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miR-18b inhibits TGF-β1-induced differentiation of hair follicle stem cells into smooth muscle cells by targeting SMAD2

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ABSTRACT

Human hair follicle mesenchymal stem cells (hHF-MSCs) are capable of differentiating into smooth muscle cells (SMCs) in response to transforming growth factor-β (TGF-β), and thus can be used for cardiovascular tissue engineering and regenerative medicine. However, the precise molecular mechanisms underlying SMC conversion of hHF-MSCs are still undefined. MicroRNAs (miRNAs) are small noncoding RNAs that modulate gene expression post-transcriptionally by binding to the complementary sequences of targeted mRNAs. Accumulating evidence indicates that miRNAs are associated with SMC differentiation *in vitro* and*in vivo*. In this study, we revealed that miR-18b was significantly downregulated during TGF-β1-induced hHF-MSCs differentiation into SMC using miRNA array profiling and quantitative RT-PCR (qRT-PCR). Over-expression of miR-18b in hHF-MSCs led to remarkable downregulation of SMC-specific markers such as SMA and calponin proteins. On the contrary, inhibition of endogenous miR-18b by its antisense oligonucleotide antagomir-18b reversed the changes of SMA and calponin proteins. We also showed that SMAD2, a key transcription regulator in TGF-β signaling which was involved in SMC differentiation, is regulated by miR-18b. miR-18b could suppress the expression of SMAD2 protein by targeting the 3′UTR of SMAD2 gene without affecting its mRNA level in hHF-MSCs. Moreover, knockdown of SMAD2 by RNA interference could block the effect of inhibition of miR-18b on SMC differentiation, indicating that SMAD2 contributed to miR-18b mediated regulation of TGF-β-induced SMC differentiation. In conclusion, this study demonstrated that miR-18b regulated the TGF-β1-induced differentiation of hHF-MSCs into SMCs by targeting SMAD2 gene, and provided novel insights into the regulatory mechanisms of TGF-β-induced SMC differentiation.

1. Introduction

hHF-MSCs can be easily obtained from hair follicle and may be an applicable source of autologous smooth muscle cells (SMCs). We have previously reported that hHF-MSCs can differentiate in response to transforming growth factor-β1 (TGF-β1) and display phenotypes of mature smooth muscle cells (SMCs) [1]. TGF-β1 is one of the members of the TGF-β super family, which consists of various multifunctional cytokines related to a wide range of cellular processes such as adhesion, proliferation, differentiation, and apoptosis [2–5]. It has been shown that TGF-β1 plays a pivotal role in vasculogenesis and SMC specification during embryonic development [6], and potently promotes differentiations of various stem cell types into SMCs or SMC-like cells *in vitro* [7–11]. TGF-β1 mediates signaling by binding to cell surface TGF-β type I and type II Ser/Thr kinase receptors, and leads to the phosphorylation of the receptor-regulated SMADs, i.e., SMAD2 and SMAD3 [12]. The phosphorylated SMAD2 and SMAD3 bind to SMAD4 and form a hetero-oligomer, and the hetero-oligomer then translocates into the nucleus, where it regulates the expression of TGF-β1 target genes [13]. However, the precise regulatory mechanisms underlying this process are still undefined, and further investigations about the regulation of TGF-β1-induced differentiation are imminently required.

MicroRNAs (miRNAs) are 18-25-nucleotide small non coding RNAs. To date, more than 1,400 human miRNAs have been identified and they participate in diverse physiologic and pathophysiologic

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processes [14,15]. Interestingly, recent studies have revealed that miRNAs are critical regulators of SMC differentiation. For example, miR-24 suppresses the expression of contractile proteins during the induction of platelet-derived growth factor (PDGF) [16], whereas miR-1 promotes retinoid acid-induced SMC differentiation by targeting Kruppel-like factor 4 (KLF4) [17]. *In vitro* over-expression of miR-143/miR-145 caused upregulation of SMC-specific markers such as smooth muscle actin (SMA) and calponin [18].

