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# MiR-124 regulates transforming growth factor-β1 induced differentiation of lung resident mesenchymal stem cells to myofibroblast by repressing Wnt/β-catenin signaling

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ABSTRACT

Lung resident mesenchymal stem cells (LR-MSCs) contribute to the progression of idiopathic pulmonary fibrosis (IPF). We aimed to investigate the molecular mechanism underlying LR-MSCs regulation upon transforming growth factor (TGF)-β1 stimulation. We induced fibrogenic differentiation of LR-MSCs isolated from mice by TGF-β1. Several stem cell markers were detected by flow cytometric analysis. Protein expression level was tested by Western blotting and mRNA level was detected by quantitative real-time polymerase chain reaction (qRT-PCR). Cell viability, proliferation and apoptosis were measured. TGF-β1 promoted fibrogenic differentiation of LR-MSCs and upregulated β-catenin and p-glycogen synthase kinase-3β, suggesting the activation of Wnt signaling. MicroRNA (MiR)-124-3p was significantly upregulated in TGF-β1 treated LR-MSCs compared to untreated cells. Intriguingly, silence of miR-124 reversed the TGF-β1-induced changes in cell viability and proliferation, and also led to a decrease of cell apoptosis. Additionally, in miR-124 silenced cells, α-smooth muscle actin, collagen I and fibronectin were downregulated compared to control cells. We ultimately identified a new target of miR-124, AXIN1, which was repressed by miR-124. In conclusion, miR-124 regulates AXIN1 to activate Wnt signaling and therefore plays a crucial role in the TGF-β1-induced fibrogenic differentiation.

## 1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive lung disease of unclear etiology. It can seriously affect the human respiratory function. Less than 50% of patients survived for 5 years (Caminati et al., 2017), which is mainly due to poorly understood pathogenesis, late diagnosis, inadequate therapeutic methods and poor prognosis (Kolb et al., 2017). To date, there are a series of novel developed therapeutic targets, some of which are undergoing clinical trials. However, no effective treatment has been approved. Therefore, to explore more other treatments, it is important to obtain deeper insights into the mechanism of IPF.

It is believed that genetic and non-genetic risk factors including smoking, infection and aging may contribute to the development of IPF. Accumulatively, these multiple factors may give rise to damage of fibroblasts and alveolar epithelial cells, aberrant wound healing, extracellular matrix deposition, and formation of fibroblastic/myofibroblastic foci (Selman et al., 2001, 2006; Thannickal et al., 2004), ultimately resulting in disrupted gas exchange, deterioration of respiratory function and dyspnea. In this process, activation of mesenchymal stem cells (MSCs) plays essential roles (Tzouvelekis et al., 2011). MSCs are a class of pluripotent stem cells that exist in bone marrow and numerous organs including the lung, liver and brain. These tissue-resident MSCs could differentiate into organ-specific progenitor cells. Evidence suggest that lung-resident MSCs (LR-MSCs) act as multipotent vascular precursors and are associated with microvascular remodeling in the lung (Chow et al., 2013a). In addition, these cells were indicated to be capable of differentiating into alveolar epithelial type II cells (Gong et al., 2014) and

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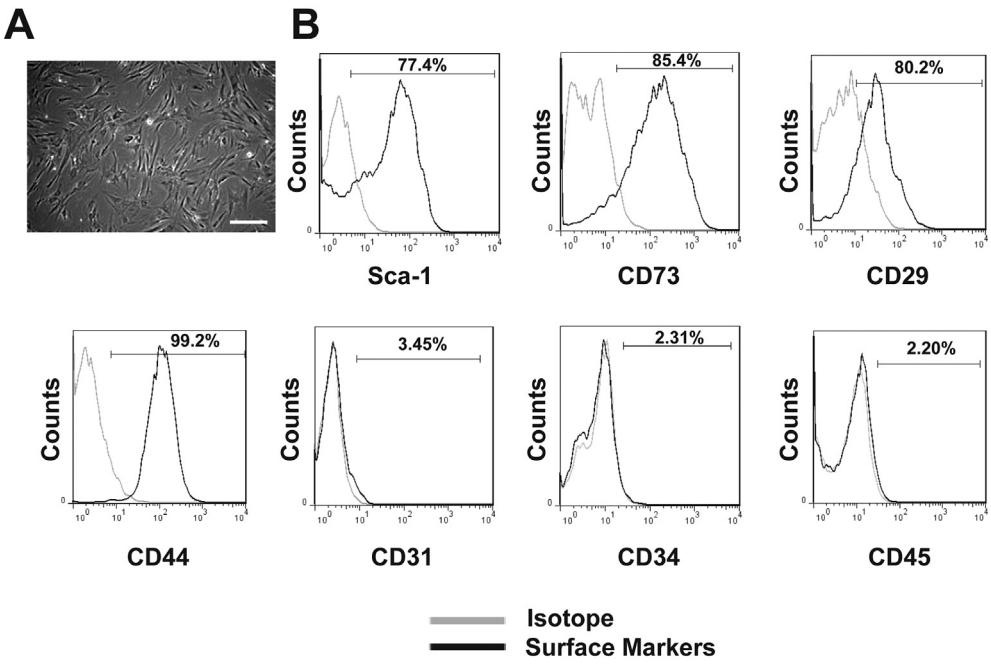


