

1 α ,25-Dihydroxyvitamin D₃ prevents the differentiation of human lung fibroblasts via microRNA-27b targeting the vitamin D receptor

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Abstract. Pulmonary fibroblasts have key roles in the formation and maintenance of lung structure and function, and are involved in tissue repair and remodeling. Transforming growth factor- β 1 (TGF- β 1) induces differentiation of fibroblasts into myofibroblasts, the key effector cells in fibrotic states, which are characterized by the expression of α -smooth muscle actin (α -SMA) markers. 1 α ,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] has been implicated in regulating differentiation, and the vitamin D receptor (VDR) may be a regulator of TGF- β signaling. In addition, there is presently only limited information regarding microRNA (miRNA) regulation of lung fibroblast differentiation. To determine the role of 1,25(OH)₂D₃ in regulating the differentiation of fibroblasts induced by TGF- β 1 and the functional importance of *miR-27b*, cell culture systems, cell transfection and the 3' untranslated region (3'UTR) luciferase assay were employed. 1,25(OH)₂D₃ inhibited differentiation and downregulated *miR-27b* expression in human lung fibroblasts induced by TGF- β 1. In addition, human lung fibroblasts were transfected with *miR-27b* mimic or *miR-27b* inhibitor, and demonstrated that the overexpression of *miR-27b* decreased the VDR protein expression and

increased the expression of α -SMA, while reducing levels of *miR-27b* had opposing effects. Finally, the luciferase reporter assays were performed to confirm that *miR-27b* directly targeted VDR 3'UTR. Taken together, these results suggest that 1,25(OH)₂D₃ inhibits lung fibroblast differentiation induced by TGF- β 1 via *miR-27b* targeting VDR 3'UTR, which may be used as a novel treatment strategy in differentiation pathways.

Introduction

1 α ,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃], the active form of vitamin D, has long been known for its effects on bone mineralization and calcium homeostasis. However, its physiological importance outside of bone health and calcium homeostasis effects has received increasing attention and it has been recognized as a key regulator of cell proliferation, differentiation, apoptosis and immunomodulation (1-4). In this regard, several studies have identified that vitamin D may have a role in multiple chronic lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), respiratory infections, lung cancer, interstitial lung disease, cystic fibrosis and pulmonary arterial hypertension (PAH) (5-12).

Vitamin D₃ is a steroid prehormone that requires two hydroxylation steps in the liver and kidney to generate the biological active form, 1,25(OH)₂D₃. The biological effects of 1,25(OH)₂D₃ are mediated through the vitamin D receptors (VDRs), which belong to the superfamily of steroid/thyroid nuclear hormone receptors and are expressed widely in the body, including the lungs and cells of the immune system (8). Following ligand binding, the VDR forms a heterodimer with retinoid X receptor (RXR) and binds to the vitamin D responsive element in the regulatory region of the target genes to activate or repress their transcription (13,14).

Pulmonary fibroblasts have key roles in the formation and maintenance of lung structure and function, and are involved in tissue repair and remodeling, which is a key feature of a number of chronic lung diseases such as asthma, COPD, pulmonary fibrosis and PAH (15-20). Transforming growth factor- β 1 (TGF- β 1) is secreted by numerous cell types and implicated in a wide range of cell functions, critically

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Abbreviations: 1,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; α -SMA, α -smooth muscle actin; RXR, retinoid X receptor; TGF- β , transforming growth factor- β ; miRNA, microRNA; UTR, untranslated region; qPCR, quantitative polymerase chain reaction

Key words: 1 α ,25-dihydroxyvitamin D₃, α -smooth muscle actin, vitamin D receptor, transforming growth factor- β 1, fibroblast, *miR-27b*, differentiation

regulating cell proliferation, growth, differentiation, apoptosis, cell movement, and extracellular matrix secretion and deposition (21-24). Additionally, TGF- β 1 is a crucial regulator of fibroblast phenotype and function. TGF- β 1-stimulated fibroblasts undergo phenotypic transition and differentiate into myofibroblasts, the key effector cells in fibrotic states, which are characterized by the expression of α -smooth muscle actin (α -SMA) fibers, contributing to the progression of pulmonary fibrogenesis (25,26). Therefore, inhibition of fibroblast proliferation and differentiation may prove to be a common and effective approach to attenuate pulmonary fibrosis.