Although TGF-β1 is a robust inductor of stem cells, little is known about the involvement of miRNAs in TGF-β/SMAD signaling during SMC differentiation. In the present study, we analyzed miRNA expression during TGF-β1-induced SMC differentiation of hHF-MSCs and focused on miR-18b as a potential inhibitor of SMC differentiation. Our data demonstrated that miR-18b was downregulated during hHF-MSCs differentiation into SMCs, and the differentiation was repressed when miR-18b was binding to its target sequence in the 3′UTR of SMAD2.

2. Materials and methods

2.1. Cell isolation and culture

hHF-MSCs were isolated and characterized as previously described [19]. The investigation conformed to the principles outlined in the Declaration of Helsinki. Isolated hHF-MSCs were cultured in growth medium (GM). To induce SMC differentiation, hHF-MSCs were shifted from GM into differentiation medium (DM) and cultured for 7 days. GM consisted of DMEM/F12 and bFGF (10 ng/μL) supplemented with 10% fetal bovine serum (Thermo, Waltham, USA), and DM comprised of DMEM/F12, 10% fetal bovine serum and TGF-β1 (5 ng/μL).

HEK-293 cells were routinely cultured in RPMI 1640 medium (Gibco, California, USA) supplemented with 10% FBS, 100 units/mL penicillin and 100 g/mL streptomycin.

2.2. Lentivirus and oligonucleotide transfection

Human Smad2 shRNA lentiviral particles and the control cop-GFP lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and stored at −80 °C. According to the manufacturer's protocol, hHF-MSCs were seeded in a 24-well plate, grown overnight in GM, and exposed for 12 h to the shRNA lentiviral particles in the presence of polybrene (5 μg/mL). After the exposure, transduced cells were selected with puromycin (8 ng/mL) (Santa Cruz, CA, USA). The resistant clones were expanded and assayed for stable shRNA expression using fluorescent microscopy (Leica, Wetzlar, Germany).

The hHF-MSCs and HEK-293 cells were transfected with miRNA negative control, miR-18b mimics or miR-18b inhibitors (RiboBio, Guangzhou, China), respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. To investigate the effect of miR-18b on the differentiation of hHF-MSCs or SMAD2-deficient hHF-MSCs, 200 nM of agomir-18b, agomir-NC, antagomir-18b or antagomir-NC was added into DM before induction. Agomir and antagomir were chemically modified and cholesterol-conjugated RNA molecules that could be easily transfected into cells without Lipofectamine 2000, and activated and inhibited endogenous miRNA, respectively.

2.3. Quantitative RT-PCR analysis

Total RNAs (miRNA and mRNA) were reverse transcribed into cDNAs using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. To determine the expression levels of miR-18b, SMA, calponin and SMAD2, real-time quantitative-PCR was performed using TransStartTM SYBR Green qPCR Supermix (TransGen Biotech, Beijing, China). GAPDH and U6 small nuclear RNA was used as an internal normalized reference of mRNAs and miRNAs, respectively. The PCR reactions were performed in PCR System 7300 (ABI, Carlsbad, USA). The expressions of mRNAs and miRNAs were measured using the CT (cycle threshold) values and then the results were converted to fold changes.

2.4. Immunofluorescent staining

Cultured hHF-MSCs were fixed in 4% paraformaldehyde for 10 min. After cell membrane was permeablized with 0.1% Triton X-100 for 10 min, followed by incubation with anti-SMA or anti-calponin primary antibody (Abcam, Cambridge, UK) (1:100 dilution) overnight. After washes, the cells were incubated with Alexa fluor 488-conjugated goat anti-mouse or anti-rabbit secondary antibody (Abcam, Cambridge, UK) (1:200 dilution) for another hour. The cells were then stained with Hoechst 33342 (BD, New-York, USA) for 2 min, mounted on glass slides with glycerol, and observed under a fluorescent microscope.