**Fig. 1.** Mouse lung resident mesenchymal stem cells (LR-MSCs) exhibit MSC properties. A. Mouse LR-MSCs morphology after 7 days of culture was obtained by a standard light microscope. Scale bar = 200 μm. B. The expression of the surface markers Sca-1, CD73, CD29, CD44, CD31, CD34 and CD45 on mouse LR-MSCs was determined by flow cytometric analysis. The Sca-1, CD73, CD29 and CD44 were positive while CD31, CD34 and CD45 were negative in mouse LR-MSCs.

involved in injury repair in the lung (Jones and McGonagle, 2007), which on the other hand may contribute to pulmonary disorders (Tzouvelekis et al., 2011).

Previous studies have found the importance of Wnt/β-catenin signaling in the lung diseases. Aberrant activation of the signaling may lead to pulmonary fibrosis and lung cancer (Königshoff et al., 2008; Uematsu et al., 2003). Accumulating recent evidence demonstrated that self-renewal and differentiation of MSCs are regulated by Wnt/β-catenin signaling, suggesting that Wnt signaling may contribute to the lung diseases via regulating MSCs fate. Therefore, in this study, we aim to obtain deeper insights into the molecular mechanism of IPF and how LR-MSCs are involved.

## 2. Methods

### 2.1. Isolation of mouse LR-MSCs

All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published by The Ministry of Science and Technology of China in 2006. All experimental protocols were approved by Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. C57BL/6 mice aged 4–6 weeks were purchased from Shanghai Laboratory Animal Center (Shanghai, China). LR-MSCs were isolated and cultured as previous studies (Shi et al., 2015). Briefly, the mouse lung parenchyma was obtained and cut by a scissor. Then the small pieces of tissues were digested and red blood cells were removed using ammonium-chloride-potassium lysis buffer. LR-MSCs were sorted by Sca-1 and CD45 using the magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), and then cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 4% L-glutamine, 1% nonessential amino acids, and 1% penicillin and streptomycin. The isolated LR-MSCs were incubated in a humidified atmosphere supplied with 5% $CO_2$ at 37 °C. LR-MSCs were passaged as 1:2 when the confluence reached 70–90%.

### 2.2. Flow cytometric analysis

Cells were collected and resuspended in phosphate-buffered saline (PBS). Then, they were stained with fluorescent antibodies including PE-conjugated anti-CD29, CD73, CD31 and 34 and FITC-conjugated anti-Ly-6A/E (Sca-1) that were purchased from Becton Dickinson as well as CD44 and CD45 purchased from eBioscience. All antibodies were incubated with cells for 1 h at 37 °C and detected using a FACS CaliburTM flow cytometer (Becton Dickinson) after 2 times of PBS wash. The results were analyzed by FlowJo software (Tree star, Ashland, Oregon).

### 2.3. Western blotting

The total protein from LR-MSCs was extracted by RIPA buffer (Cell Signaling Technology Inc., Danvers, MA). Equal amounts of proteins were separated by 10% sodium dodecyl sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, polyvinylidene difluoride membranes (Bio-Rad) were used and blocked by 8% non-fat milk. Primary antibodies were applied including anti-α-smooth muscle actin (α-SMA), collagen I, fibronectin, β-catenin, phosphorylated glycogen synthase kinase (p-GSK)-3β, GSK-3β and GAPDH as a loading control. All antibodies were purchased from Abcam (Cambridge, MA,) and diluted at 1:1000. The immunoreactive signals were detected using an Odyssey Scanning System (LI-COR, Inc., Lincoln, NE) after incubating the membranes with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (Boster, Wuhan, China).

