. PASMCs transfected with miR-1260b or control mimic for 24 h **under normoxia conditions** were immunostained with anti-Ki-67 antibody to measure the number of proliferating cells. Hoechst dye was used to stain DNA in cells. Ki-67 positive proliferating cells were approximately 8% of total cells in the control mimic-transfected cells and increased to approximately 16.2% in the miR-1260b mimic-transfected cells. Quantitative analysis of Ki-67 immunostaining demonstrates that miR-1260b increases the number of proliferating cells approximately 2-fold compared with the control (Figure 1B), which suggests that miR-1260b promotes the proliferation of VSMCs. In contrast, when miR-1260b is downregulated using a hsa-miR-1260b inhibitor (anti-miR-1260b) **under normoxia conditions**, the percentage of proliferating cells was decreased to approximately 47% of the control mimic-transfected cells. These findings suggest that the downregulation of miR-1260b inhibits VSMC proliferation (Figure 1C). Therefore, a

hypoxia-induced increase of miR-1260b expression might stimulate VSMC proliferation.

GDF11 is downregulated by hypoxia

To further elucidate the molecular function of miR-1260b in VSMCs, we first checked whether known target mRNAs of miR-1260b, such as G-protein signaling 22, PTPRT, sFRP1, and Smad4, are downregulated by hypoxia from our NGS-based RNA sequencing data (11). None of genes investigated showed significant changes in hypoxic conditions, suggesting that hypoxia-induced miR-1260b promotes VSMC proliferation through regulation of novel targets. Thus, we searched predicted target mRNA candidates that have a conserved miRNA recognition element (MRE) sequence within their 3'UTR using a target prediction algorithm, TargetScan. We then investigated whether the expressions of the predicted target genes, such as chromosome transmission fidelity factor 8 (CHTF8), DiGeorge syndrome critical region gene 2 (DGCR2), GDF11, MAF BZIP transcription factor G (MAFG), nuclear factor I C (NFIC), RNA polymerase II subunit F (POLR2F), transmembrane protein 104 (TMEM104), and ubiquitin conjugating enzyme E2 H (UBE2H), are downregulated by miR-1260b via qRT-PCR analysis. Among these 8 genes, DGCR2, GDF11 and POLR2F mRNA levels were reduced by approximately 70%, 60% and 37%, respectively, following the overexpression of miR-1260b, which suggests that miR-1260b might regulate the expression of DGCR2, GDF11 and POLR2F (Figure 2A). As the expression of miR-1260b is increased under hypoxia, if target genes are posttranscriptionally repressed by miR-1260b, decreased target gene expression levels would be expected under hypoxia. Thus, we examined the mRNA levels of the

predicted target genes in PASMCs after 24 hours exposure to hypoxia via qRT-PCR. Under hypoxia, the transcript level of GDF11 was downregulated by approximately 58%; however, no other transcript levels were significantly changed (Figure 2B). Similar to the qRT-PCR results, GDF11 protein levels were decreased by approximately 57% in the hypoxia-exposed cells for 24 h (Figure 2C). These results suggest the possibility that GDF11 might be posttranscriptionally regulated by miR-1260b under hypoxia. The overexpression of miR-1260b by the exogenous miR-1260b mimic was confirmed by qRT-PCR (Figure 2D).

GDF11 is a direct target of miR-1260b

We examined whether endogenous GDF11 protein levels are regulated by miR-1260b. Immunoblot analyses indicated that the exogenic miR-1260b mimic significantly reduced the endogenous GDF11 protein level by approximately 43% (Figure 2E). In contrast, miR-1260b inhibition by an anti-miR-1260b elevated the basal level of GDF11 protein by approximately 1.7-fold (Figure 2F). The expression level of *GDF11* transcripts was also elevated by approximately 43% in PASMCs transfected with an anti-miR-1260b (Figure 2G), indicating that endogenous miR-1260b represses GDF11 expression. The inhibition of miR-1260b by anti-miR-1260b was confirmed via qRT-PCR (Figure 2H).

