miR-30a as Potential Therapeutic by Targeting TET1 through Regulation of the Hydroxymethylation of Drp-1 Promoter in IPF

Songzi Zhang ¹²⁺, Huizhu Liu ¹⁺, Yuxia Liu ¹⁺, Jie Zhang ¹, Hongbo Li ³, Weili Liu ³, Guohong Cao ³, Pan Xv ³, Jinjin Zhang ¹, Changjun Lv ¹³,+ and Xiaodong Song ¹⁺⁺

¹ Department of Cellular and Genetic Medicine, School of Pharmaceutical Sciences, Binzhou Medical University, Yantai 264003, China; 18865675850@163.com (H.L.); jjinzhang@126.com (J.Z.)
² Department of Clinical Pharmacology, School of Pharmaceutical Sciences, Taishan Medical University, Taishan 271016, China
³ Department of Respiratory Medicine, Affiliated Hospital to Binzhou Medical University, Binzhou 256602, China; lihongbo0516@sina.com (H.L.); weililiu0543@sina.com (W.L.); xupan_333@126.com (P.X.)

* Correspondence: lucky_lej@sina.com (C.L.); songxd71@163.com (X.S.)
⁺ These authors contributed equally to this work.

Abstract: Several recent studies have indicated that miR-30a plays critical roles in various biological processes and diseases. However, the mechanism of miR-30a participation in the regulation of idiopathic pulmonary fibrosis (IPF) is ambiguous. Our previous study demonstrated that miR-30a may function as a novel therapeutic target for lung fibrosis by blocking mitochondrial fission, which is dependent on dynamin-related protein-1 (Drp-1). However, the regulatory mechanism between miR-30a and Drp-1 has yet to be investigated. In addition, whether miR-30a can act
as a potential therapeutic has not been verified in vivo. In this study, the miR-30a expression in IPF patients was evaluated. Computational analysis and a dual luciferase reporter system assay were used to identify the target gene of miR-30a, and cell transfection was used to confirm this relationship. Ten-eleven translocation 1 (TET1) was validated as a direct target of miR-30a, and the transfection of miR-30a mimic/inhibitor significantly reduced/increased the expression of TET1 protein. Further experiment verified that the interference on TET1(siRNA) could inhibit the hydroxymethylation of the Drp-1 promoter. Finally, miR-30a agomir was designed and applied to identify and validate the therapeutic effect of miR-30a in vivo. Our study demonstrated that miR-30a could inhibit the TET1 expression by base pairing with complementary sites in the 3’ untranslated region to regulate the hydroxymethylation of the Drp-1 promoter. Furthermore, miR-30a could act as a potential therapeutic target for IPF.

**Key Words:** idiopathic pulmonary fibrosis; miR-30a; TET1; Drp-1

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and lethal fibrotic lung disease [1]. IPF is characterized by alveolar epithelial cell injury and activation, the formation of myofibroblast foci, and the exaggerated accumulation of extracellular matrix in the lung parenchyma. Despite significant progress, the etiology and molecular mechanisms underlying IPF are largely unknown [2].

MicroRNAs (miRNAs) constitute a large family of regulatory RNAs that inhibit
target expression by base pairing with complementary sites in the 3’ untranslated region (3’ UTR) to promote mRNA decay and translational repression [3]. The critical roles of miRNAs in development and that their dysregulation causes diseases have been increasingly known [4]. Therefore, identifying disease-specific miRNAs can reveal novel mRNA target pathways and provide insight into the pathogenesis of specific diseases and therapeutic targets [5]. Recent research reported that circulating miRNAs are very stable in plasma and serum and remain stable when free from RNases under conditions of long-term storage or repeated freezing and thawing; these features show that the circulation miRNAs from patients can be used as a biomarker for diagnosis and treatment [6–8]. However, the mechanisms of circulating miRNAs controlling the IPF progression remain poorly understood.