### 2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

To test the levels of microRNA (miRNA) expression, we firstly extracted total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Next, 2 μg of total RNA was used to synthesize cNDA by using a specific stem-loop primer (Guangzhou RiboBio Co., Ltd.). PCR was conducted with SYBR Green master mix on

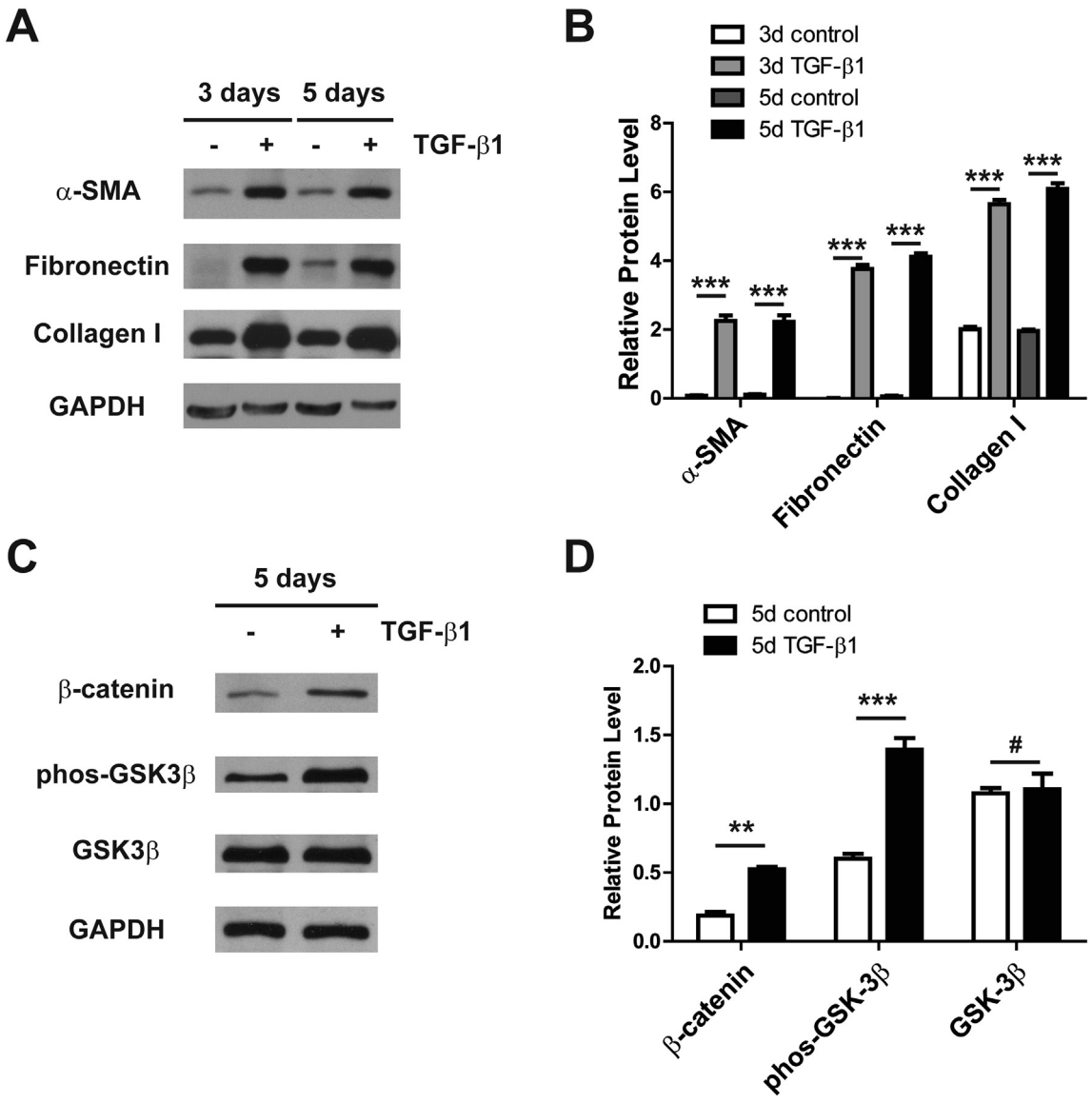


**Fig. 2.** Transforming growth factor-β1 (TGF-β1) induces fibrogenic differentiation and activates Wnt/β-catenin signaling in mouse LR-MSCs. A. The mouse LR-MSCs were cultured in the presence or absence of TGF-β1 (10 ng/mL) and the protein levels of α-smooth muscle actin (α-SMA), collagen I and fibronectin were determined by Western blot. n = 3 independent experiments and this panel presented one of these repeats. B. Statistic of the relative level of α-SMA, collagen I and fibronectin in panel (A). Data were presented by mean ± standard deviation (S.D.). n = 3 independent experiments, *** indicated $p < 0.001$. C. The mouse LR-MSCs were cultured in the presence or absence of TGF- β1 (10 ng/mL) for 5 days and the protein levels of β-catenin, phos-GSK-3β and GSK-3β were determined by Western blot. n = 3 independent experiments and this panel presented one of these repeats. D. Statistic of the relative level of β-catenin, phos-GSK-3β and GSK-3β in panel (C). Data were presented by mean ± S.D. n = 3 independent experiments, ** indicated $p < 0.01$, *** indicated $p < 0.001$ and # indicated $p > 0.05$.

the ABI7000 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). All primers for the microRNAs were purchased from Exiqon Inc.

