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MiR-34b-5p knockdown attenuates bleomycin-induced pulmonary fibrosis by targeting tissue inhibitor of metalloproteinase 3 (TIMP3)

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Abstract. – OBJECTIVE: To examine the effects of microRNA-34b-5p (miR-34b-5p) on bleomycin-induced lung fibrosis in mice and the underlying mechanism.

MATERIALS AND METHODS: TIMP3-deficient (Timp3–/–) and wild-type mice were administered with bleomycin before to detect the miR-34b-5p expression using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Luciferase reporter assay was used to identify the target of miR-34b-5p in human lung fibroblast MRC-5. MiR-34b-5p was then silenced in vivo before lung histologic analysis and evaluation of extracellular matrix (ECM) genes as well as myofibroblast marker. The protein and mRNA expression levels were detected by Western blot and qRT-PCR, respectively.

RESULTS: We found that miR-34b-5p was significantly increased in lung tissues from bleomycin-stimulated mice. TIMP3 was identified as a direct target of miR-34b-5p by using dual-luciferase reporter assay, and enhanced expression of miR-34b-5p led to a decrease in TIMP3 whereas miR-34b-5p knockdown was responsible for TIMP3 elevation in MRC-5 cells. MiR-34b-5p knockdown in vivo attenuated the bleomycin-induced pulmonary fibrosis in wild-type mice, displayed by a reduced expression of Col1A1, fibronectin (Fn), and α-SMA. Furthermore, histological examination of lung sections also verified a diminishing fibrotic phenotype caused by the miR-34b-5p knockdown. But in Timp3-/- mice, down-regulation of miR-34b-5p did not exert an effect on the severe fibrotic lung injury after bleomycin exposure.

CONCLUSIONS: MiR-34b-5p knockdown appears to enhance the resistance to bleomycin by regulating its target gene TIMP3 during the pathogenesis of lung fibrosis.

Key Words:
Idiopathic pulmonary fibrosis (IPF), MiR-34b-5p, Bleomycin, Tissue inhibitor of metalloproteinase 3 (TIMP3).

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease with an estimated prevalence of 0.03-0.05‰, leading to death within 2-3 years[1]. It is characterized by a continuum of clinical and pathological changes including uncontrolled production of extracellular matrix (ECM), inflammatory cell infiltration, and fibroblast recruitment in the alveoli, resulting in impaired lung function, and ultimately, respiratory failure[2,3]. Many of the features of IPF, as described in most clinical cases, can be imitated by animal models of bleomycin-induced pulmonary fibrosis displaying subpleural scarring[4]. Bleomycin treatment induces alveolar epithelial apoptosis, secretion of various chemokines, inflammatory cell recruitment, and subsequent elevated expression of profibrotic hallmarks such as transforming growth factor (TGF)-β1, fibronectin, and procollagen-1[5-7], which ultimately results in excessive activation of fibroblasts and interstitial ECM deposition.

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs encompassing ~22 nucleotides that suppress the translation of mRNAs by targeting 3'-untranslated regions (UTR)[8]. Increasing evidence has shown that miRNAs are implicated in a wide array of pathophysiological processes and diseases[9]. For instance, miR-34a was found significantly increased in the alveolar epithelial cells from patients with IPF[10]. Enhanced expression of miR-34 was reported[11] to suppress cell proliferation and induces a senescent phenotype in lung fibroblasts, thereafter reducing fibrotic lung injury in mice. However, there are few researches focusing on the role of other miR-34 family members in the pathogenesis of IPF. In this study, we examined the effects of miR-34b-5p on

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bleomycin-induced lung fibrosis in mice, which provides insight into the mechanism underlying how this miRNA participates in human IPF.

Materials and Methods

Experimental Pulmonary Fibrosis Model

C57BL/6 mice (6-8 weeks old) were obtained from the Laboratory Animal Center of Shandong University (Jinan, China). The bleomycin-provoked lung fibrosis model was described previously[12]. In brief, *Timp3*$^{-/-}$ and wild-type mice were anesthetized with isoflurane, before bleomycin (1-1.5 U/kg) was intratracheally injected through the nasal passage. Mice were sacrificed 18 days after injection of bleomycin for further analysis.