2.5. Luciferase reporter assay

The 3′UTR sequence of the human SMAD2 gene containing the miR-18b binding sites was PCR amplified using the SMAD2 primers 5′-TCT AGA GTA GCC TCA TAC TCA TAA GGT GC-3′ and 5′-GCG GCC GCC ATG GTA AAC AAC TCA AAT GGC-3′, and was cloned into the *Hind*III and *Sac*I sites downstream of the luciferase reporter gene on the pMIR-REPORT miRNA expression reporter vector (Ambion, CA, USA). The resultant plasmid was named SMAD2-3′UTR-wt. Mutagenesis of different nucleotides within the predicted miR-18b binding sites was performed using an Easy Mutagenesis System kit (TransGen Biotech, Beijing, China), and the resultant plasmids were named SMAD2-3′UTR-mut1, SMAD2-3′UTR-mut2 and SMAD2-3′UTR-mut3.

HEK 293T cells were seeded in 24-well plates and transfected with a mixture of 400 ng of luciferase reporter vector and 20 ng of pRL-TK following the recommended protocol for the Lipofectamine 2000 transfection system (Invitrogen, Carlsbad, CA). After 48 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The experiments were performed in triplicate.

2.6. Western blot analysis

hHF-MSCs were lysed and the protein concentration was quantified with BCA assay reagent (Pierce Chemical Co., Rockford, IL). Protein samples were separated in 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocked in phosphate-buffered saline/Tween-20 containing 5% nonfat milk, the membranes were incubated with antibodies for SMAD2 or GAPDH (Santa Cruz, CA, USA). GAPDH was used as an internal control. The blots were then incubated with peroxidase-conjugated secondary antibodies, and then the protein signals were developed with an enhanced chemiluminescent method (Pierce Chemical Co., Rockford, IL).

2.7. Statistical analysis

Data are expressed as mean values ± standard deviations. The differences between groups were assessed using two-tailed student's *t* test. A difference with $p < 0.05$ was considered significant.

3. Results

3.1. miR-18b level was developmentally regulated during hHF-MSCs differentiation

As described previously, after 7 days of induction, the hHF-MSCs treated with TGF-β1, but not the untreated control cells, were positively stained by SMC-specific makers SMA and calponin proteins (Fig. 1A), indicating that TGF-β1 potently induced the differentiation of hHF-MSCs into SMCs. In an attempt to examine miRNAs involved in regulating TGF-β-induced SMC differentiation, we performed miRNA microarray analysis using total RNA harvested from hHF-MSCs treated with TGF-β1 at 5 ng/μL for 7 days. Several established SMC differentiation-related miRNAs such as miR-143, miR-145, and miR-146a were present, other miRNAs were also highly regulated, including miR-18b, which is functional but has not yet been studied in SMC differentiation. In order to confirm miR-18b expression changes observed in the microarray analysis, we performed quantitative RT-PCR assay to measure the dynamic expression of miR-18b at different time points during the differentiation process. The result showed that the level of miR-18b decreased for 3-fold 3 days after TGF-β1 induction in the differentiated SMCs, and kept decreasing as the incubation time extended (Fig. 1B). These findings suggested that miR-18b might play a role in the differentiation of hHF-MSCs into SMCs.

3.2. miR-18b inhibited TGF-β1-induced differentiation of HFMSCs

To investigate whether forced expression of miR-18b is able to modulate TGF-β1-induced SMC differentiation, hHF-MSCs were transfected with agomir-18b or antagomir-18b, and stimulated with TGF-β1 (5 ng/μl) for 7 days. (Fig. 2A) showed that *in vitro* overexpression of miR-18b obviously inhibited the expression of SMA and calponin during SMC differentiation of hHF-MSCs induced by TGF-β1, whereas inhibition of miR-18b with its complementary RNA analogs (antagomir-18b) resulted in enhancing SMC differentiation by a significant increase in SMA and calponin expression. Moreover, accompanied by the changes in proteins levels, SMA and calponin mRNAs levels were also markedly decreased or increased in hHF-MSCs transfected with agomir-18b or antagmir-18b, respectively (Fig. 2B). In addition, cells transfected with negative control miRNAs failed to show changes in mRNA and protein expressions of SMA and calponin. These results suggested that miR-18b was a key negative regulator of TGF-β1-induced hHF-MSCs differentiation into SMC.