Primers for miRNAs: mmu-mir-410: Forward: GGAGAGGTTGTCTGTGATGAGTTCGCTTTATTAATGACGAATATAACACAGATG; Reverse: GGTCCAGTTTTTTTTTTTTTTTGGT. mmu-mir-133a: Forward: GTAAAATGGAACCAAATCGCCTCTTCAATGGA; Reverse: CCAGTTTTTTTTTTTTTTGCTACAG. mmu-miR-144-5p: Forward: GCGCAGGGATATCATCATATAC; Reverse: GTCCAGTTTTTTTTTTTTTTACTTACAG. mmu-miR-124-3p: Forward: AGGCACGCGGTGA; Reverse: TCCAGTTTTTTTTTTTTTTGGCA. mmu-miR-142a-3p: Forward: CGCAGTGTAGTGTTTCCT; Reverse: GGTCCAGTTTTTTTTTTTTTTTCCA. mmu-mir-30b: Forward: TTTCAGTTCATGTAAACATCCTACACTCAGCTGTCATACATGCGTTGGCTGGGATGTG; Reverse: GGTCCAGTTTTTTTTTTTTTTTATACTC.

### 2.5. Cell viability, proliferation and apoptosis assays

Cell viability and proliferation were detected using bromodeoxyuridine (BrdU, Abcam) labeling and Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Cell apoptosis was measured by annexin V-FITC and propidium iodide (PI) staining kit (Vazyme, Nanjing, China) according to manufacturer's instructions.

### 2.6. Statistical analysis

All the data were shown as the mean ± standard deviation (S.D.). SPSS version 19.0 (IBM SPSS, Amronk, NY, USA) was employed to analyze statistical differences. All the experiments were performed in 3 independent biological repeats. Statistical differences were determined using Student's *t*-test or one-way ANOVA analysis. A value of $p < 0.05$

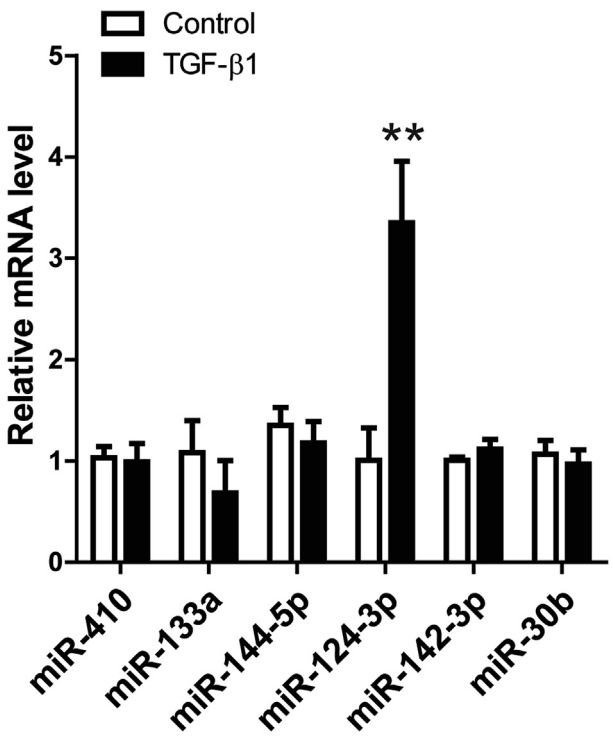


**Fig. 3.** miR-124-3p is up-regulated in LR-MSCs stimulated by TGF-β1. LR-MSCs were treated with or without 10 ng/mL were lysed and the miRNA levels were determined by qPCR. n = 3 independent experiments, data were presented as mean ± standard deviation (S.D.), ** indicated $p < 0.01$.

was considered to be statistically significant difference.