In vivo transfection was carried out by use of the anti-miR-34b-5p oligonucleotide (GenePharma, Shanghai, China) accompanied by Invivofectamine (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Normal saline was used for sham. All animal experiments were carried out under a protocol approved by the Laboratory Animal Ethics Committee of Liaocheng People's Hospital (Liaocheng, China).

Cell Culture and Transfection

Human lung fibroblast line MRC-5 and HEK 293 cell line were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). MRC-5 cells were cultured in Opti-MEM medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and HEK 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA). Cells were cultured at 37°C in a humidified environment containing 5% CO_2.

Transfections of miR-34b-5p mimics and inhibitor, as well as control mimic and control inhibitor (synthesized by GenePharma, Shanghai, China), were performed with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. The transfection efficiency was measured by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Detailed information is provided as follows: miR-34b-5p mimic (sense) 5′-AGGCAGUGUAAUUAGCUGAUUGU-3′; miR-34b-5p mimic (antisense) 5′-AAUCAGCUAAUUACACUGCCUUU-3′; miR-34b-5p inhibitor (sense) 5′-ACAAUCAGCUAAUUACACUGCCU-3′; miR-34b-5p inhibitor (antisense) 5′-CGAGGCAGTGTAATTAGCTGATTGT-3′.

QRT-PCR

The right lung was immediately homogenized after the mice were euthanized and total RNA was isolated using miRNeasy Mini kits (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. MiR-34b-5p expression was measured using TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA), where small nucleolar RNAs sno135 was used as an internal reference. mRNA levels were evaluated by using the SYBR Green Master Mix Kit (Roche, Mannheim, Germany), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (human) or Tubulin α1 (mouse) as the internal reference. Primer sequences are listed in Table I.

Luciferase Reporter Assay

The 3′-UTR of human TIMP3 mRNA was cloned into a psiCHECK2 vector and co-transfected into HEK-293 cells with miR-34b-5p mimics cloned into LentiLox 3.7 (pll3.7) using the Lipofectamine 3000. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions, after 48 h of incubation.

Table I. The primers used in this experiment.

Gene or oligonucleotide	Sequence (5′-3′)
Fn (sense)	TCTGGGAAATGGAAAAGGGGAATGG
Fn (antisense)	CACTGAAGCAGGTTTCCTCGGTTGT
Col1A1 (sense)	GGAGGGCGAGTGCTGTGCTTT
Col1A1 (antisense)	GGGACCAGGAGGACCAGGAAGT
α-SMA (sense)	CATCACCAACTGGGACGACATGGAA
α-SMA (antisense)	GCATAGCCCTCATAGATGGGGACATTG
Tubulin α1 (sense)	GGATGCTGCCAATAACTATGCTCGT
Tubulin α1 (antisense)	GCCAAAGCTGTGGAAAACCAAGAAG

Hydroxyproline Content Determination

For the measurement of the lung hydroxyproline content, the mouse's right lung were homogenized and hydrolyzed with 12N HCl, and the samples were incubated at 120°C for 3 hours. Hydroxyproline contents were then measured in triplicate and expressed as μg/lung.

Lung Histologic Analysis

The left lung of each mouse was fixed with 4% paraformaldehyde, embedded, and cut into ~4 μm sections for histological processing. The histological examination was then performed by staining the sections with hematoxylin and eosin (HE; Boster, Wuhan, China).

Western Blotting

Mouse lung tissues were homogenized and lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China), before centrifugation at 15,000 g for 10 minutes, and the protein lysate were separated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were incubated at 4°C overnight with anti-TIMP3 or anti-beta-actin (Cell Signaling, Danvers, MA, USA) primary antibodies, after blocking with 5% silk milk for 1 hour. Blots were then incubated with horseradish peroxidase (HRP)-linked anti-IgG conjugates for 1 hour at room temperature. Proteins were visualized by enhanced chemiluminescence (Millipore, Billerica, MA, USA).