3.3. The SMAD2 gene was the target of miR-18b

In order to explore the underlying molecular mechanism by which miR-18b promoted TGF-β1-induced hHF-MSCs differentiation into SMC, we performed a computational analysis using Targetscan (http://www.targetscan.org/) and miRBase (http://www.mirbase.org) to predict the possible target gene of miR-18b. We found that the human SMAD-2 gene contained two theoretic miR-18b binding sites in its 3′ UTR (Fig. 3A). To confirm that these sites contributed to the regulation of SMAD2 mRNA, we constructed a luciferase reporter vector with the SMAD2 3′UTR and performed the luciferase reporter activity assay. We also constructed plasmids in which the conserved miR-18b binding sequence, the poorly conserved miR-18b binding sequence, or both were mutated. When a wild type SMAD2 3′-UTR luciferase reporter was co-transfected with miR-18b mimics into HEK-293 cells, the relative luciferase activity was significantly reduced by almost 60% compared with that of the negative control, whereas mutation of one sequence or the other attenuated the repression mediated by the miR-18b mimics. In contrast, the luciferase activity in cells transfected with empty vector or the reporter carrying both mutant sequences was not affected (Fig. 3B).

Furthermore, we found that transfection of hHF-MSCs with miR-18b mimics efficiently reduced SMAD2 protein levels, whereas transfection with miR-18b inhibitors notably increased the expression of SMAD2 (Fig. 3C). On the contrary, there was no significant alteration in SMAD2 mRNA expression in cells transfected with both miR-18b mimics and inhibitors (Fig. 3D). These data indicated that miR-18b directly bound to the 3′UTR of SMAD2 mRNA and inhibited translation.

3.4. SMAD2. silencing abrogated the effect of antagomir-18b

To further investigate whether SMAD2 contributed to the effect of mir-18b in regulating hHF-MSCs differentiation, we transfected antagomir-18b into hHF-MSCs after SMAD2 knockdown (KD) and then performed TGF-β1-induced differentiation. Results of immunofluorescent staining (Fig. 4A) and qRT-PCR analysis (Fig. 4B) showed that adoption of antagomir-18b after transfection of NC could improve the TGF-β1-induced differentiation, whereas knockdown of SMAD2 inhibits the effects of antagomir-18b in promoting SMC differentiation. From a sidewise approach, these results demonstrated that depletion of the target could abrogate the effect of antagomir-18b, further implicating that the negative role of

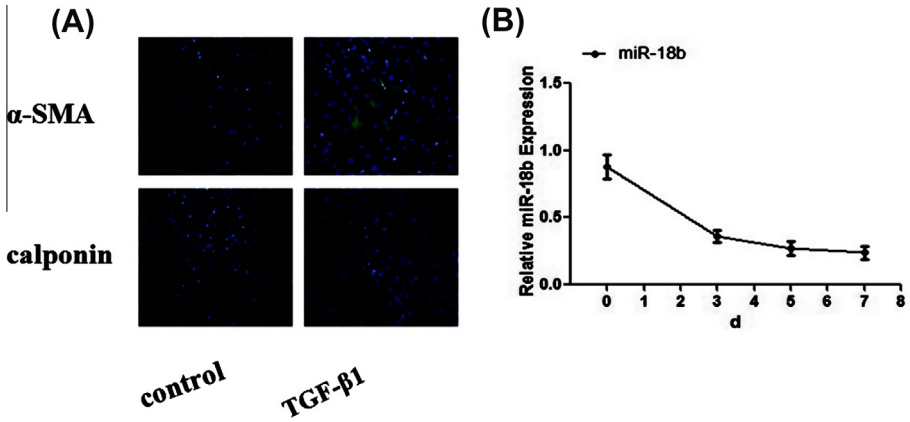

Fig. 1. miR-18b level was developmentally regulated during hHF-MSCs differentiation. (A) The expression of the SMC specific markers SMA and calponin in hHF-MSCs were detected by immunofluorescence staining over 7-day period after culture in GM and DM. (B) Downregulated expression of miR-18b in hHF-MSCs induced by TGF-β1 was determined by qRT-PCR at different stage of SMC differentiation (0 d, 3 d, 5 d, 7 d). Data were presented as means ± SD values from at least three independent experiments. Bars indicate SD. *Compared to the untreated hHF-MSCs (0 d), $p < 0.05$.