## 3. Results

### 3.1. Identification of MSC properties in the mouse lung resident (LR)-MSCs

We firstly isolated lung resident (LR)-MSCs from mice. After 7 days of culture, the stellate, spindle and thin morphology was displayed and recorded using a standard light microscope (Fig. 1A). To identify whether these cells possess MSC characteristics, we used flow cytometry to analyze their surface markers. The isolated cells expressed markers of Sca-1, CD73, CD29 and CD44 but not CD31, CD34 and CD45 (Fig. 1B), whose profile of antigen expression resembles the typical pattern of MSC surface marker. Together, this evidence showed that the isolated LR-MSCs exhibited MSC properties.

### 3.2. Transforming growth factor-β1 (TGF-β1) induces fibrogenic differentiation and activates Wnt/β-catenin signaling in LR-MSCs

As TGF-β1 was reported to induce the fibrogenic differentiation of MSCs, we confirm these findings in our study. By incubating LR-MSCs with 3 and 5 days of TGF-β1, it was observed that α-SMA, collagen I and fibronectin were all upregulated (Fig. 2A and B). We further tested the change in Wnt/β-catenin signaling. As expected, 5 days of TGF-β1 treatment significantly activated Wnt by enhancing the levels of β-catenin and phos-GSK-3β (Fig. 2C and D).

### 3.3. The effects of miR-124-3p on LR-MSCs

Next, we tested several miRNAs that are related to differentiation of LR-MSCs. It was found that only miR-124-3p was remarkably increased in TGF-β1 treated cells than untreated cells (Fig. 3). To investigate whether miR-124 could affect the TGF-β1 induced fibrogenic differentiation of LR-MSCs, we silenced miR-124 using a synthesized antago-miR-124. Interestingly, use of antago-miR-124 significantly repressed cell proliferation (Fig. 4A), viability (Fig. 4B) and apoptosis (Fig. 4C) compared to those of TGF-β1 treated cells.

### 3.4. MiR-124 attenuates TGF-β1 induced fibrogenic differentiation by downregulating AXIN1

Aforementioned results demonstrate the involvement of miR-124 in TGF-β1 induced LR-MSCs growth. We next sought for whether miR-124 affects fibrogenic differentiation of LR-MSCs. Surprisingly, silence of miR-124 significantly suppressed the upregulated expression of α-SMA, collagen I and fibronectin by TGF-β1 in LR-MSCs (Fig. 5A and B). To identify which gene could be regulated by miR-124, we employed TargetScan (v7.2) to predict potential gene target (Agarwal et al., 2015). Among all the predicted target, we found that AXIN, an important protein participating in Wnt signaling, was a possible target of miR-124 (Fig. 5C). By treating the LR-MSCs with TGF-β1, AXIN1 expression was markedly declined compared to untreated cells. However, after silencing miR-124 in LR-MSCs, its expression recovered back as untreated cells (Fig. 5D and E). These results indicate that miR-124 plays an important role in TGF-β1 induced fibrogenic differentiation of LR-MSCs via regulating AXIN1.

## 4. Discussion

Currently, pirfenidone and nintedani are two approved compounds that have been frequently used for IPF treatment. These two compounds served as antifibrotic that may target TGF-β and tumor necrosis factor-α (Kolb et al., 2017), and tyrosine-kinase inhibitor targeting fibroblast growth factor receptor and platelet derived growth factor receptor (Fletcher et al., 2016), to alleviate the progression of IPF, respectively. However, they could not cure IPF and often are accompanies with numerous side effects (Tzouvelekis et al., 2017, 2018). Identification of novel targets is urgently needed. The use of MSCs in this circumstance has drawn the attention from numerous researchers for its potential immune-regulation and anti-inflammatory properties (Uccelli et al., 2007). Interestingly, although the cytokine and gene expression profiles of LR-MSCs and bone marrow-derived MSCs are distinguished (Badri et al., 2011), studies demonstrated that LR-MSCs also possess immunosuppressive capacity (Jarvinen et al., 2008). Loss of LR-MSCs was stated to be associated with bleomycin-induced fibrosis (Jun et al., 2011). However, excessive other evidences implicated a different role of LR-MSCs in IPF. They indicated that LR-MSCs activities were highly affected by the local microenvironment, in which could drastically impair the proper differentiation and proliferation of LR-MSCs, eliciting the progression of IPF (Foronjy and Majka, 2012; Volckaert et al., 2011). It therefore has become important to reveal the molecular of LR-MSCs.