Statistical Analysis

One-way analysis of variance, followed by the Bonferroni test, was used for multiple group comparisons. The Student's ***t***-test was used for the comparison between the two groups. Data were presented as mean ± standard deviation, and $p<0.05$ was considered statistically significant. Statistical analysis of the data was performed with ***t***-test or analysis of variance (ANOVA) followed by Tukey's post-hoc test.

Results

MiR-34b-5p is Markedly Increased in Mice With Bleomycin-Induced Lung Fibrosis

To explore the potential role of the miR-34b-5p in IPF, a mouse model of lung fibrosis was successfully induced by bleomycin injection. We first evaluated miR-34b-5p expression level in lung tissue of mice with lung fibrosis using qRT-PCR. As shown in Figure 1, miR-34b-5p was significantly increased in lung tissue of those mice which were intratracheally injected with bleomycin compared to the control.

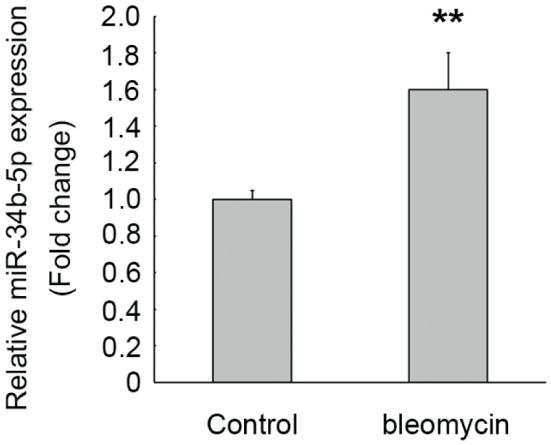

Figure 1. MiR-34b-5p is increased in bleomycin-induced lung fibrosis mouse models. Quantitative RT-PCR was conducted to assess the expression of miR-34b-5p in lung tissues from C57BL/6 mice exposed to bleomycin. Results are means ± SD for independent experiments conducted in triplicate. *$p<0.05$.

MiR-34b-5p Binds to the 3′-UTR of TIMP3 and Inhibits Its Expression

By virtue of miRNA target prediction programs (RegRNA and Targetscan), we first identified TIMP3 as a potential target gene of miR-34b-5p, with a complementary site for human miR-34b-5p in the region from 1282 to 1288 at the 3′-UTR of TIMP3 mRNA (shown in Figure 2A). To confirm that miR-34b-5p directly binds to TIMP3 mRNA, a luciferase reporter construct was generated by cloning the 3'-UTR of TIMP3 into the psiCHECK2 vector and transfected into HEK-293 cells. As expected, transfection with miR-34b-5p mimics significantly suppressed the luciferase activity of TIMP3 (Figure 2B).

Next, human lung fibroblasts were transfected with miR-34b-5p mimics or inhibitor, before the expression of TIMP3 was measured by using quantitative RT-PCR. The expression of miR-34b-5p was successfully enhanced or suppressed by transfection with mimics or inhibitor, respectively (Figure 2C). However, the overexpression of miR-34b-5p failed to significantly alter TIMP3 mRNA level in comparison to the control (data