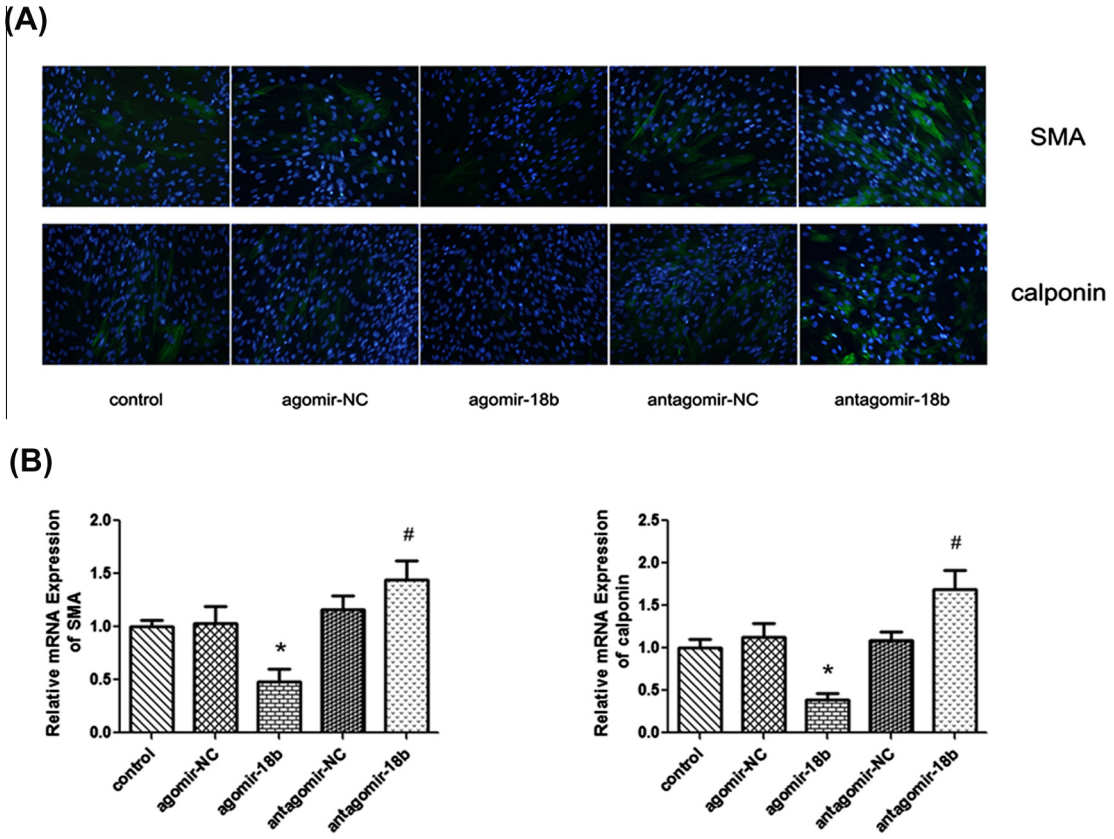

Fig. 2. The regulatory role of miR-18b in the expression of SMA and calponin in differentiated hHF-MSCs. (A) After 7 days treatment with TGF-β1 (5 ng/μl), the detection of SMA and calponin in hHF-MSCs transfected with agomir-NC, agomir-18b, antagomir-NC and antagomir-18b by immunofluorescence staining. (B) Quantitative PCR analysis of the effects of miR-18b on the levels of SMA and calponin mRNAs in hHF-MSCs treated with TGF-β1 (5 ng/μl) at day 7. Data were presented as means ± SD values from at least three in dependent experiments. Bars indicate SD. *Compared to the agomir-NC group, $p < 0.05$, #compared to the antagomir-NC group, $p < 0.05$.

miR-18b in the TGF-β1-induced SMC differentiation of hHF-MSCs is at least partially mediated via repressing SMAD2.

