

Regulatory effect and mechanism of LncRNA SOX2OT in idiopathic pulmonary fibrosis

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ABSTRACT

This study discusses the role played by long noncoding RNA (lncRNA) SOX2OT (SOX2OT) in idiopathic pulmonary fibrosis (IPF). By inducing human embryonic lung fibroblasts (MRC5) through hypoxia, the researchers observed changes in SOX2OT expression and fibrotic processes during hypoxia. Moreover, SOX2OT abnormal expression vectors were constructed and transfected into MRC5 to analyze the effect of SOX2OT on MRC5. The results showed that the expression levels of SOX2OT and α -SMA were elevated under hypoxic conditions and were positively correlated ($P < 0.05$). α -SMA, Collagen I and Collagen III protein expression and SOX2OT levels all increased under hypoxia ($P < 0.05$). Finally, silencing SOX2OT expression led to weakened MRC5 proliferation, inhibited fibrosis process, and reduced inflammation ($P < 0.05$). In conclusion, SOX2OT is closely related to the occurrence and development of IPF, and silencing its expression can inhibit fibrosis progression.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and fibrotic interstitial pulmonary disease in which lesions are confined to the lungs, with undefined etiology and a predilection for middle-aged and elderly people (1). Worldwide, the incidence of IPF ranges from 2 to 29 per 100,000 people and is increasing at an annual rate of 11% (2). In China, due to the large population base and the aggravation of population aging, the average number of new IPF cases is more than 500,000 per year (3). Approximately 15% of the cases have acute exacerbation of IPF with progressive dyspnea aggravated due to upper respiratory tract infection, and most of them die of respiratory and circulatory failure within 6 months (4). IPF is affected by smoking, environment, heredity, bacterial (viral) infection and many other reasons, and a thorough understanding of the causes of IPF is of great significance for finding a new diagnosis and treatment scheme in the future (5).

In recent years, the deepening of research has led researchers to gradually focus on the study of the molecular pathogenic mechanism of IPF (6). Among them, long noncoding RNAs (lncRNAs) are one of the research hotspots. LncRNAs have been shown to be important regulators of multiple diseases, and their abnormal expression is associated with the pathophysiology of fibrosis, including IPF (7). In recent years, evidence has demonstrated that activating transcription factor 3 (ATF3) activates LINC00941/lncIAPF to differentiate fibroblasts (FBs) into myoblasts by blocking ELAVL1/HuR-dependent autophagy in pulmonary fibrosis (8) while silencing lncRNA SNHG6 alleviates bleomycin-induced pulmonary fibrosis in mice through the miR-26a-5p/TGF- β 1-smads axis (9). All the

preceding studies demonstrate the potentially important role of lncRNAs in IPF, which is of great significance for the future diagnosis and treatment of the disease. In an analysis by Poulet C et al. on the potentially abnormal expression of lncRNAs in IPF, lncRNA SOX2OT (SOX2OT) has caught our attention (10). SOX2OT has been confirmed to play a vital part in mesangial cell and cardiomyocyte proliferation and fibrosis in diabetic nephropathy (11, 12), suggesting the potentially important effect of SOX2OT on the fibrosis process of IPF. However, no studies have confirmed our view. Correspondingly, by analyzing the influence of SOX2OT on IPF under hypoxia conditions, this study aims to provide new references and guidance for the future diagnosis and treatment of IPF.

Materials and Methods

Cell data

Human embryonic lung FBs MRC5, ordered from the American Type Culture Collection (ATCC), were cultured in the supporting culture medium. Trypsin was added for digestion when the cells were 80-90% confluent, followed by supernatant removal via centrifugation and the addition of 10% FBS-supplemented MEM medium for passage at a 1:2 ratio and further culture.