Previous studies demonstrated that 1,25(OH)₂D₃ was capable of inhibiting the TGF- β -mediated tissue remodeling responses in cultured lung fibroblasts and blocking myofibroblastic transformation of fibroblasts by TGF- β 1 (18,27). In addition, several studies showed cross-talk between the TGF- β and vitamin D signaling pathways. VDR binds to the MH1 domain of Smad3, which belongs to a receptor of the TGF- β superfamily, thereby enhancing Smad3 ligand-induced transactivation (28-31). However, the molecular mechanisms are far from understood. MicroRNAs (miRNAs) have attracted increasing attention due to their significant roles in diverse biological processes, including developmental processes, cell proliferation, differentiation, apoptosis, stress responses, and cancer initiation and progression (32-37). miRNAs are short non-coding RNAs with wide gene regulatory activity at the post-transcriptional level, which forms RNA silencing complexes with several proteins to cause mRNA degradation or translation inhibition, or both processes (32,33). The relatively few studies of miRNAs in lung fibrogenesis include *miR-155*, *Let-7* family, *miR-29* family, *miR-21*, *miR-30* family, *miR-145* and *miR-27b* (38-42). The present study aimed to evaluate the ability of 1,25(OH)₂D₃ to inhibit the differentiation of lung fibroblasts and the participation of *miR-27b* in this process. The first aim of the study was to determine the role of 1,25(OH)₂D₃ in regulating the differentiation of human lung fibroblasts induced by TGF- β 1. Furthermore, whether *miR-27b* may be relevant for the regulating effect of 1,25(OH)₂D₃ was also determined.

Materials and methods

Materials and reagents. The α -SMA (cat. no. sc-53015), VDR (cat. no. sc-13133) and β -actin antibodies (cat. no. sc-47778) were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Secondary antibodies for goat anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) and goat anti-mouse IgG labeled with Cy3 were sourced from Boster (Hubei, China). 1,25(OH)₂D₃ (cat. no. D1530) and human recombinant TGF- β 1 (cat. no. 240-B-002/CF) were obtained from Sigma-Aldrich (St. Louis, MI, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Scrambles, miRNA mimics and inhibitors were from Ambion (Austin, TX, USA). α -SMA, VDR and β -actin primers were produced by BGI-Beijing (Beijing, China).

Cell culture. MRC5 human lung fibroblasts and 293A cells were obtained from the Cell Resource Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). These two types of cells were incubated in Dulbecco's modified Eagle's medium (DMEM)

with 4.5 g/l of glucose supplemented (Gibco, Carlsbad, CA, USA), supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified atmosphere with 5% CO₂. MRC5 cells were cultured to ~80% confluence, serum-starved for 24 h, and were treated for 48 h in 2% FBS medium with an ethanol vehicle or 1,25(OH)₂D₃ (100 nM) in the absence or presence of rhTGF- β 1 (10 ng/ml).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was generated from total RNA using PrimeScript RT Master mix (5X) (Takara, Dalian, China), according to the manufacturing instructions. To quantify mRNA expression, SYBR Premix Ex Taq (2X) (Takara) was used. The qPCR thermal cycling protocol was programmed in the CFX96™ Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA) and consisted of an initial denaturation step at 95°C for 30 sec, followed by 40 cycles of denaturation for 5 sec at 95°C, and annealing and extension for 30 sec at 60°C. A primer pair for the detection of human β -actin was used as the internal control. RT-qPCR primers were based on GenBank published sequences and were as follows: α -SMA sense, 5'-GGC GGT GCT GTC TCT CTA TG-3' and antisense, 5'-CCC ATC AGG CAA CTC GTA AC-3'; VDR sense, 5'-GGC CGG ACC AGA AGC CTT T-3' and antisense, 5'-CAG CCT TCA CAG GTC ATA GCA-3'; β -actin sense, 5'-ACT GGA ACG GTG AAG GTG AC-3' antisense, 5'-GGC ACG AAG GCT CAT CAT-3'.

miRNA extracts were prepared using an mirVana™ miRNA Isolation kit (Ambion) and subsequently reverse transcribed using a TaqMan miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. miRNA expression levels were quantified using a TaqMan Universal Master mix II (2X) (Applied Biosystems), normalized to U6 snRNA. Results are presented as fold changes in gene expression calculated using the $\Delta\Delta$ Ct method.

Western blotting. Cells were plated in 60-mm dishes in 10% FBS-DMEM, cultured until nearly 80% confluence and serum-starved for 24 h, and subsequently the cells were treated as indicated. After 48 h culture, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and were subsequently harvested with cell lysis buffer with protease inhibitors [20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l EDTA, 1 mmol/l EGTA, 20 mol/l Na₄P₂O₇, 2 mol/l Na₃VO₄, 0.1% SDS, 10% glycerol, 2 μ g/ml leupeptin and 1 mmol/l PMSF]. Upon centrifugation at 13,000 \times g for 5 min at 4°C, the supernatants were collected and the total protein concentrations were determined by the bicinchoninic acid protein assay kit (Beyotime, Shanghai, China) following the manufacturer's instructions. SDS-PAGE (10%) gels were prepared and 10-20 μ g/lane of cellular proteins was loaded. The resolved proteins were transferred to a polyvinylidene difluoride membrane and incubated with primary antibodies (α -SMA, 1:100 dilution; VDR, 1:50 dilution) following the manufacturer's instructions. Following incubation with HRP-conjugated secondary antibodies

(1:1,000 dilution), the blotting bands were visualized with ECL chemiluminescent kit (Thermo Scientific, Rockford, IL, USA) and quantified with a ChemiDoc™ XRS⁺ Imaging System (Bio-Rad).