4. Discussion

Accumulating evidences have supported the idea that miRNAs play a crucial role in modulating stem cell self-renewal and differentiation by repressing the post-transcriptional regulation of target genes in stem cells and differentiating daughter cells. In this study, we focused on miR-18b, a miRNA markedly downregulated in response to TGF-β1 treatment and subsequently, quantitative RT-PCR analysis confirmed expression changes of miR-18b, implicating its potential negative role in SMC differentiation.

miR-18b belongs to the miR106a-363 cluster. Lately, it has been shown that miR-18b is also associated with germ cell maturation, embryonic development and stem cells differentiation. For instance, miR-18 directly targets heat shock factor 2 (HSF2) and regulates spermatogenesis [20]. Using microarray profiling of microRNAs, Knelangen et al. have uncovered that miR-18b is significantly downregulated during adipogenic differentiation of the mouse embryonic stem cells [21]. Additionally, the level of miR-18b is markedly increased after hormonal stimulation and accelerated adipogenic differentiation of mouse preadipocytes 3T3L1 [22]. Here, we explored a new role of miR-18b in hHF-MSCs differentiation. We found that cells transfected with miR-18b had reduced levels of SMC-specific markers, and that the antagonism of miR-18b with antagmir-18b promoted TGF-β1-induced conversion of hHF-MSCs into SMCs. Interestingly, our data showed that miR-18b was significantly downregulated upon TGF-β1 treatment, which created a positive feedback loop of TGF-β1/SMADs signaling, and therefore facilitated SMC differentiation.

To identify the potential miR-18b targeting gene that is related to TGF-β1-induced differentiation of hHF-MSCs into SMCs, a search with Targetscan and miRBase online software for prediction were performed. It was revealed that SMAD2, a transcription regulator in the TGF-β signaling cascade, might be a possible target with duple match sites (one conserved and one poorly conserved) complementary to miR-18b in the 3′UTR of its mRNA. Our luciferase reporter analysis demonstrated that over-expression of miR-18b downregulated the luciferase activity when miR-18b miRs regulatory elements (MREs) from SMAD2 3′UTR was inserted into the luciferase reporter vector, and that the effect was reduced when mutated MREs sequence was utilized. Furthermore, we examined the effect of miR-18b on the mRNA and protein levels of SMAD2. Our data showed that both miR-18b mimic and miR-18b inhibitor regulated SMAD2 expression at the protein level without markedly affecting mRNA levels, suggesting that miR-18b inhibited SMAD2 at the post-translational level.

SMAD proteins are the classical intracellular mediators of TGF-β signaling, and can be divided into three functional subclasses: receptor-activated SMADs (R-SMADs), common partner SMADs (Co-SMADs) and inhibitory SMADs (I-SMADs) [23,24]. After the ligands bind to the TGF-β receptors, R-SMADs are directly activated via phosphorylation, and the phosphorylated R-SMADs bind to Co-SMADs to form a homomeric complex [25]. The hetero-oligomer translocates to the nucleus and then positively or negatively regulates the transcription of target genes. SMAD2 and SMAD3 are R-SMADs that corporately involved in TGF-β1-induced differentiation of various types of stem cells into SMCs. Chen et al. have