Oxidative stress is an important phenomenon in the pathological processes of IPF and is commonly associated with the oxidative damage of epithelial cells, which is believed to be the initial event that triggers a series of aberrant repair pathways that lead to inappropriate fibrosis [9]. Du Bois once proposed that targeting the manipulation apoptosis of epithelial cell appears to be an advantageous approach to treating IPF. Considering novel antioxidant approaches is also recommended [10]. Therefore, our study focused on miR-30a regulation on the epithelial cell apoptosis during H2O2-induced IPF. Pandit et al. reported that the miR-30 family significantly decreased in IPF patients [11]. However, the regulation mechanism of miR-30 in IPF has never been systematically explored until now. Our previous study demonstrated that miR-30a may function as a novel therapeutic target for lung fibrosis by blocking
mitochondrial fission, which is dependent on dynamin-related protein-1 (Drp-1) [12]. However, three critical issues must be addressed. First, the expression of miR-30a in IPF patients has not been evaluated. Second, the target gene of miR-30a and the regulatory mode between the target gene and Drp-1 has yet to be investigated. Third, whether miR-30a can act as a potential therapeutic has not been verified in vivo. In this study, we further evaluated the circulating miR-30a expression in IPF patients and explored the miR-30a target gene of methylcytosine dioxygenase ten-eleven translocation 1 (TET1) and the regulatory mode between TET1 and Drp-1. Finally, we designed and applied the miR-30a agomir to identify and validate the therapeutic effect of miR-30a in vivo.

2. Results

2.1. miRNA-30a expression in patients and analysis of the possible target gene

Our previous study reported that miR-30a decreases in the in vivo and in vitro models of lung fibrosis [12]. In this study, we further evaluated the miR-30a expression in IPF patients to assess the significance of miR-30a in clinical research. The qRT-PCR result shows that the level of circulating miR-30a expression decreases in IPF patients compared with the normal level. This result is in agreement with that of our previous work with in vivo and in vitro models.

miRNAs exert their regulatory functions through specific interactions with their target genes. Therefore, the target genes of miR-30a were first predicted based on TargetScan, miRanda data, and miRbase. The computational analysis data showed that binding sites with miR-30a may exist in the 3'UTR of TET1 and TET3. Thus,
TET1 and TET3 are potential candidate target genes. qRT-PCR was used to further identify TET1 and TET3 as possible miR-30a targets. The results indicate that the expression of TET1 increased with the increase in H2O2 treatment time, suggesting the time dependence of the TET1 expression. Meanwhile, TET3 expression showed a disordered condition. Further analysis of the Pearson correlation coefficient showed that miR-30a is inversely correlated with TET1. Therefore, only TET1 was selected as miR-30a target for further study.

2.2. Confirmation of TET1 as a target gene of miR-30a

To verify TET1 as a target gene of miR-30a, we set up a dual luciferase assay reporter system by amplifying and inserting the 3′ UTR of TET1 into the pMIR-REPORT vector, which contained downstream firefly luciferase. The results show that the luciferase activity of the wild-type (WT) 3′ UTR-TET1 significantly decreased in the cells transfected with the miR-30a mimic, whereas the miR-30a mimic could not inhibit the luciferase activities of the mutant-type (MT) 3′ UTR-TET1. The data suggest that TET1 may be a miR-30a target.

To confirm TET1 as a target gene of miR-30a, we used cell transfection with miR-30a mimic or inhibitor to identify the relationship. The results show that the TET1 expression levels increased in the H2O2-treated group compared with that in the control group, whereas the miR-30a mimic could inhibit the TET1 expression and the miR-30a inhibitor could improve the TET1 expression. The data further confirm that TET1 is a target gene of miR-30a.

2.3. Interference on TET1 inhibited hydroxymethylation of Drp-1 promoter
Our previous study showed that miR-30a could block the apoptosis of pulmonary epithelial cells by repressing mitochondrial fission, which is dependent on Drp-1. However, the regulatory mode between TET1 and Drp-1 is not clear. We further explored the regulated pattern between TET1 and Drp-1. We designed three specific primers for the predicted methylation sites in the Drp-1 promoter region and interference on TET1(siRNA). The results of the hydroxymethylation experiment show that the hydroxymethylation of the Drp-1 expression increased in the A549 cells treated with H2O2 for 24 h. However, the hydroxymethylation of the Drp-1 expression decreased in the A549 cells treated with H2O2 for 24 h after being treated with the siRNA of TET1. This finding indicates that interference on TET1 could significantly inhibit the hydroxymethylation of the Drp-1 expression. Mutual regulation may be correlated to the hydroxymethylation mode between TET1 and Drp-1, while the expression of the tested Drp-1 decreased significantly after the interference on TET1.