We next determined whether GDF11 is targeted by a direct binding of miR-1260b via luciferase assay. Three potential miRNA recognition elements (MREs) in the *GDF11* 3'UTR, which are partially complementary to the miR-1260b, were found based on the prediction by TargetScan (Figure 2I). Thus, we assessed luciferase activities of

the construct that contained a partial 3' UTR of *GDF11*, including all three MREs, following the overexpression of the miR-1260b (Figure 2J). The luciferase activity of the *GDF11* 3' UTR construct was reduced by approximately 63% in the presence of miR-1260b mimic, which indicates that *GDF11* is a novel direct target of miR-1260b. To determine which of these predicted MREs are necessary for the repression by miR-1260b, luciferase reporter constructs that contained individual MREs and miR-1260b were transfected into cells. The transcriptional activity of a luciferase reporter construct that included the MRE1 or MRE3 sequence, but not MRE2, was significantly reduced by miR-1260b, which suggested that MRE1 and MRE3 are essential for the target recognition by miR-1260b. To further support that the identified MRE sequences are critical for recognition by miR-1260b, we made a mutant 3'UTR luciferase reporter construct (3'UTR mut), which disrupted both MRE1 and MRE3, and then measured the luciferase activity. The inhibition of the luciferase activity of miR-1260b is abrogated in the mutant 3'UTR construct, which suggests that miR-1260b targets *GDF11* by direct binding with MRE1 and MRE3 (Figure 2J).

GDF11 regulates VSMC phenotypes

As miR-1260b promotes VSMC proliferation, we hypothesized that a target of miR-1260b, *GDF11*, might be linked to the modulation of cell proliferation. To examine whether the *GDF11* expression affects VSMC proliferation, the number of proliferating cells was measured by immunostaining with an antibody against Ki-67 following transfection of *GDF11* siRNA into PASCs. Using siRNA, the levels of *GDF11* mRNAs and proteins were reduced by approximately 78% and 60%, respectively (Figure 3A and 3B). Quantitative analysis of Ki-67 immunostaining

demonstrates that knockdown of GDF11 by siRNA increased the percentage of Ki-67 positive proliferating cells approximately 2.2-fold compared with the control, which implies that the targeting of GDF11 by miR-1260b enhances the proliferation of VSMCs under hypoxia (Figure 3C). We subsequently ascertained that GDF11 inhibits the proliferation of VSMCs. PSMCs were stimulated with 10 ng of GDF11 for 24 h and subjected to immunostaining with an antibody against Ki-67. The number of Ki-67 positive proliferating cells was reduced by approximately 35% following GDF11 stimulation (Figure 3D).

As GDF11 is a secreted protein, we confirmed that secretion of GDF11 is affected by the intracellular miR-1260b levels via measuring the secreted GDF11 levels. Medium of PSMCs transfected with control miRNA or miR-1260b was used for trichloroacetic acid (TCA)-induced protein precipitation and pellets were subjected to western blotting using a GDF11 antibody. The level of GDF11 in the medium of miR-1260b-transfected cells was reduced by approximately 40% compared to that of the control miRNA-transfected cells, suggesting that exogenous miR-1260b suppresses GDF11 expression and less GDF11 is secreted (Figure 3E). These results further support that GDF11 is a key regulator of miR-1260b-mediated VSMC proliferation.

Given that proliferation is a critical feature between synthetic and contractile phenotypes of VSMCs, we investigated whether GDF11 also modulates the expression of SMC contractile-associated genes and leads to phenotypic changes of VSMCs. PSMCs were stimulated with GDF11 for 24 h, and the mRNA levels of smooth muscle genes, such as α -SMA, SM22 α and calponin, were measured via qRT-PCR. The expressions of all three smooth muscle cell-specific contractile genes were significantly increased approximately 3.7 to 6-fold following GDF11 stimulation,

which suggests that GDF11 promotes the contractile phenotype (Figure 3F). The protein levels of α -SMA and calponin were increased approximately 5.4-fold and 3-fold, respectively, after in response to GDF11 stimulation (Figure 3G). Considering that GDF11 inhibits the proliferation of VSMCs and enhances the expression of SMC contractile-associated genes, GDF11 induces the contractile state. Therefore, miR-1260b-mediated downregulation of GDF11 under hypoxia might induce the synthetic state by modulating specific aspects of the VSMC phenotype.