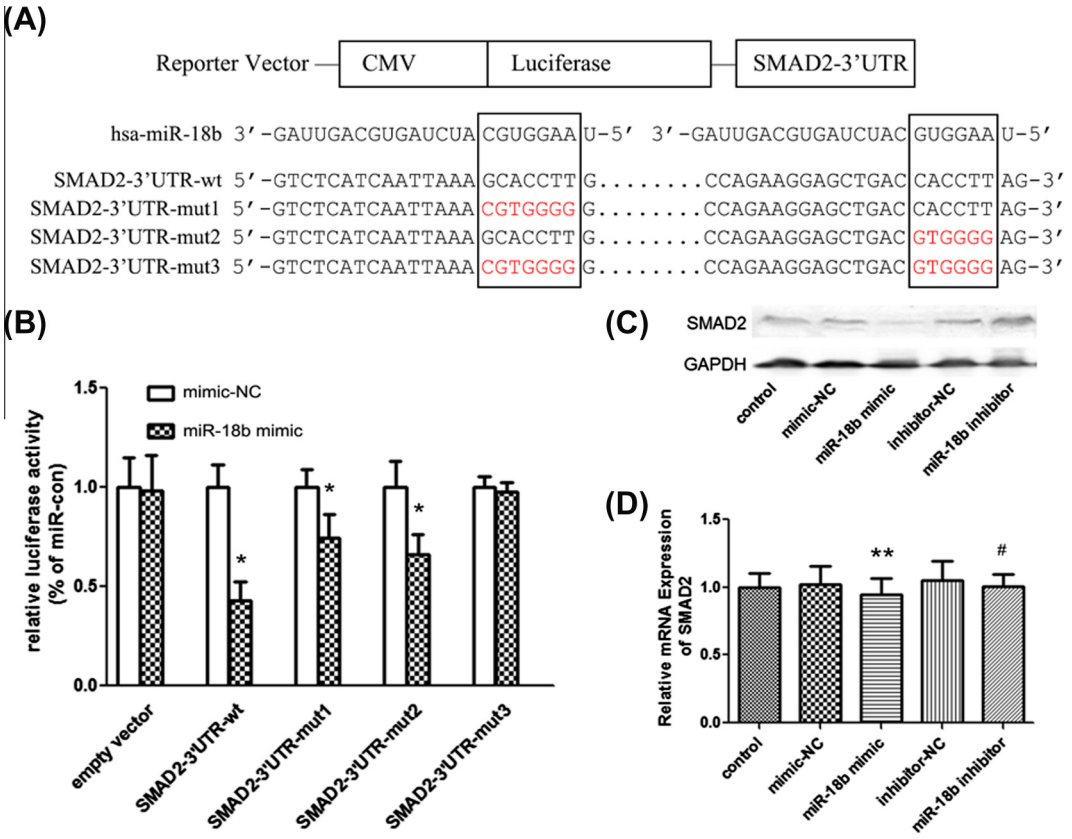

Fig. 3. miR-18b posttranscriptionally downregulates SMAD2 expression by directly targeting its 3′-UTR. (A) The two predicted miR-18b binding sites within the SMAD2 3′-UTR, including a conserved sequence and a poorly conserved sequence. The wild type SMAD2 3′UTR and 3 different mutated sequences (mut1, mut2 and mut3) were cloned into the region downstream of the luciferase gene to construct the luciferase assay reporter. (B) Dual luciferase assay was performed on HEK293 cells transfected with empty vector (control), SMAD2-3′UTR-wt, SMAD2-3′UTR-mut1, SMAD2-3′UTR-mut2 and SMAD2-3′UTR-mut3. (C) Western blot assays of the SMAD2 protein levels in hHF-MSCs (control) or those infected with miR-18b mimic, miR-18b inhibitor or miR negative control. (D) The influences of miR-18b mimic or miR-18b inhibitor on the mRNA level of SMAD2 gene. Data were presented as means ± SD values from at least three in dependent experiments. Bars indicate SD. *Compared to the mimic-NC group, $p < 0.05$, **compared to the agomir-NC group, $p > 0.05$, #compared to the antagomir-NC group, $p > 0.05$.