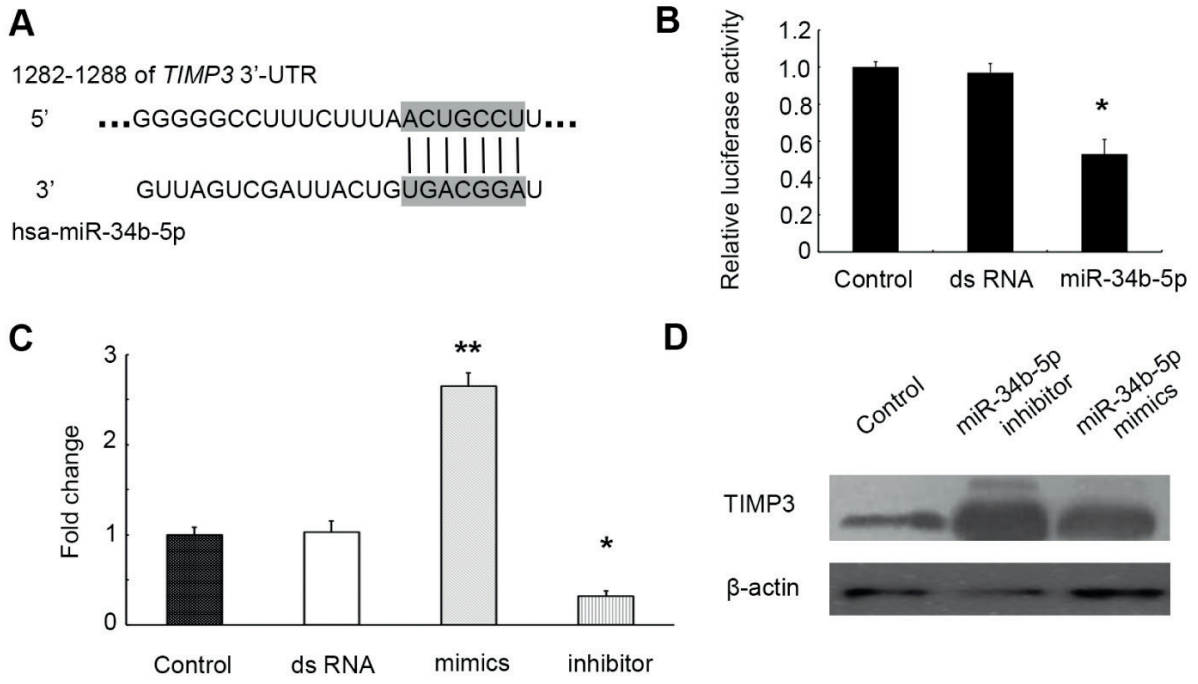

Figure 2. MiR-34b-5p binds to the 3'-UTR of TIMP3 and inhibits TIMP3 expression. ***A,*** The putative binding site of human miR-34b-5p in the 3'-UTR of TIMP3 gene. ***B,*** Luciferase reporter assays were performed by transiently transfecting HEK-293 cells with psiCHECK2 vector carrying TIMP3 3'-UTR together with control dsRNA or miR-34b-5p mimics. Quantitative RT-PCR (***C***) and Western blot (***D***) were performed to measure the mRNA and protein level of TIMP3, respectively. Results are means ± SD for independent experiments conducted in triplicate. *$p<0.05$.

not shown). We then evaluated the effect of miR-34b-5p on the protein expression of TIMP3 by Western blot analysis. As shown in Figure 2D, the endogenous expression of TIMP3 was remarkably decreased due to the transfection with miR-34b-5p mimics. In contrast, the miR-34b-5p knockdown significantly enhanced the TIMP3 protein level in the lung fibroblasts. These results imply that miR-34b-5p may directly target TIMP3 and function as a suppressor of it at the translational level.

Bleomycin-Induced Lung Fibrosis is Attenuated by miR-34b-5p Knockdown but Unaltered in TIMP3-null (Timp3$^{-/-}$) Mice

To determine whether miR-34b-5p is implicated in the pathogenesis of pulmonary fibrosis, mice were pre-treated with anti-miR-34b-5p and the severity of bleomycin-induced lung fibrosis in these mice was compared with the control. As shown in Figure 3A-3C, the increased production of hydroxyproline, collagen type I α 1 (Col1A1), fibronectin (Fn), as well as alpha-smooth muscle actin (α-SMA), was observed in the lungs upon bleomycin stimulation. However, anti-miR-34b-5p reduced the bleomycin-induced augment in the expression of above-mentioned genes. These indicated that miR-34b-5p knockdown alleviated the bleomycin-induced lung fibrosis, which was further confirmed by the histological examination of the lung sections (Figure 3D). Intriguingly, the down-regulation of miR-34b-5p in mice with TIMP3 ablation (*Timp3*$^{-/-}$) did not significantly change the fibrotic phenotypes and related gene expression, compared to the wild-type mice that were exposed to bleomycin treatment. Given that miR-34b-5p suppresses TIMP3 expression by directly targeting its 3'-UTR in fibroblasts, these results show that the miR-34b-5p silencing eliminated the inhibitory effect on TIMP3 gene, thereby dampening the bleomycin-induced lung fibrosis in wild-type mice. But in mice with TIMP3 deficiency, the down-regulation of miR-34b-5p failed to abrogate the fibrotic phenotypes in mice incented by intratracheal injection of bleomycin, suggesting that miR-34b-5p may function in pulmonary fibrosis by regulating the TIMP3 gene.