miR-1260b-GDF11 axis regulates Smad signaling

As GDF11 is known to induce the phosphorylation of Smad2/3 in various cell types(21-24), we investigated whether the miR-1260b-GDF11 axis modulates Smad signaling in VSMCs. First, we examined the changes in Smad3 phosphorylation following GDF11 stimulation. Immunoblot analysis using an antibody against phosphorylated Smad3 showed that the levels of phosphorylated Smad3 were enhanced in PSMCs treated with GDF11 for 1 h, which demonstrates that GDF11 activates Smad signaling in VSMCs (Figure 4A). Next, we examined the activation of Smad signaling after transfection of miR-1260b mimic or anti-miR-1260b into PSMCs by immunoblotting. The levels of phosphorylated Smad3 were reduced when miR-1260b was overexpressed (Figure 4B), which suggests that miR-1260b inhibits Smad signaling. In contrast, the phosphorylation of Smad3 was enhanced following the transfection with anti-miR-1260b (Figure 4C), which indicates that the downregulation of miR-1260b activates the Smad signaling pathway. These results imply that miR-1260b acts as a negative regulator of Smad signaling through the suppression of GDF11 expression.

As miR-1260b is upregulated and GDF11 is consequently repressed under hypoxia, we examined whether hypoxia affects the Smad signaling pathway. Immunoblot analysis showed a decrease of the phosphorylation levels of Smad3 in cells exposed to hypoxia for 24 h compared with the normoxia controls, which suggests that Smad signaling is inhibited under hypoxia conditions (Figure 4D). Therefore, these results imply that Smad signaling, which can prevent the synthetic phenotype, might be impaired by the modulation of the miR-1260b-GDF11 axis under hypoxia and thus the proliferative response of VSMCs is induced.

As hypoxia-induced miR-1260b inhibits Smad signaling, we examined whether modulation of miR-1260b using anti-miR-1260b affects the hypoxia-mediated Smad signaling regulation (Figure 4E). PSMCs transfected with control or anti-miR-1260b for 48 h were exposed to normoxia or hypoxia for 24 h. In control-transfected PSMCs, the phosphorylated Smad3 level was reduced by hypoxia; however, the phosphorylated Smad3 level was recovered in anti-miR-1260b-transfected cells even under hypoxia conditions. These results suggest that modulation of miR-1260b might control Smad signaling of VSMCs under hypoxia conditions.

Discussion

We previously observed that miR-1260b expression is upregulated in VSMCs exposed to hypoxia(11). However, the mechanism that underlies the regulation of hypoxic responses via miR-1260b has not been investigated. In this study, we reported miR-1260b as a novel hypoxamir to regulate VSMC proliferation and

identified GDF11 as a miR-1260b target. We provided evidence that the downregulation of GDF11 by miR-1260b is crucial for promoting proliferation under hypoxia. Furthermore, we demonstrated that Smad signaling via GDF11 is modulated by miR-1260b under hypoxia, so that VSMC proliferation is promoted.

GDF11, as a TGF- β family member, is widely expressed and plays various roles, including regulating axial skeletal patterning during development (18). Recently, evidence regarding the association between GDF11 function and vascular disease has emerged. For example, the levels of plasma GDF11 were decreased in chronic obstructive pulmonary disease (COPD), in which the progression of COPD was promoted by activating the AKT signaling pathway (25). In addition, the circulating GDF11 concentration declined with age; thus GDF11 has been considered as a “rejuvenating” factor that improves cardiovascular disease, osteoporosis, and other diseases of old age, although there are conflicting reports regarding the function of GDF11 during aging (26, 27). However, the exact function of GDF11 associated with VSMC proliferation, which can lead to vascular disease, has not previously been elucidated. In this study, we observed that the repression of GDF11 expression inhibits Smad signaling and promotes VSMC proliferation. Our findings provide evidence for an anti-proliferative function of GDF11 and suggest that GDF11-Smad3-dependent signaling is an important molecular mechanism in the regulation of VSMC proliferation. Moreover, Smad signaling upon TGF- β family ligands is known to be critical to regulate VSMC phenotypes through the inhibition of cell proliferation (4).