Immunofluorescence. Fibroblasts growing on cover slides were fixed in 4% paraformaldehyde for 1 h. Following being permeabilized with 0.2% Triton X-100 in Tris-buffered saline (TBS) for 30 min, the cells were blocked in TBS containing 2% bovine serum albumin for 1 h. Cells were subsequently incubated with anti- α -SMA antibody (1:50 dilution) at 4°C overnight. Cells were washed 3 times and were incubated at room temperature with Cy3-conjugated goat anti-mouse secondary antibody (1:50 dilution) for 1 h. Following this, cells were counterstained with 4',6-diamidino-2-phenylindole (Boster) for nuclear staining, 5 min in the dark and were washed 3 times. Negative controls were carried out by omitting the primary antibody. Fluorescent images were captured with a laser scanning confocal microscope (Olympus FV1000; Olympus, Tokyo, Japan).

Cell transfection. Transfection was performed with scramble, *miR-27b* mimic (50 nM) or *miR-27b* inhibitor (100 nM) (Ambion). All the transfection experiments were conducted with Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Cells in medium containing 10% FBS were seeded in each well and incubated at 37°C. The transfection mixture was incubated at room temperature for 10 min, and was added to a 6-well plate. After 24 h, cells were serum-starved for an additional 24 h, following which they were treated with vehicle control, 1,25(OH)₂D₃ or TGF- β 1 for 48 h in 2% FBS medium. Cells were subsequently harvested and total RNA, miRNA or proteins were extracted and analyzed by RT-qPCR or western blot analysis.

3' Untranslated region (3'UTR) luciferase assay. A synthetic oligonucleotide was inserted at the 3'UTR region of the luciferase reporter gene of the pMIR-target vector. The wild-type and mutant 3'UTR of the VDR genes were amplified by PCR and subcloned in the pMIR-target vectors. The constructs were co-transfected into 293A cells along with scramble (50 nM), *miR-27b* mimic (50 nM) or *miR-27b* inhibitor (100 nM) using Lipofectamine 2000, according to the manufacturer's instructions. Luciferase activities were measured with the Dual Luciferase Reporter Assay system (Promega, Madison, WI, USA) 48 h after transfection.

Statistical analysis. Values are expressed as means \pm standard error of the mean derived from at least three independent experiments. Comparison of two groups was made with an unpaired, two-tailed Student's t-test. Comparison of multiple groups was made with a one-way analysis of variance followed by Dunnett or Tukey test. $P < 0.05$ was considered to indicate a statistically significant difference. All the statistical analyses were performed and graphs were plotted using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

Results

1,25(OH)₂D₃ inhibits differentiation and upregulates VDR protein expression of human lung fibroblasts induced by

TGF- β 1. Previous studies have documented that 1,25(OH)₂D₃ opposed the effect of TGF- β 1 on mouse lung fibroblasts differentiation, as demonstrated by its ability to inhibit TGF- β 1-induced expression of α -SMA (27). Consequently, whether 1,25(OH)₂D₃ could inhibit α -SMA expression of human lung fibroblasts induced by TGF- β 1 was examined. Based on previous experiments, the optimum concentration of 1,25(OH)₂D₃ (100 nM) was chosen as the dose for the present experiments. Notably, RT-qPCR and western blot analysis revealed that TGF- β 1 significantly upregulated α -SMA expression at the mRNA and protein levels in MRC5 cells; however, 1,25(OH)₂D₃ markedly inhibited this effect (Fig. 1A and C). Confocal immunofluorescence analysis of MRC5 fibroblasts also revealed that TGF- β 1 increased levels of α -SMA, and the process was inhibited by 1,25(OH)₂D₃ (Fig. 1D).

Several studies showed cross-talk between the TGF- β and vitamin D signaling pathways (28-31) and TGF- β contributed to the decreased expression of VDR in dermal fibroblasts of healthy volunteers (43). Furthermore, 1,25(OH)₂D₃ had a significant effect *in vivo* on the TGF- β signaling pathway by altering levels of VDR and Smad3, and subsequently affecting the bioactive of TGF- β 1 (44). Based on this evidence, we speculated that VDR may be a negative regulator of fibroblast differentiation induced by TGF- β . TGF- β significantly decreased the protein expression of VDR, and treatment of TGF- β -stimulated fibroblasts with 1,25(OH)₂D₃ effectively upregulated the VDR protein level (Fig. 1C). However, the levels of the VDR transcripts did not change (Fig. 1B).