Polymerase chain reaction (PCR) testing

Total cell RNA was extracted by Trizol, and reverse transcribed into cDNA after its purity determination by ultraviolet spectrophotometer. Reverse transcription of 20 μ g total RNA was then performed at 50°C for 2 min, 95°C for 10 min, 95°C for 15s, and 60°C for 60s, for 60 cycles. SOX2OT expression was calculated by $2^{-\Delta\Delta C_t}$ with GAPDH as an internal reference. Primer sequences, are

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Table 1. Primer sequences.

	F (5'-3')	R (5'-3')
SOX2OT	GGCTGGGAAGGACAGTTCG	AGATGATCTTGCCAGGCGATC
GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG

presented in Table. 1, were designed and constructed by Shanghai Rothen Pharma Co., Ltd. In addition, serum α -SMA levels of both groups were measured as instructed by the manuals of the ELISA kit (Beijing TransGen Biotech).

Hypoxic culture

The condition of the constant temperature incubator was set as 37°C, 1% O₂, 94% N₂, and 5% CO₂. Cells were taken out at 0, 6, 12 and 24 h after hypoxic culture, respectively, to determine SOX2OT expression according to the method described above. In addition, Western blots were performed to quantify α -SMA, Collagen I, and Collagen III protein levels. Bicinchoninic acid (BCA) was used to detect the protein concentration after cell lysis by RIPA. SDS-PAGE of 1/5 volume was then added to denature the proteins, followed by electrophoresis and the subsequent addition of primary antibodies (1:500) to seal the membrane. After incubation at 4°C overnight, the membrane was rinsed the next day and added with a secondary antibody (1:2000). Following exposure and development using enhanced chemiluminescence (ECL), ImageJ software was utilized to analyze the gray value.

Cell transfection

Additionally, normal cultured logarithmic (Log)-growth-phase MRC5 cells were collected for transfection with SOX2OT overexpression vector (SOX2OT-OV), silencing vector (SOX2OT-SI), and empty vector (SOX2OT-NC), respectively, as per the Lipofectamine™ 3000 kit instructions, with the groups named according to the corresponding transfected expression vector. The transfection success rate was verified by PCR detection of SOX2OT expression.

Influence of SOX2OT on MRC5

MTT: The Log-growth-phase cells were trypsin (0.25%) digested for plate (96-well) inoculation at 100 μ L/well. Four duplicate wells were set in each group, and 10 μ L MTT solution was added to one well every 24 hours. A microplate reader was used to determine the absorbance value (490 nm). **Detection of inflammatory factors:** 0.25% trypsin-lysed cells were collected to quantify tumor necrosis factor- α (TNF- α) and interleukin-1 β /6 (IL-1 β /6) concentrations following the instructions of enzyme-linked immunosorbent assay (ELISA) kits purchased from Beijing TransGen Biotech.

Statistical analysis

Statistical analysis was made by SPSS24.0 software. All tests were repeated three times, and the results were expressed as ($\bar{x} \pm s$). Variance analysis and LSD intra-group test were used for comparison among groups, with the presence of statistical significance indicated by a *P*-value less than 0.05.

Results

Influence of hypoxia on MRC5

α -SMA, Collagen I, and Collagen III protein levels in MRC5 were the lowest at 0 h after hypoxic culture among the four-time points, while their expression began to increase at 6 h after hypoxic treatment and reached the highest at 24 h (*P*<0.05). A consistent trend was observed in SOX2OT expression, that is, SOX2OT showed the lowest expression at 0 h after hypoxic culture and the highest level at 24 h after hypoxic culture (*P*<0.05). See Fig. 1 for details.

Impact of SOX2OT on the biological behavior of MRC5

SOX2OT expression was the highest in the SOX2OT-OV group and the lowest in the SOX2OT-SI group after transfection of MRC5 with different SOX2OT abnormal expression vectors (*P*<0.05), which confirmed the success of transfection. Further, the MTT assay indicated that the cell growth capacity of the SOX2OT-OV group was the strongest among the three groups, while that of the SOX2OT-SI group was significantly decreased compared with the SOX2OT-NC group (*P*<0.05). See Fig. 2 for details.