We previously demonstrated that BMP4-Smad signaling induces miR-21 biogenesis, which increases contractile gene expression and inhibits cell migration by targeting PDCD4 and DOCK family proteins, respectively, thus promoting the contractile

phenotype of VSMCs (28, 29). In addition, BMP4-Smad signaling downregulates miR-302 that targets the BMP receptor type II (BMPRII) as an autoregulatory mechanism, which inhibits cell migration and proliferation and accelerates the contractile phenotype of VSMCs (30). In this study, we identified an additional miRNA, miR-1260b, which regulates VSMC functions, further supporting the fact that miRNAs provide a delicate regulatory mechanism in the complex vascular system. As Smad proteins are the main signal transducers for the TGF- β signaling pathway that inhibits proliferation of VSMCs, suppression of GDF11 as a member of the TGF- β superfamily by miR-1260b impairs the TGF- β signaling pathway, and consequently VSMC proliferation is accelerated.

Several studies suggest that miR-1260b is implicated in the proliferation, migration or invasion of cancer cells (12-14); however the role of miR-1260b in VSMCs is unclear, and the function associated with hypoxia conditions has not previously been elucidated. We first report miR-1260b as a hypoxamir. miR-1260b expression is induced by hypoxia and has a pro-proliferative function in VSMCs. Hypoxia-induced miR-1260b might contribute to promoting VSMC proliferation by targeting GDF11 to inhibit Smad signaling that restrains the proliferative synthetic phenotype. As hypoxia-induced pulmonary artery hypertension and many other vascular diseases are associated with increased VSMC proliferation, miR-1260b is likely to be a crucial mediator to develop vasculopathies. Our understanding of the regulatory functions of miR-1260b in the proliferation of VSMCs under hypoxia provides new insight into the mechanisms of vascular proliferative disorders.

Conclusion

Several lines of evidence have converged to link GDF11 with vascular diseases. Our findings raise the possibility that the hypoxia-induced proliferative response of VSMCs might be mediated by modulation of the miRNA-GDF11 axis. miR-1260b appears to be of biological importance because it was upregulated by hypoxia and promoted VSMC proliferation. Our finding of GDF11 as a target of miR-1260b provides relevant insight into the mechanisms of hypoxia-induced vascular proliferative pathologies and potential future interventions.

Materials and Methods

Cell culture

Human primary pulmonary artery smooth muscle cells (PASMCs) were purchased from Lonza (CC-2581) and cultured as previously described (11). For hypoxia, PASMCs in Sm-Gm2 medium (Lonza) were grown in a sealed modular incubator chamber (Billups-rothenberg Inc.) for 24 h at 37 °C after flushing with a mixture of 5% CO₂, 1% O₂ and 94% N₂ for 4 min. For GDF11 stimulation, 10 ng of recombinant GDF11 purchased from R&D Systems was used.

Quantitative reverse transcriptase-PCR (qRT-PCR)

Real-time PCR was performed for quantitative analysis of the change in levels of mRNA or miRNA expression. Levels of mRNAs were normalized to 18S rRNA. The primers employed in this study were as follows: 18S rRNA, 5'-GTAACCCGTTGAACCCATT-3' and 5'-CCATCCAATCGGTAGTAGCG-3'; GDF11, 5'-ACCACCGAGACCGTCATTAG-3' and 5'-GGGTACAGGCCGTAGGTACA-3'; CBX6, 5'-GAACCGCGTTATAGGCAAGA-3' and 5'-GGGTCAGAGGACTGTGGTGT-3', CHTF8, 5'-ATGGAGCTACAGGGGGAGAT-3' and 5'-