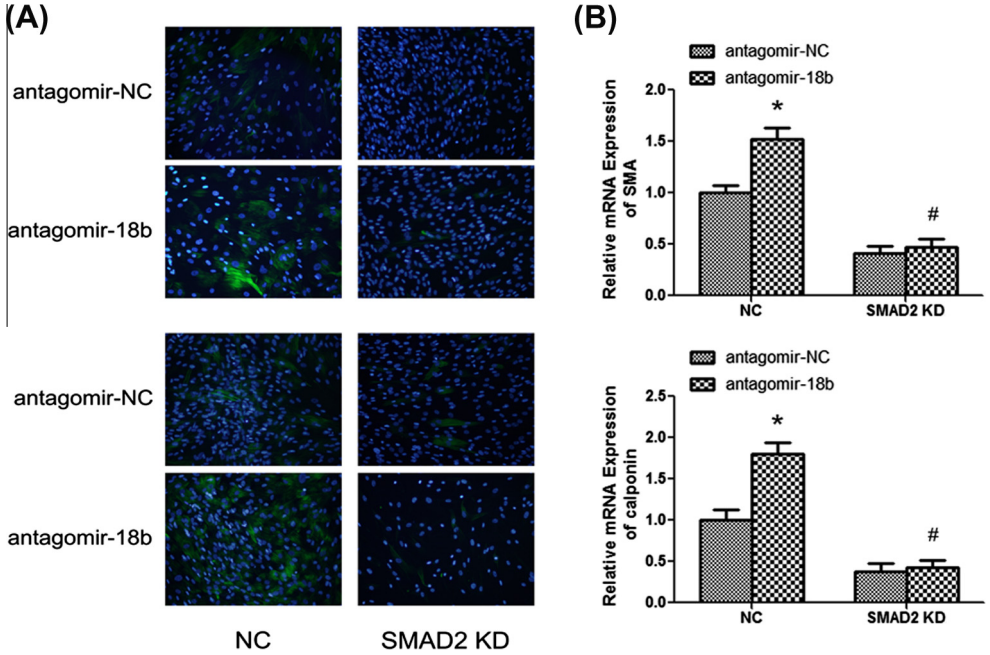

Fig. 4. SMAD2 knockdown (KD) blocked the effects of miR-18b on TGF-β-induced differentiation. (A) Transfected with antagomir-18b and antagomir-NC, wild type hHF-MSCs and SMAD2 KD hHF-MSCs were stained to show SMA (upper) and calponin (lower) expression over 7-day period after TGF-β1 (5 ng/μl) stimulation. (B) The expression of SMA and calponin were analyzed by qRT-PCR among four groups. Data were presented as means ± SD values from at least three independent experiments. Bars indicate SD. *Compared to the antagomir-NC group, $p < 0.05$, #compared to the antagomir-NC group, $p > 0.05$.

shown that SMAD2 and SMAD3 are rapidly phosphorylated after TGF-β stimulation, and that knockdown of SMAD2 or SMAD3 expression lead to a loss of α-SMA expression in neural crest stem cells [26]. Likewise, Huang et al. have demonstrated that knockdown of SMAD2 by short hairpin RNA results in downregulation of response gene to complement 32 (RGC-32) and SMC marker genes [27]. However, SMAD2 and SMAD3 have distinct roles in promoting embryonic SMC specification and in regulating SMC differentiation. Smad2-deficient mice embryos die early at embryonic day 7.5–12.5 due to defective primitive streak and mesoderm formation [28,29]. In comparison, SMAD3 knockout mice are born with a normal vasculature [30]. Furthermore, Sinha et al., have found that the SMA activity is dependent on both SMAD2 and SMAD3, whereas the SM-MHC activity is only SMAD2-dependent during SMC differentiation of mouse embryonic stem cells [31]. All these reports suggest that SMAD2, but not SMAD3, is indispensable for SMC development and gene expression. In this study, we performed SMAD2 knockdown and detected significant downregulation of SMC-specific makers, supporting the key role of SMAD2 in the differentiation of hHF-MSCs into SMCs. Besides, deletion of SMAD2 significantly abrogated the effect of miR-18b inhibition, which suggested that SMAD2 was a major, if not the only, downstream target of miR-18b that contributed to miR-18b mediated regulation of TGF-β1-induced SMC differentiation.

In conclusion, for the first time we demonstrated that miR-18b was downregulated during TGF-β1-induced differentiation of hHF-MSCs into SMCs, and that the downregulation of miR-18b positively regulated its direct target gene SMAD2 and promoted the differentiation of hHF-MSCs into SMCs. These findings indicate the key role of miR-18b in TGF-β1-induced SMC differentiation, reveal the molecular mechanism of directed differentiation of stem cells and provide new insights into the differentiation mechanism.

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