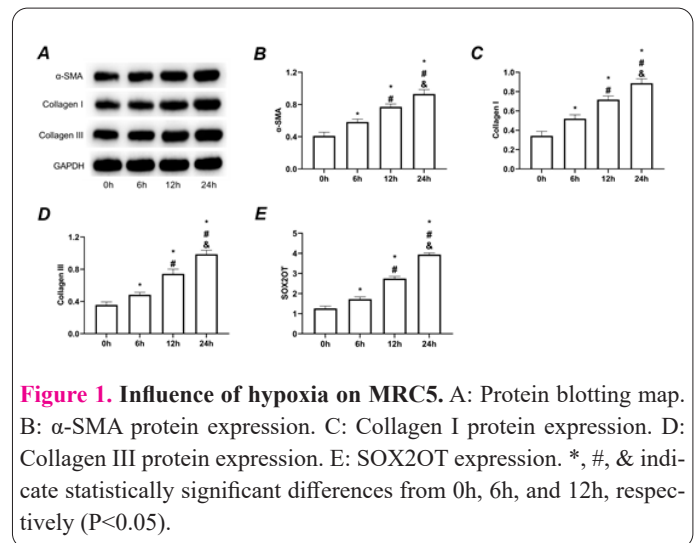


Figure 1. Influence of hypoxia on MRC5. A: Protein blotting map. B: α -SMA protein expression. C: Collagen I protein expression. D: Collagen III protein expression. E: SOX2OT expression. *, #, & indicate statistically significant differences from 0h, 6h, and 12h, respectively (*P*<0.05).

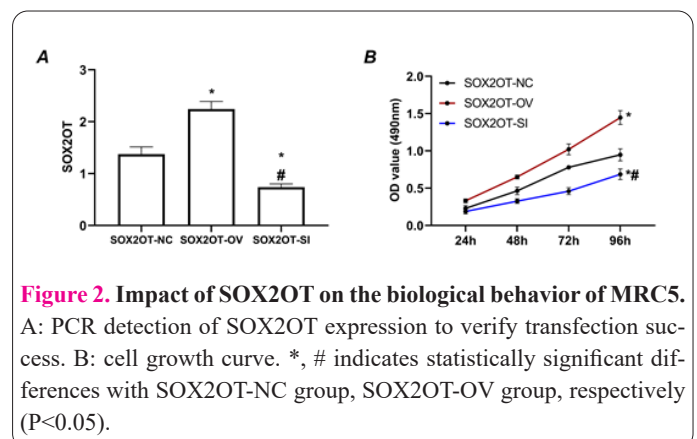


Figure 2. Impact of SOX2OT on the biological behavior of MRC5. A: PCR detection of SOX2OT expression to verify transfection success. B: cell growth curve. *, # indicates statistically significant differences with SOX2OT-NC group, SOX2OT-OV group, respectively (*P*<0.05).

Impact of SOX2OT on the fibrosis process of MRC5

Finally, the detection of cell fibrosis progression in the three groups showed that α -SMA, Collagen I and III protein levels were the highest in the SOX2OT-OV group, followed in descending order by the SOX2OT-NC group and the SOX2OT-SI group ($P < 0.05$). See Fig. 3 for details.

Effect of SOX2OT on the inflammatory response of MRC5

Similarly, the detection of inflammatory factors revealed that IL-1 β , IL-6 and TNF- α were the highest in the SOX2OT-OV group and the lowest in the SOX2OT-SI group among the three groups ($P < 0.05$). See Fig. 4 for details.

Discussion

IPF, as one of the most serious and recurrent types of idiopathic interstitial pneumonia, accounts for 20-50% of all interstitial diseases (13). As the pathogenesis has not been fully defined, IPF treatment focuses on relieving clinical symptoms, which can not avoid the continuous progression of the disease, eventually resulting in an adverse prognosis and a huge burden for patients and their families (14). In this study, we found that the hypoxic environment can promote the expression of α -SMA, Collagen I, Collagen III and SOX2OT in MRC5, and silencing SOX2OT alleviated the fibrotic process of MRC5. With further studies on the mechanisms of lncRNAs in IPF, molecular targeted therapy from the perspective of lncRNAs may be a breakthrough in future IPF therapies.