1,25(OH)₂D₃ downregulates TGF- β 1-induced miR-27b expression in human lung fibroblasts. As TGF- β 1 is a critical cytokine in the pathogenesis of human lung fibroblast differentiation, and a previous study reported that *miR-27b* was downregulated in the lungs of mice that were administered with bleomycin, a widely used animal model of lung fibrosis (45), we hypothesized that *miR-27b* participates in this process and could be relevant for the inhibitory effect of 1,25(OH)₂D₃ on the differentiation of lung fibroblasts induced by TGF- β 1. Therefore, the levels of *miR-27b* were examined in MRC5 cells treated with the ethanol vehicle or 1,25(OH)₂D₃ in the absence or presence of TGF- β 1. In the present study, *miR-27b* expression levels were significantly higher in MRC5 cells induced by TGF- β 1. However, treatment of TGF- β -stimulated fibroblasts with 1,25(OH)₂D₃ effectively decreased *miR-27b* expression (Fig. 2). These data suggest that *miR-27b* may have a role in regulating the differentiation phenotype of the pulmonary fibroblasts.

miR-27b regulates differentiation and VDR expression of human lung fibroblasts. To determine whether *miR-27b* regulates the differentiation phenotype of the pulmonary fibroblasts, human lung fibroblasts were transfected with scramble, *miR-27b* mimic or *miR-27b* inhibitor and evaluated α -SMA levels in these cells. Overexpression of *miR-27b* markedly increased the baseline levels of the α -SMA transcripts and α -SMA proteins in lung fibroblasts (Fig. 3A-a and c). The *miR-27b* inhibitor decreased the α -SMA levels in lung fibroblasts (Fig. 3B-a and c). Given that TGF- β 1 upregulated *miR-27b* in lung fibroblasts, these data suggest that *miR-27b* may mediate TGF- β 1-induced α -SMA expression. TGF- β 1-treated