2.4. miR-30a as a potential therapeutic target for IPF

To explore whether miR-30a can act as a potential therapeutic in vivo, we synthesized a miR-30a agomir and sprayed it across the mouse lung using a Penn-Century MicroSprayer (Penn-Century Inc., Wyndmoor, USA). The fibrosis and the collagen content were evaluated using hematoxylin and eosin (H&E) and Masson’s staining. The results show that the mice in the miR-30a agomir group presented a more continuous structure with a better intact wall of bronchial mucous membrane than the BLM group. The alveoli in the miR-30a agomir group showed clearer hollow cavities with thinner alveolar walls than the BLM group. In addition,
the lung mesenchyme in the agomir group showed fewer collagen fibers, indicating that the hallmark of the fibroblastic foci decreased distinctly decreased. These findings suggest that miR-30a agomir could improve pulmonary fibrosis. The miR-30a level decreased significantly and TET1 increased significantly in the BLM group compared with those in the sham group, and miR-30a agomir could improve the miR-30a expression and inhibit the TET1 expression in the agomir group compared with those in the BLM group.

To further investigate the anti-fibrosis action of miR-30a agomir in vivo, we tested the expression levels of hydroxyproline (HYP), a-SMA, E-cadherin, and vimentin, which are indicators of pulmonary fibrosis, after spraying with miR-30a agomir in vivo. The results demonstrate that agomir could reduce the HYP content, a-SMA, and vimentin expression compared with those in the BLM group but improved the E-cadherin expression.

3. Discussion

Currently, IPF is the most life-threatening idiopathic disease, presenting a mortality rate that exceeds those of numerous cancers [10,13]. Although several genetic, epigenetic, and proteomic studies have been conducted to date, studies investigating miRNA regulatory networks in IPF have only recently gained significant attention [2]. Our previous study revealed that miR-30a can block mitochondrial fission, which is dependent on Drp-1, to regulate the development of IPF. However, the regulatory mechanism between miR-30a and Drp-1 has yet to be investigated. The miR-30 family includes miR-30a, b, c, d, and e and serves important functions in
various life activities and the development of various illnesses, such as epithelial-to-mesenchymal transition, cellular differentiation, and senescence, and cancer [14,15]. The family members share the same seed sequence and are encoded by six genes located in human chromosomes 1, 6, and 8. The genes identified as targets of one or more miR-30 family members include Xlim1/Lhx1 [16], Snail1 [17], BCL6 [18], p53 [19], and RUNX2 [20]. In the present study, we identified and validated TET1 as a target of miR-30a and the regulatory mode between TET1 and Drp-1. Furthermore, we demonstrated that miR-30a could act as a potential therapeutic by targeting TET1 through regulating the hydroxymethylation of Drp-1 promoter in IPF.

The TET protein family includes three members (TET1–3), all of which are capable of converting 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC) in a 2-oxoglutarate- and Fe(II)-dependent manner [21,22]. In addition, the DNA methylation at the 5-position of cytosine is a key epigenetic mark, which is critical for various biological and pathological processes [23,24]. Genome-wide studies demonstrated the enrichment of 5-hmC at the enhancers, promoters, and gene bodies of actively expressed genes [25,26]. The presence of 5-hmC may contribute to both passive and passive DNA demethylation. TET1 is required to maintain the normal abundance and distribution of 5-hmC, which prevents the hypermethylation of DNA and promotes the demethylation of DNA by a sequential process involving the deamination of 5-hmC to 5-hmU; moreover, TET1 is required for the regulation of genes-encoding molecules involved in chromosome maintenance and DNA repair.
TET1 expression is the highest in long-term hematopoietic stem cells, multipotent progenitors, common lymphoid progenitors, and common myeloid progenitors, and decreases during B lineage commitment and becomes undetectable in immature and mature myeloid cells. The loss of TET1 function predisposes HSCs to malignancy and, more specifically, B cell lymphoma [28]. In drug addiction, TET1 normally regulates cocaine reward negatively, and the cocaine-induced suppression of TET1 in nucleus accumbens contributes to enhanced drug sensitivity [29]. This epigenetic lesion has also been extended to the DNA methylation-mediated silencing of miRNA [30-32]. miR-22 was recently found to exert its metastatic potential by silencing antmitotic miR-200 through the direct targeting of the TET family of methycytosine dioxygenases, thereby inhibiting the demethylation of the miR-200 promoter [33]. However, the mechanism by which this epigenetic mark of 5-hmC and the related enzyme of TET participate in IPF progression remains unknown. Our data illustrate that the decrease in the 5-hmC of Drp-1 was caused, at least in part, by the decreased expression level of the key enzyme TET1, which controls the 5-hmC production.