CTGATCCCCAGGAGTGTGTT-3'; DGCR2, 5'-CTGAGATGTGTGTGGCTGCT-3'
 and 5'-CAGCAGTGACAGGATGAGGA-3'; MAFG, 5'-
 CGACCCCCAATAAAGGAAAC-3' and 5'-TTCTGCTTCTCCAGCTCCTC-3'; NFIC,
 5'-ACCTGGCATAACGACCTGAAC-3' and 5'-GGGCTGTTGAATGGTGACTT-3';
 POLR2F, 5'-CGACGACTTTGATGATGTGG-3' and 5'-
 GCTCGCTCGTACTTGGTCAT-3'; TMEM104, 5'-CTTGCGGATCTTCACTCTCC-3'
 and 5'-AGTAGACGCACACCCCAAAC-3'; and UBE2H, 5'-
 TGAAGGCGGAGTATGGAAAG-3' and 5'-GCCAATAACTGAGGCAGGAA-3'. We
 used miScript SYBR Green PCR kit and miScript primer assays from Qiagen to
 measure the levels of mature miR-1260b expression. The levels of U6 small nuclear
 RNA as the control were measured to calculate the relative expression level of miR-
 1260b. Data was analyzed using a comparative C_T method in Bio-Rad software. All
 procedures were carried out at least three times and the average results with
 standard errors are presented.

Transfection of miRNAs

The miR-1260b mimic and negative control miRNA were purchased from Genolution
 Pharmaceuticals. PSMCs were transfected with 5 nM miRNA mimics using RNAi
 Max (Invitrogen) according to the manufacturer's protocol. The hsa-miR-1260b
 inhibitor (anti-miR-1260b) was purchased from Ambion and was transfected at 50
 nM using G-fectin (Genolution Pharmaceuticals) according to the manufacturer's
 instructions.

RNA interference

For knockdown of *GDF11*, small interfering RNAs (siGDF11) synthesized by
 Genolution Pharmaceuticals were used. The target sequence of siGDF11 was 5'-
 CAAUGACAAGCAGCAGAUUUAU-3'. As a control, negative control siRNA from
 Genolution Pharmaceuticals was used.

Luciferase reporter constructs

A part of the 3'UTR sequence of *GDF11* and a *GDF11* 3'UTR sequence containing mutations within the predicted MRE1 were cloned into the pIS0 vector (Addgene) that included the luciferase gene. To amplify the 3'UTR sequence of *GDF11*, RT-PCR was performed using mRNA isolated from PSMCs. The primers used were 5'-TTAGAGCTCCGTGTGCAATACAACAGAGG-3' and 5'-ATTGGCCGGCCGCCTCACATCTCTTCACCACT-3'. For the 3'UTR mutant construct including mutated MRE sequences, an upstream region that contained a mutated sequence in MRE1 and a downstream region that contained a mutated sequence in MRE3 were amplified separately using primers containing the *XhoI* restriction site. Two PCR products were cleaved by *XhoI* restriction enzyme and ligated. The ligated DNA fragment was subsequently cloned into the pIS0 vector. To amplify the upstream region, 5'-ATGGAGCTCGGGAGGCAGGACCCTATTGAGGG-3' and 5'-TTCCTCGAG CTTGGTCTGCTGGCTC-3' were used. To amplify the downstream region, 5'-ATGCTCGAGAGGATTCTGGAAGGGGGACA-3' and 5'-ATTGGCCGGCCGCCTCACATCTCTTCACCACT-3' were used. Each predicted MRE sequence was cloned into the pIS0 vector. The primers were as follows: MRE1, 5'-CAACAGAGGGAGGCAGGTGGGAACCGG-3' and 5'-TTCCCACC TGCCTCCCTCTGTTGAGCT-3'; MRE2, 5'-AGCTCCCCATGCGGGGGTGGGAG CCGG-3' and 5'-CTCCCACCCCCGCATGGGGAGCTAGCT-3'; and MRE3, 5'-GAGCCAGCAGACCAAGGTGGGAACCGG-3' and 5'-TTCCCACC TTGGTCTGCTGGCTCAGCT-3'.

Luciferase assay

Cotransfection of control or miR-1260b mimics and luciferase reporter constructs into Cos7 cells was performed using Lipofectamine 2000 (Life technologies). As an internal transfection control, a β -galactosidase expression plasmid was used. After 24 h, the luciferase activities were measured and shown after normalization to the β -galactosidase activity.