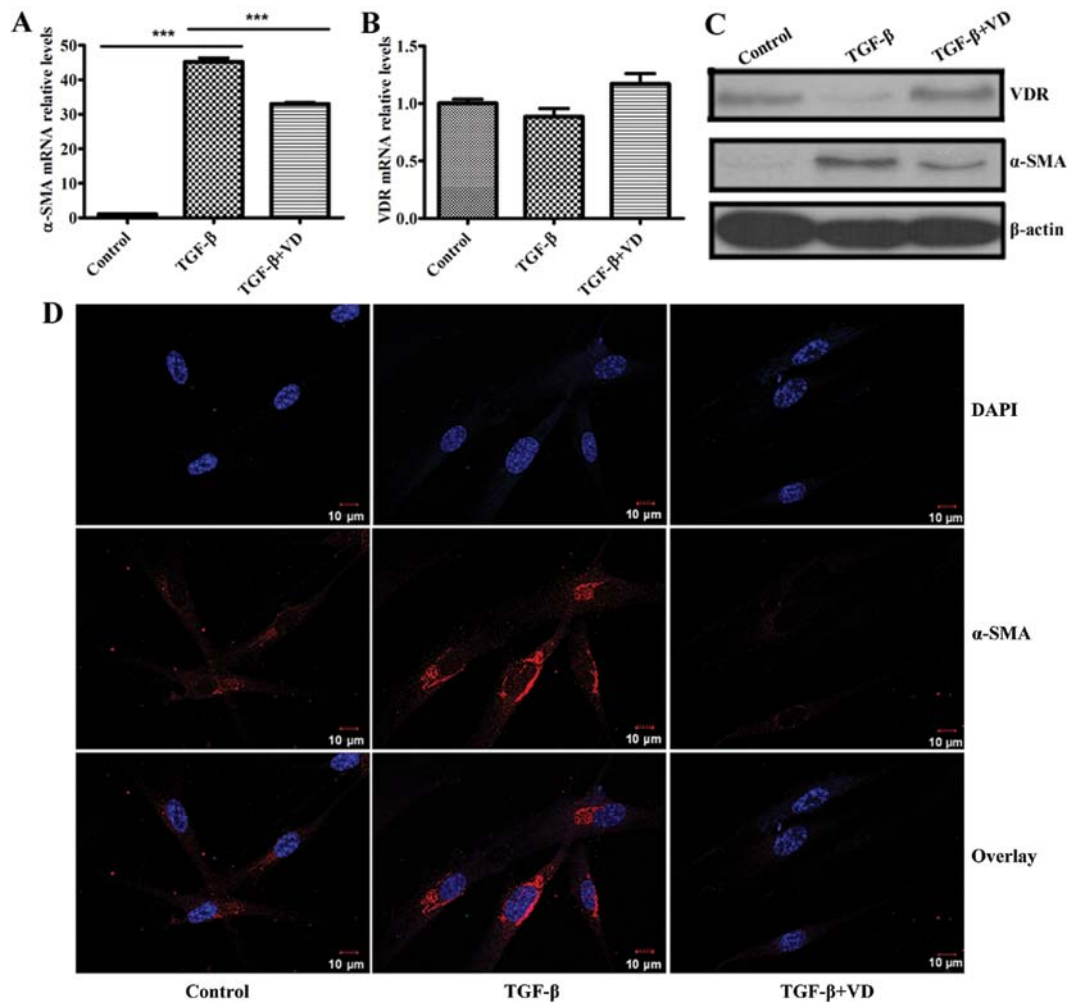


Figure 1. 1 α ,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃; VD] downregulates α -smooth muscle actin (α -SMA) expression at the mRNA and protein levels, and upregulates vitamin D receptor (VDR) protein expression of human lung fibroblasts induced by transforming growth factor- β 1 (TGF- β 1). The human lung fibroblast MRC-5 cell line was treated with an ethanol vehicle (control) or 100 nM 1,25(OH)₂D₃ in the absence or presence of 10 ng/ml TGF- β 1 (TGF- β) for 48 h. (A) α -SMA mRNA expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was detected and normalized to β -actin expression. ***P<0.001 TGF- β vs. control; ***P<0.001 TGF- β + VD vs. TGF- β . (B) VDR mRNA expression was assessed by RT-qPCR and normalized to β -actin expression. No statistically significant differences exist between the three groups of control, TGF- β and TGF- β + VD. (C) Levels of α -SMA, VDR and β -actin protein were determined by western blot analysis. α -SMA protein expression analysis was consistent with the mRNA expression analysis in (A). (D) Immunofluorescence microscopy was used to assess the degree of α -SMA (red).

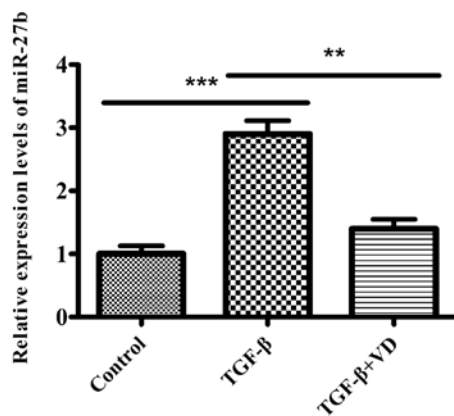


Figure 2. 1 α ,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] downregulates transforming growth factor- β 1 (TGF- β 1)-induced *miR-27b* expression in lung fibroblasts. The human lung fibroblast MRC-5 cell line was treated with an ethanol vehicle (control), 10 ng/ml TGF- β 1 (TGF- β) or 10 ng/ml TGF- β 1 plus 100 nM 1,25(OH)₂D₃ (VD) for 48 h. The expression of *miR-27b* was assessed by reverse transcription-quantitative polymerase chain reaction and normalized to U6 expression. ***P<0.001 TGF- β vs. control; **P<0.01 TGF- β + VD vs. TGF- β .

lung fibroblasts were transfected with *miR-27b* inhibitor and subsequently evaluated α -SMA levels in the cells. *miR-27b* inhibitor attenuated TGF- β 1-induced α -SMA expression at the mRNA and protein levels in lung fibroblasts (Fig. 3C-a and c). These data indicate that TGF- β 1-mediated α -SMA expression requires, at least in part, the induction of *miR-27b*. In addition, these initial experiments have demonstrated that 1,25(OH)₂D₃ reduced α -SMA expression and downregulated *miR-27b* expression in human lung fibroblasts induced by TGF- β 1. When the *miR-27b* mimic was transfected into the human lung fibroblasts, the reduced expression of α -SMA protein in 1,25(OH)₂D₃-treated cells was attenuated by the *miR-27b* mimic (Fig. 3D-a and c).

Given that VDR can be a negative regulator of fibroblast differentiation induced by TGF- β , whether *miR-27b* regulates VDR gene expression was further investigated. MRC5 cells were transfected with scramble, *miR-27b* mimic or *miR-27b* inhibitor, and VDR levels were evaluated in these cells. The *miR-27b* mimic reduced VDR protein levels in lung