Discussion

In mammals, there are three miR-34 family members generated from two transcriptional units: miR-34a is transcribed from its own transcript, while miR-34b and miR-34c share a common primary transcript. Being identified as a direct transcriptional target of p53, miR-34 family members are implicated in the induction of G1 cell cycle arrest, senescence, and apoptosis in response to the DNA damage and oncogenic stress[13]. Although the role of miR-34a-mediated senescence in the pathogenesis of pulmonary fibrosis remains controversial[11,14], it is aberrantly expressed in lungs with bleomycin treatment[15,16]. However, studies addressing the role of miR-34b in the pathogenesis of pulmonary fibrosis are limited. MiR-34b-5p is predominantly expressed in lung tissue[17] and dysregulation of it is linked to lung carcinoma[18]. Moreover, miR-34b-5p is recently reported to be significantly elevated during the occurrence of inflammation-related diseases, including acute graft-versus-host reaction and intracranial aneurysm[19,20]. Through intravenous injection of miRNA antagomir into mice after LPS administration, Xie et al[21] found that the inhibition of miR-34b-5p dampened lung inflammation by targeting PGRN during lung injury. These all suggest that miR-34b-5p may play a critical role in inflammatory responses.

Fibrotic pathobiological process provoked by bleomycin is generally divided into three stages: epithelial cell apoptosis, inflammation, and excessive deposition of ECM. Thus, we assume that miR-34b-5p may participate in the bleomycin-stimulated lung fibrosis. Our results show that miR-34b-5p was markedly elevated in lung tissues of mice upon bleomycin incitement. Further, miR-34b-5p was found to directly target the 3'-UTR of TIMP3 and suppress the endogenous expression of TIMP3.

Matrix metalloproteinases (MMPs) comprise a class of endopeptidases that can degrade all ECM components as well as divers nonmatrix proteins including cytokines, chemokines, and receptors. As a result, MMPs are conventionally deemed as suppressors in the pathogenesis of lung fibrosis based on their role in degrading ECM components, which was also supported by previous studies[22] reporting increased levels of several MMPs in plasma and lung tissues from patients with