In the present study, we isolated LR-MSCs from mice and stimulated those cells with TGF-β1. Previous studies suggested that early alveolar injury could stimulate the productions of mediators including TGF-β1 from the alveolar epithelial cells, which led to the releases of cytokines and chemokines and abnormal wound healing (Bagnato and Harari, 2015; Papaioannou et al., 2018). TGF-β1 was proved to participate as a critical factor in LR-MSCs differentiation into myofibroblasts (Popova et al., 2010). It is well known that myofibroblasts is associated with dysfunctional matrix remodeling which induces chronic lung diseases (Toti et al., 1997). This study provided further evidence showing the sensitivity of LR-MSCs to microenvironment. Here, we found that TGF-β1 stimulation successfully triggered fibrogenic differentiation of LR-MSCs with significantly improved expression of α-SMA, collagen I and fibronectin compared to untreated cells. These results are consistent with other previous studies (Walker et al., 2011).

Wnt proteins belong to a group of highly conserved proteins, whose signaling is involved in a series of fundamental biological process such as tissue repair, wound healing, fibrosis and tissue remodeling (Cheon et al., 2004). On the other hand, aberrant activation of Wnt signaling has been

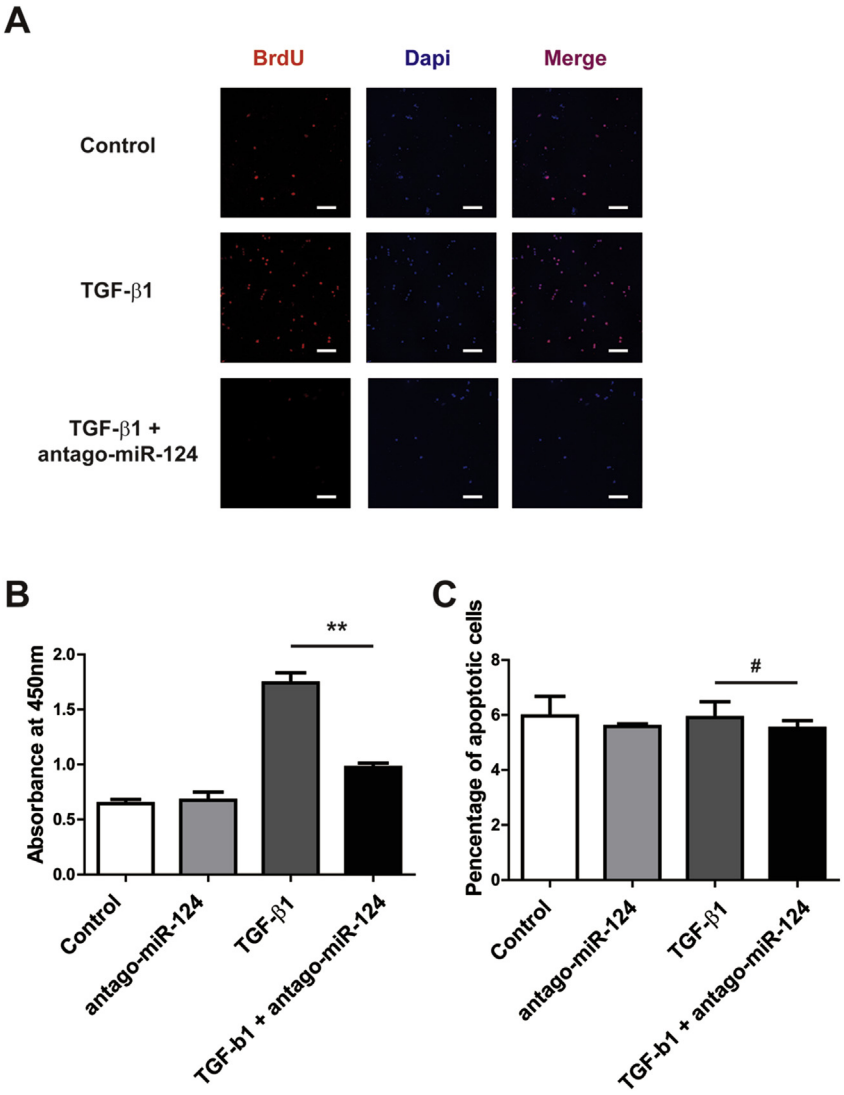


**Fig. 4.** Downregulation of miR-124 inhibits the proliferation of mouse LR-MSCs. A. Mouse LR-MSCs were treated with TGF-β1 (10 ng/ml) for 48 h in the absence or presence of antago-miR-124. Culture medium were pulsed with bromodeoxyuridine (BrdU,) for 4 h before test and BrdU signal was obtained by the immune-staining of BrdU antibody. Scale bar was 200 μm. B. Cell viability was determined by Counting Kit-8 (CCK-8) assay. Data were presented by mean ± standard deviation (S.D.). n = 3 independent experiments, *** indicated $p < 0.001$. C. Cell apoptosis was evaluated by flow cytometry by Annexin V & PI staining, the AnnexinV and PI double positive cells were calculated as the apoptotic cells. Data were presented by mean ± S.D. n = 3 independent experiments, *** indicated $p < 0.001$.