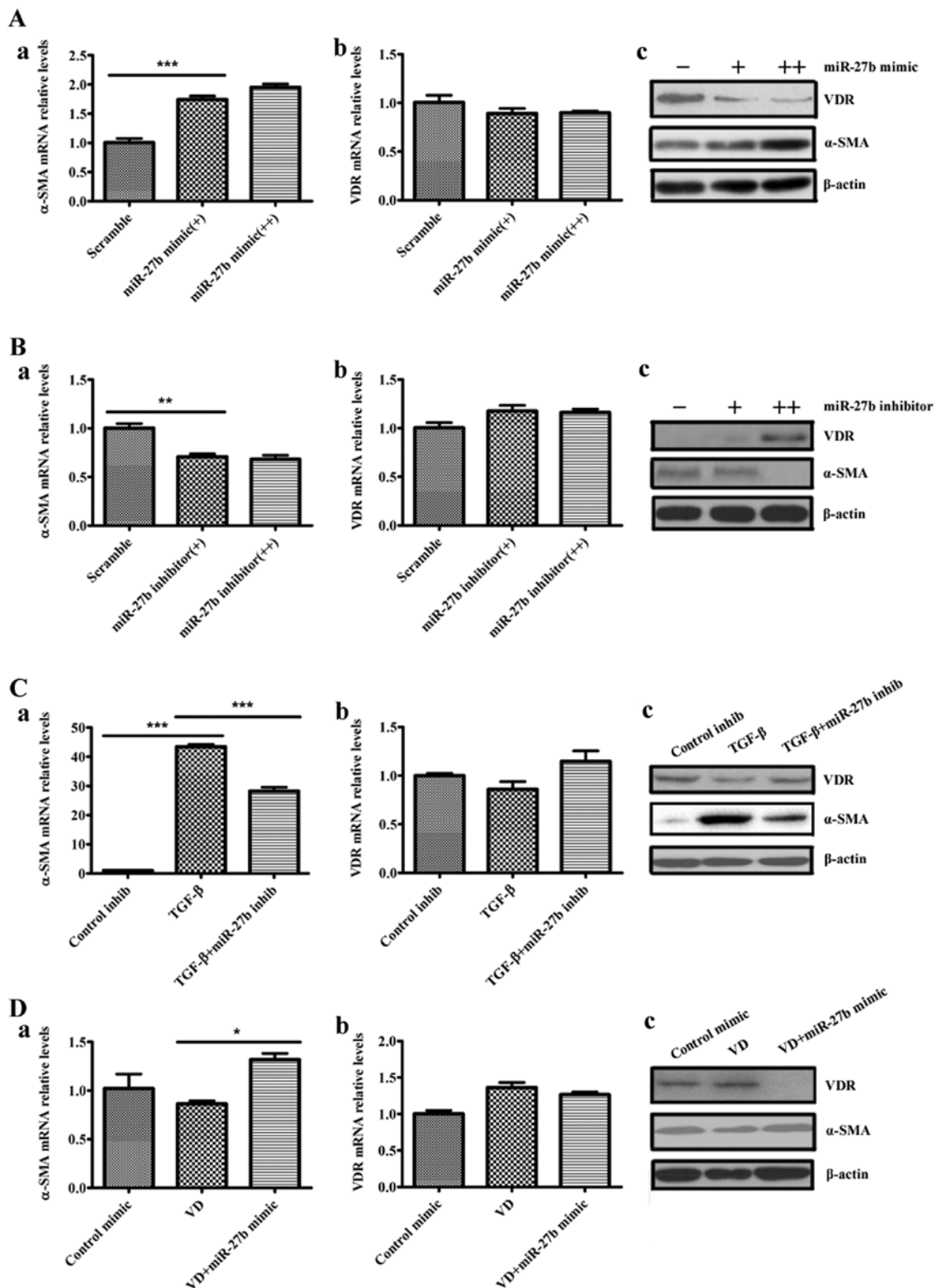


Figure 3. *miR-27b* regulates differentiation and vitamin D receptor (VDR) expression of human lung fibroblasts. (A) Human lung fibroblasts were transfected with 50 nM scramble, 50 nM *miR-27b* mimic (+) or 100 nM *miR-27b* mimic (++). At 48 h after transfection, levels of α -smooth muscle actin (α -SMA) and VDR were determined by (a and b) reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and (c) western blot analysis, normalized to β -actin expression. *** P <0.001 *miR-27b* mimic (+) vs. scramble. (B) Human lung fibroblasts were transfected with 100 nM scramble, 100 nM *miR-27b* inhibitor (+) or 200 nM *miR-27b* inhibitor (++), and 48 h post transfection, RNA or whole cell lysates were harvested. The expression levels of α -SMA and VDR were determined by (a and b) RT-qPCR and (c) western blot analysis, normalized to β -actin expression. ** P <0.01 *miR-27b* inhibitor (+) vs. scramble. (C) Human lung fibroblasts were transfected with 100 nM control inhibitor (control inhib) or 100 nM *miR-27b* inhibitor (*miR-27b* inhib). At 24 h after transfection, cells were serum-starved for an additional 24 h, after which they were treated with transforming growth factor- β 1 (TGF- β 1) for 48 h. RNA or whole cell lysates were subsequently harvested. The expression levels of α -SMA and VDR were determined by (a and b) RT-qPCR and (c) western blot analysis, normalized to β -actin expression. *** P <0.001 TGF- β vs. control inhib; *** P <0.001 TGF- β + *miR-27b* inhib vs. TGF- β . (D) Human lung fibroblasts were transfected with 50 nM control mimic or 50 nM *miR-27b* mimic. At 24 h after transfection, cells were serum-starved for an additional 24 h, after which they were treated with 100 nM $1\alpha,25$ -dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (VD) for 48 h. RNAs or whole cell lysates were subsequently harvested. The expression levels of α -SMA and VDR were determined by (a and b) RT-qPCR and (c) western blot analysis, normalized to β -actin expression. * P <0.05 VD + *miR-27b* mimic vs. VD.