As we all know, hypoxia is one of the important and key factors of pathological changes of fibrosis. In previous studies, hypoxia was often used to induce fibrosis changes in tissues and cells (15, 16). Hence, hypoxic treatment was further carried out on MRC5 to confirm the relationship between SOX2OT and the fibrosis process. α -SMA, Collagen I and III proteins in cells, as well as SOX2OT expression, were found to be elevated gradually with the extension of hypoxia time, demonstrating the participation of SOX2OT in the process of hypoxia-induced cell fibrosis. In previous studies, SOX2OT was also highly expressed in hypoxia-induced human pulmonary artery smooth muscle cells and promoted the enhancement of inflammatory reaction and apoptosis (17), which can validate our experimental results and views.

Finally, we constructed SOX2OT abnormal expression vectors for MRC5 transfection to confirm the effect of SOX2OT on MRC5. The experimental results revealed enhanced proliferation, accelerated fibrosis process, and intensified inflammation of MRC5 after increasing SOX2OT expression, while the opposite was true when SOX2OT was silenced. Therefore, the highly expressed SOX2OT in IPF can promote the fibrosis process; conversely, the silencing of its expression can inhibit fibrosis progression. Therefore, SOX2OT is expected to be a potential molecular target of IPF in the future. In the research of Yang G, SOX2OT was also proposed to be an important research direction for reversing myocardial cell fibrosis (18), which not only confirms our experimental results but also further demonstrates the important influence of SOX2OT on organ, tissue and cell fibrosis. Combining our findings with previous studies, we believe that SOX2OT may exert an anti-fibrosis effect in IPF and play an impor-

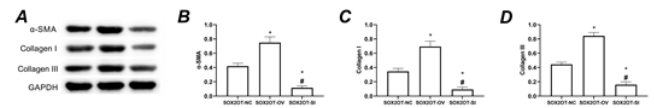


Figure 3. Impact of SOX2OT on the fibrosis process of MRC5. A: Protein blotting map. B: α -SMA protein expression. C: Collagen I protein expression. D: Collagen III protein expression. *, # indicates statistically significant differences with SOX2OT-NC group, SOX2OT-OV group, respectively ($P < 0.05$).

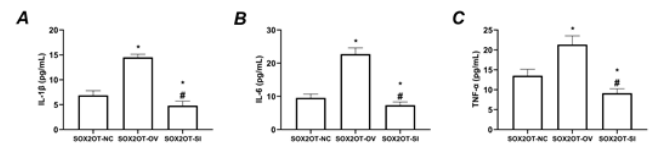


Figure 4. Effect of SOX2OT on the inflammatory response of MRC5. A: IL-1 β expression. B: IL-6 expression. C: TNF- α expression. *, # indicates statistically significant differences with SOX2OT-NC group, SOX2OT-OV group, respectively ($P < 0.05$).

tant role in the fibrosis process of other organs. Of course, more experiments are needed to confirm this.

Limited by experimental conditions, there are still many shortcomings to be addressed in this study. For example, we have not conducted more detailed detection of other biological behaviors of cells, such as cell apoptosis and cycle changes, which needs to be supplemented and confirmed by subsequent analysis. Second, in vivo, experiments are needed to confirm the influence of abnormally expressed SOX2OT on IPF.

SOX2OT is highly expressed in IPF and closely related to the fibrosis progression of MRC5; silencing SOX2OT can inhibit fibrosis and inflammation of MRC5. Therefore, SOX2OT is expected to be a molecular therapeutic target for future IPF therapies, providing a more reliable safety guarantee for the prognosis of IPF patients.

Conflict interest

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

The data in this article can be obtained from the corresponding author under reasonable circumstances.

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