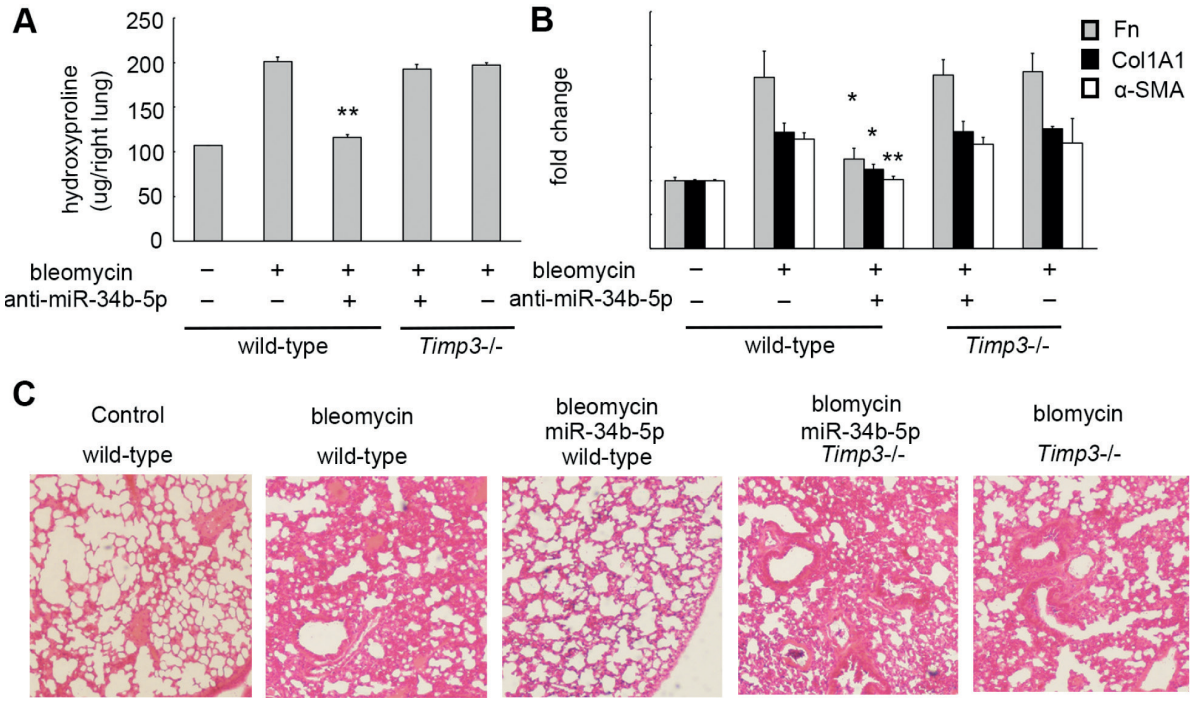

Figure 3. MiR-34b-5p down-regulation reduces pulmonary fibrosis in bleomycin-treated wild-type mice, but not in TIMP3-null mice. Wild-type (WT) or TIMP3-null (*Timp3*$^{-/-}$) mice were pretreated with anti-miR-34b-5p and then exposed to bleomycin. ***A,*** Total collagen was assessed by the hydroxyproline levels in lung tissues. ***B,*** Real Time-PCR was performed to determine mRNA levels of ECM genes Col1A1, Fn, and myofibroblast marker α-SMA. ***C,*** Histological examination of lung sections was conducted by H&E staining (magnification: 200×). n=3-6 mice in each group. Mean ± SD. *p<0.05; **p<0.01.

IPF. Actually, the catalytic activity of MMPs can be compromised by their endogenous inhibitors – four members of the tissue inhibitor of metalloproteinases (TIMP) family containing TIMP1, TIMP2, TIMP3, and TIMP4[23] – that bind to the active site of MMPs through the amino-terminal domain. Among them, TIMP3 has been recognized as a key regulator in lung homeostasis since Leco et al[24] found that the alveolar spaces were continually enlarged in parallel with aging of mice with TIMP3 deficiency. As we previously know, a more severe fibrosis occurs in bleomycin-injured *Timp3*$^{-/-}$ lungs[25]. This was also highlighted by the observation of an augment in the circulating level of active TNFα in TIMP3-null mice after LPS incitement compared to the wild-type[26]. Indeed, TIMP3 is unique from other TIMPs because it can also antagonize several ADAM domain family members, such as ADAM17 or TNFα-converting enzyme, other than most MMPs[27]. Therefore, it seems that TIMP3 plays a versatile part in the development of inflammation as well as fibrosis, rather than merely acting through the restriction of ECM degradation[28].

In this work, we examined the effects of miR-34b-5p on bleomycin-induced lung fibrosis in mice, and the data demonstrated that miR-34b-5p knockdown *in vivo* compromised the enhanced lung fibrotic status resulting from bleomycin exposure. However, the down-regulation of miR-34b-5p in mice with TIMP3 ablation did not attenuate the lung fibrosis in the presence of bleomycin compared to the wild-type mice. Given that miR-34b-5p was up-regulated in bleomycin-provoked mouse fibrotic lungs, these data together suggest that it may serve to dampen pulmonary fibrosis by directly targeting TIMP3. This will provide new insight into the mechanism underlying how miR-34b-5p is involved in human IPF.

On the other hand, there are only four TIMPs but more than 20 MMPs, and thus, each TIMP can block multiple MMPs, suggesting a complicated network between the pro-fibrotic and anti-fibrotic functions of specific MMPs and TIMPs in the process of fibrosis. Thus, further research should be conducted to explore how miR-34b-5p regulates the MMPs- and TIMP3-mediated IPF pathobiology.

Conclusions

We found that the miR-34b-5p knockdown appears to enhance the resistance to bleomycin by regulating its target gene TIMP3 during the pathogenesis of lung fibrosis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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