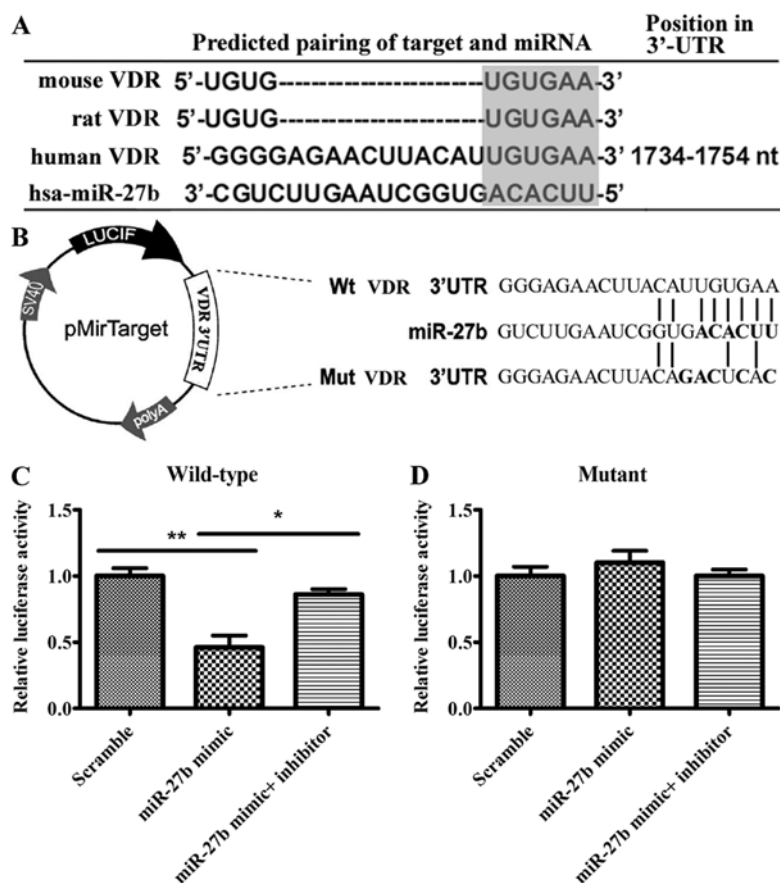


Figure 4. *miR-27b* directly targets vitamin D receptor (VDR) 3' untranslated region (3'UTR). (A) *miR-27b* is predicted to target VDR. Conservation among mammals of the *miR-27b* binding site sequence located within the VDR 3'UTR is highlighted in gray. The VDR 3'UTR has an exact match to the critical seed sequence of *miR-27b*. (B) Schematic of the pMirTarget reporter construct. The sequences of the wild-type (wt) VDR 3'UTR, *miR-27b* and the mutated (mut) VDR 3'UTR are shown to the right, with bars indicating a sequence match. (C) Luciferase reporter activity in 293A cells co-transfected with 50 nM scramble, 50 nM *miR-27b* mimic or 50 nM *miR-27b* mimic plus 100 nM *miR-27b* inhibitor and a vector containing the wild-type VDR 3'UTR. Data are expressed as luciferase values relative to the scramble. ** $P < 0.01$ *miR-27b* mimic vs. scramble; * $P < 0.05$ *miR-27b* mimic/*miR-27b* inhibitor vs. *miR-27b* mimic. (D) Luciferase reporter activity in 293A cells co-transfected with 50 nM scramble, 50 nM *miR-27b* mimic or 50 nM *miR-27b* mimic plus 100 nM *miR-27b* inhibitor and a vector containing the mutated VDR 3'UTR. Data are expressed as luciferase values relative to the scramble. No statistically significant differences exist between the three groups.

fibroblasts (Fig. 3A-c). The *miR-27b* inhibitor increased the VDR protein levels in these cells (Fig. 3B-c). However, *miR-27b* had no effect on the levels of the VDR transcripts (Fig. 3A-b and B-b), suggesting that *miR-27b* may affect the VDR translation, but not the transcripts in lung fibroblasts. These initial experiments have demonstrated that TGF- β 1 upregulates *miR-27b* and decreases VDR protein expression, but 1,25(OH)₂D₃ opposed the above effects of TGF- β 1. The reduced expression of VDR protein in TGF- β 1-treated cells was attenuated by the *miR-27b* inhibitor (Fig. 3C-c). Furthermore, the *miR-27b* mimic resulted in a decrease of VDR protein expression in lung fibroblasts treated with 1,25(OH)₂D₃ (Fig. 3D-c). Similarly, *miR-27b* had no effect on the levels of the VDR transcripts (Fig. 3C-b and D-b). Taken together, these data suggest that *miR-27b* promotes the differentiation of human lung fibroblasts by suppressing VDR protein expression.

***miR-27b* directly targets VDR 3'UTR.** The aforementioned results demonstrate that *miR-27b* can regulate VDR expression; however, they do not prove that there is a direct interaction between *miR-27b* and the mRNA of VDR. To further substantiate that *miR-27b* targets VDR directly, whether there was

a perfect match between the seed sequence of *miR-27b* and a region in the 3'UTR of VDR was verified. In addition, this binding site is identical among mammals (Fig. 4A). These results suggested that VDR may be regulated by *miR-27b*, thus this prediction was evaluated. Luciferase reporter constructs were used, incorporating a wild-type or mutant 3'UTR of VDR, in which the sequence corresponding to the seed region was altered (Fig. 4B). The reporter vectors were subsequently co-transfected into 293A cells with scramble, *miR-27b* mimic or *miR-27b* mimic/*miR-27b* inhibitor. The *miR-27b* mimic significantly decreased luciferase activity and introduction of the *miR-27b* inhibitor increased luciferase activity (Fig. 4C). The *miR-27b* target site was subsequently mutated to confirm that *miR-27b* was binding to this sequence. Of note, the effects of the *miR-27b* mimic or *miR-27b* inhibitor were abolished (Fig. 4D). Taken together, the results confirm that *miR-27b* targets VDR 3'UTR specifically and directly.