linked to various human diseases such as pulmonary fibrosis. Activation of Wnt signaling is characterized by the binding of Wnt proteins to its coreceptor complex. Then, β-catenin protein, as a central mediator of canonical Wnt signaling is released from Axin/GSK3β/APC complex, which in a steady state without Wnt stimulation normally phosphorylates β-catenin and leads to ubiquitin-mediated proteolysis of β-catenin. These released β-catenin proteins accumulate in the nucleus and interact with T-cell factor/lymphoid enhancer factor 1 (TCF/LEF1) to initiate the transcription of Wnt target genes. However, mutations of the proteins in Wnt signaling may result in constitutive activation of the pathway and contribute to the development of various diseases. In the context of IPF, Wnt/β-catenin signaling is a key pathway involved in a series of processes. In IPF tissue sections, β-catenin proteins were accumulated in the nucleus, suggesting the activation of Wnt signaling in the IPF patients (Chilosi et al., 2003). Sustained activation of Wnt signaling was found in the proliferative myofibroblast lesions and to contribute to the fibrosis of the lung (Königshoff et al., 2009). Additionally, it has been stated that Wnt signaling controls the differentiation of LR-MSCs (Chow et al., 2013b; Wang et al., 2009). In the present study, we found that the expression level of β-catenin was significantly upregulated after 5 days of TGF-β1 stimulation as well as the level of phosphorylated GSK3β. The activated Wnt signaling pathway may elicit a set of changes in gene expression in MSCs and affect MSC differentiation to myofibroblasts. It is known that miRNA is an important mechanism of the regulation of gene expression. The changes of miRNA may play significant roles in the fibrogenic differentiation of LR-MSCs. In this study, we analyzed the miRNAs that could be involved and found that only miR-124-3p was significantly upregulated in TGF-β1 stimulated LR-MSCs compared to untreated cells. Silence of miR-124 reversed the TGF-β1-induced changes in cell viability and proliferation and also repressed cell apoptosis. In addition, the fibrogenic differentiation of LR-MSCs was prevented after miR-123 silence and the protein levels of α-SMA, collagen I and fibronectin were diminished. Interestingly, we identified that AXIN1, an important factor in Wnt signaling, is a potential target of miR-124-3p. This could be the molecular mechanism how Wnt signaling is controlled in patients with IPF. After the use of antago-miR-124, it was observed that AXIN1 protein level was strikingly elevated, suggesting that β-catenin proteins were sequentially inhibited.