Western blot

Total cell lysates were prepared in TNE buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1 mM EDTA), and proteins were separated in SDS-PAGE and transferred onto PVDF membranes. Transferred membranes were probed for GDF11 (Abcam, ab71347), β -actin (Santa Cruz, sc47778), phospho-Smad 3 (Cell Signaling) and Smad 3 (Cell Signaling) antibodies, followed by anti-rabbit secondary antibodies from Santa Cruz Biotechnology, Inc.

Immunofluorescence staining

Equal amounts of PSMCs placed in chamber well slides were transfected with miR-1260b, control mimic or siGDF11. The cells were fixed using 2% paraformaldehyde in PBS and incubated with 3% BSA in PBS to block unspecific binding of the antibodies. After permeabilization in 0.1% Triton X-100 in PBS, the cells were probed for Ki-67 antibody (Abcam, #ab16667), followed by goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Flour 488 (Thermo Fisher Scientific, #A-11008). Hoechst 33342 (Thermo Fisher Scientific, #62249) was used to stain nuclei. Images were obtained using a Zeiss Axio Imager Z1 microscope. We counted at least 2000

cells per condition and show the percentages of Ki-67 positive cells. The graphs showed the average \pm S.E. of triplicate experiments.

Trichloroacetic acid (TCA)-induced protein precipitation

PASMCs were transfected with control or miR-1260b for 48 h. Media was collected and mixed with one volume of 20% TCA. After precipitation, the pellet was washed with cold acetone and dissolved in 1X SDS sample buffer.

Statistical Analysis

All experimental procedures were carried out in triplicate and analyzed with unpaired Student's *t*-test. Data are presented as the average with standard errors. Statistical significance is defined as $p < 0.05$.

Acknowledgments

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Conflicts of interest

There is no conflict of interest.

Figure Legends

Figure 1. Hypoxia-induced miR-1260b promotes proliferation of VSMCs. A.

Levels of miR-1260b relative to U6 snRNA measured by qRT-PCR in PSMCs 24 h after exposure to normoxia or hypoxia. Data represent the means \pm S.E. of triplicates. *, $p < 0.05$. **B-C.** Representative microphotographs of Ki-67 immunostaining. PSMCs transfected with control mimic, miR-1260b (B) or anti-miR-1260b (C) were subjected to immunofluorescence staining with anti-Ki-67 antibody. Approximately 200 cells from at least 10 independent fields were counted for each condition, and Ki-67 positive cells were presented as a percentage of the total population. Scale bar represents 50 μ m. *, $p < 0.05$.

Figure 2. Identification of target mRNAs for miR-1260b. **A.** Endogenous levels of predicted target candidate mRNAs relative to 18S rRNA were quantified by qRT-PCR analyses in PSMCs transfected with control or miR-1260b mimic. Data represent the means \pm S.E. of triplicates. *, $p < 0.05$. **B.** Levels of predicted target candidate mRNA relative to 18S rRNA measured by qRT-PCR 24 h after exposure to normoxia or hypoxia. Data represent the means \pm S.E. of triplicates. *, $p < 0.05$. **C.** Total cell lysates from PSMCs exposed to normoxia or hypoxia for 24 h were subjected to immunoblot analysis with anti-GDF11 or anti- β -actin antibodies. Relative amounts of GDF11 proteins normalized to β -actin were quantitated by densitometry. *, $p < 0.05$. **D.** Mature miR-1260b levels relative to U6 snRNA measured by qRT-PCR 24 h after transfection with control or miR-1260b mimic. Data represent the means \pm S.E. of triplicates. *, $p < 0.05$. **E-F.** Immunoblot analysis of GDF11 and β -actin using cell lysates of PSMCs transfected with control, miR-1260b mimic (E) or anti-miR-1260b (F). Protein bands were quantitated by densitometry, and relative amounts of GDF11 proteins normalized to β -actin were presented. *, $p < 0.05$. **G**