Discussion

Pulmonary fibroblasts have important roles in lung tissue repair and remodeling. Fibroblasts often differentiate into

myofibroblasts that possess enhanced fibrotic, contractile and migratory activities. TGF- β 1 stimulated the proliferation of lung fibroblasts and their differentiation, as highlighted by increased expression and organization of α -SMA, a marker of myofibroblasts and a primary contributor to the contractile force in myofibroblasts (46,47). New emerging studies regarding the roles of miRNAs in pulmonary fibrosis and in their regulation of TGF- β signaling are increasing (38,48-50). *miR-27b* has been studied in various cancer cells (51-55), however, *miR-27b* has not been well studied in lung fibroblasts. Previous studies suggested that *miR-27b* was identified as a pro-angiogenic miRNA (56) and modulated fibrotic responses (41). The aim of the present study was to investigate the role and mechanism of *miR-27b* regulating human lung fibroblasts differentiation induced by TGF- β 1.

Firstly, 1,25(OH) $_2$ D $_3$ inhibited the effect of TGF- β 1 on human lung fibroblast differentiation, as demonstrated by its ability to inhibit TGF- β 1-induced expression of α -SMA. These results are consistent with findings from a study of vitamin D inhibition of pro-fibrotic effects of TGF- β 1 in mouse lung fibroblasts (27). The present study identified that *miR-27b* expression levels were significantly higher in MRC5 cells induced by TGF- β 1, however, treatment of TGF- β -stimulated fibroblasts with 1,25(OH) $_2$ D $_3$ effectively decreased *miR-27b* expression. This suggests that *miR-27b* may have a role in regulating the differentiation phenotype of the pulmonary fibroblasts. By contrast, two studies observed that TGF- β treatment downregulated *miR-27b* expression (41,57), which may be caused by different types of cells, and additional investigations are required to demonstrate the function.

There is evidence showing that VDR is involved in pathological fibrogenesis. VDR belongs to the superfamily of steroid/thyroid nuclear hormone receptors. Following ligand binding, the VDR forms a heterodimer with RXR, or Smad3, a receptor of TGF- β /Smad signalling. Previous studies showed cross-talk between the TGF- β and vitamin D signaling pathways and VDR binds to the MH1 domain of Smad3, enhancing Smad3 ligand-induced transactivation (28-31). 1,25(OH) $_2$ D $_3$ had a significant effect *in vivo* on the TGF- β signaling pathway by altering levels of VDR and Smad3, and subsequently affecting the bioactive of TGF- β (44). Additionally, a recent study identified VDR as a negative regulator of fibroblast activation that interfered with the pro-fibrotic effects of TGF- β (43). The present study further showed that reduction of the VDR protein was mediated by TGF- β 1 in human lung fibroblasts, whereas 1,25(OH) $_2$ D $_3$ effectively upregulated VDR protein expression, which was in accordance with the study of activation of VDR by paricalcitol reducing the stimulatory effects of TGF- β on skin fibroblasts (43).

In addition, the experiments of lung fibroblasts transfected with *miR-27b* mimic or *miR-27b* inhibitor and luciferase reporter assays reveal that *miR-27b* directly targets VDR 3'UTR and inhibits VDR gene expression to promote differentiation of human lung fibroblasts characterized by expression of α -SMA. The present *in vitro* study showed that the overexpression of *miR-27b* decreased VDR protein expression and increased expression of fibroblast differentiation marker, α -SMA, while reducing levels of *miR-27b* had opposing effects. Studies of fibrosis have not reached a consensus on the importance of *miR-27b* in disease pathology. An *in vitro* study

suggested that *miR-27b* may be profibrotic in activated hepatic stellate cells (58). Additionally, cardiomyocyte overexpression of *miR-27b* induces cardiac hypertrophy in mice (57). These two studies were similar to the present results. By contrast, a recent study suggested that *miR-27b* overexpression markedly repressed fibrotic responses in pulmonary epithelial cells (41).

In conclusion, 1,25(OH) $_2$ D $_3$ inhibits differentiation and downregulates *miR-27b* expression in human lung fibroblasts induced by TGF- β 1. Furthermore, *miR-27b* overexpression decreased the expression of VDR protein and increased the expression of α -SMA, while reducing levels of *miR-27b* had opposing effects. Notably, *miR-27b* has abilities for targeting the 3'UTR of VDR and negatively regulating VDR protein expression, which effects differentiation of human lung fibroblasts. Thus, 1,25(OH) $_2$ D $_3$ inhibits lung fibroblast differentiation induced by TGF- β 1 via *miR-27b* targeting VDR 3'UTR, and this may be used as a novel treatment strategy in differentiation pathways.

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