## 5. Conclusion

In conclusion, we isolated LR-MSCs from mice and stimulated those cells with TGF-β1. We confirmed that TGF-β1 promoted fibrogenic differentiation of MSCs and activated Wnt signaling by upregulating the

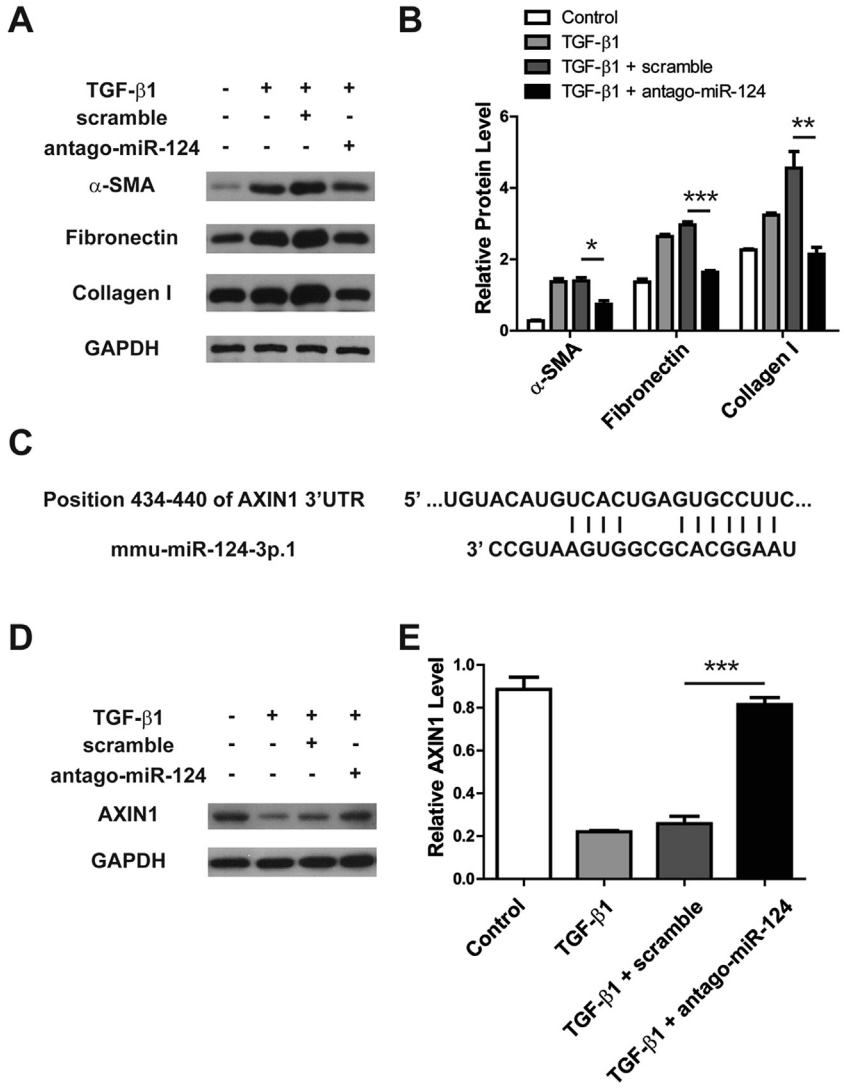


**Fig. 5.** miR-124 attenuates TGF-β1 induced fibrogenic differentiation by downregulating AXIN1. A. Mouse LR-MSCs were treated with TGF- β1 (10 ng/ml) for 48 h in the absence or presence of antago-miR-124 and the protein levels of α-smooth muscle actin (α-SMA), collagen I and fibronectin were determined by Western blot. n = 3 independent experiments and this panel presented one of these repeats. B. Statistic of the relative level of α-SMA, collagen I and fibronectin in panel (A). Data were presented by mean ± standard deviation (S.D.). n = 3 independent experiments, * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$. C. The targeting sites between miR-124-3p and AXIN1 mRNA. D. Mouse LR-MSCs were treated with TGF- β1 (10 ng/ml) for 48 h in the absence or presence of antago-miR-124 and the protein level of AXIN1 was determined by Western blot. n = 3 independent experiments and this panel presented one of these repeats. E. Statistic of the relative level of AXIN1 in panel (D). Data were presented by mean ± S.D. n = 3 independent experiments, *** indicated $p < 0.001$.

expression of β-catenin and phos-GSK-3β. Several miRNA expression levels were detected. Ultimately, we found that miR-124 regulates AXIN1 to activate Wnt signaling and therefore participates in the TGF-β1 induced fibrogenic differentiation.

## Declarations of interest

The authors declare that there is no conflict of interests.

## Significance statement

This study presents evidence indicating that miR-124 regulates AXIN1 to activate Wnt signaling and therefore plays a crucial role in the TGF-β1-induced fibrogenic differentiation.

## Acknowledgment

None.

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