Levels of endogenous GDF11 mRNA relative to 18S rRNA were quantified by qRT-PCR analyses in PSMCs transfected with control or anti-miR-1260b. Data represent the means \pm S.E. of triplicates. *, $p < 0.05$. **H.** Mature miR-1260b levels relative to U6 snRNA were measured by qRT-PCR 24 h after transfection with control or anti-miR-1260b. Data represent the means \pm S.E. of triplicates. *, $p < 0.05$. **I.** (Upper panel) Schematic diagram of predicted miR-1260b MREs in the 3'UTR of *GDF11* transcripts and luciferase reporter constructs used for luciferase assays. CDS and AAA represent the protein coding sequence and poly(A) tail, respectively. Mutations introduced in the MRE to disrupt a base pairing with the miR-1260b sequence are indicated as X. (Bottom panel) Sequences of the predicted miR-1260b MREs in the 3'UTR of *GDF11* transcripts. **J.** Luciferase activities of constructs with the 3'UTR of *GDF11*, mutant 3'UTR and MRE sequences were examined in Cos7 cells by transfecting control or miR-1260b mimic. A luciferase vector without the 3' UTR sequence (Vector) was used as a negative control. Data represent the means \pm S.E. of triplicates. *, $p < 0.05$.

Figure 3. GDF11 modulates VSMC proliferation. **A.** Levels of endogenous GDF11 mRNA relative to 18S rRNA measured by qRT-PCR analyses in PSMCs transfected with control or siGDF11. Data represent the means \pm S.E. of triplicates. *, $p < 0.05$. **B.** Total cell lysates from PSMCs transfected with control or siGDF11 were subjected to immunoblot analysis with anti-GDF11 or anti- β -actin antibodies. Relative amounts of the GDF11 proteins normalized to β -actin were quantitated by densitometry. *, $p < 0.05$. **C-D.** Representative microphotographs of Ki-67 immunostaining. PSMCs transfected with control or siGDF11 (C) or treated with 10

ng GDF11 (D) were subjected to immunofluorescence staining with anti-Ki-67 antibody. Approximately 200 cells from at least 10 independent fields were counted for each condition, and Ki-67 positive cells were presented as a percentage of the total population. Scale bar represents 50 μ m. *, $p < 0.05$. **E. Proteins in the media of PSMCs transfected with control miRNA or miR-1260b for 48 h were precipitated by TCA-induced protein precipitation approach and subjected to western blotting using a GDF11 antibody. Bands of secreted GDF11 protein were quantitated by densitometry. *, $p < 0.05$. F. Levels of smooth muscle genes relative to 18S rRNA measured by qRT-PCR analyses in PSMCs treated with 10 ng GDF11. Data represent the means \pm S.E. of triplicates. *, $p < 0.05$. G. Protein levels of α -SMA and calponin were measured by immunoblot analysis. Protein bands were quantitated by densitometry. *, $p < 0.05$.**

Figure 4. Modulation of GDF11 by miR-1260b regulates Smad signaling. A. Total cell lysates from PSMCs exposed to GDF11 for 1 h were subjected to immunoblot analysis with anti-phospho-Smad3, anti-Smad3 or anti- β -actin antibodies. Relative amounts of phosphorylated Smad3 normalized to total Smad3 were quantitated by densitometry. *, $p < 0.05$. **B-C.** Total cell lysates from PSMCs transfected with control, miR-1260b mimic (B), or anti-miR-1260b (C) were subjected to immunoblot analysis with antibodies against pSmad3, Smad3 or β -actin. By densitometry, relative amounts of phosphorylated Smad3 protein normalized to total Smad3 were quantitated. *, $p < 0.05$. **D.** Total cell lysates from PSMCs exposed to normoxia or hypoxia for 24 h were subjected to immunoblot analysis with anti-phospho-Smad3, Smad3 or anti- β -actin antibodies. By densitometry, relative amounts of

phosphorylated Smad3 normalized to total Smad3 were quantitated. *, $p < 0.05$. **E.** PASMCs transfected with control or anti-miR-1260b were exposed to normoxia or hypoxia for 24 h. Total cell lysates were subjected to immunoblot analysis with antibodies against pSmad3, Smad3 or β -actin. By densitometry, relative amounts of phosphorylated Smad3 normalized to total Smad3 were quantitated. *, $p < 0.05$.

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