Several recent studies indicated that miR-30a performs critical roles in various biological processes. For example, the expression of Ubc9 is regulated by miRNA-30a in human subcutaneous adipocytes [34]. miR-30a reduces IRF4 expression through the specific binding with the 3′ UTR, thus suppressing the Th17 differentiation and preventing the full development of autoimmune encephalomyelitis [35]. miR-30a targets the DNA replication protein RPA1, hinders the replication of
DNA, and induces DNA fragmentation [36]. These studies lack information on the therapeutic effect of miR-30a in vivo. Moreover, pulmonary fibrosis data have yet to be reported. To verify the therapeutic effect of miR-30a further, the miR-30a agomir was designed and applied in vivo. Here, we found that injection of miR-30a agomir to overexpressed miR-30a leads to thinner walls of alveoli and fewer fibers, which could speed up the diffusion of oxygen into the adjacent blood capillaries and the opposite movement of carbon dioxide compared with the BLM group.

In addition, we assessed the significance of circulating miR-30a in clinical application. Future studies need to expand the analysis of miR-30a to a larger cohort of patients with IPF to determine with statistical confidence whether reduced miR-30a expression levels are correlated with poor patient outcome. In summary, our findings in vivo and in vitro together with the clinical data of patients with IPF provide evidence that the upregulation of miR-30a could downregulate the expression level of its target gene TET1 and exert anti-fibrotic effect and protection function against pulmonary injury. Our work suggests a novel approach to prevent pulmonary fibrosis damage and provides a potential therapeutic target for pulmonary fibrosis treatment.

4. Materials and methods

4.1. IPF patients

IPF was diagnosed in accordance with the American Thoracic Society/European Respiratory Society consensus criteria [1], which include clinical, radiographic, and characteristic histopathological features (n=40). 5 mL of blood sample was obtained from each participant and prepared for testing. Matched plasma samples from healthy
volunteers (n=40) were selected corresponding to the IPF patients’ sex and age. As stated in the agreement, a written informed consent was obtained by the doctors from each participant. The ethics committee of Binzhou Medical University approved this study.

4.2. Cell transfection

A549 cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell culture was as our previously described [37,38]. miR-30a mimic, inhibitor and TET1 siRNA were synthesized by RiboBio Co. Ltd (Guangzhou, China). An amount of $1 \times 10^5$ cells were seeded in 24-well plates and cultivated with 1640 medium containing 10% newborn calf serum for 24 hours. 1.25 ul of 20 uM miR-30a mimic/inhibitor or 1.25 ul of 20 uM TET1 siRNA was dilute with 50 ul 1× riboFECT™ CP buffer and incubated for 5 min. at room temperature. Five microlitres riboFECT™ CP reagents were added and incubated for 15 min. at room temperature. The mixed liquid was added to 443.75 ul 1640 medium without 10% newborn calf serum. Cells were incubated with the mixed liquor for 48 hrs.

4.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRizol reagent. RNA quantity and quality were measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Complementary DNA synthesis was performed with the M-MLV reverse transcriptase kit following the manufacturer’s instructions. qRT-PCR was performed with a SYBR green-based PCR master mix kit on a Rotor Gene 3000 real-time PCR system.
4.4. Animal model and ethic statement

C57BL/6 mice (8 weeks) were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). All animal experiments were performed in accordance with the regulations established by the Committee on the Ethics of Animal Experiments of Binzhou Medical University. The mice were housed under a 12-h light/dark cycle and were allowed free access to food and water and randomly divided into four groups (10 mice each) including the sham group, bleomycin (BLM)-treated group (BLM group), BLM+ negative control of miR-30a agomir (NC group) and BLM+ miR-30a agomir. The dosage of miR-30a agomir was 10 nmol for each mouse and be sprayed into the lung twice per week for 4 weeks by using Penn-Century MicroSprayer (Penn-Century Inc., Wyndmoor, USA). On day 28, all mice were killed, and lung tissue sections were collected and immediately frozen in liquid nitrogen for further studies. The BLM animal model was administered 5 mg/kg BLM dissolved in saline via a single intratracheal instillation under anaesthesia as our previously described [39,40].

4.5. Dual luciferase assays

The wild type and mutation type 3’UTR of TET1 containing miR-30a-binding site were synthesized by Obio Technology Co., Ltd (Shanghai, China). 293T cells were plated at 50–60% confluence and transfected with miR-30 mimics as well as the 3’UTR luciferase vector using Lipofectamine™ 3000 transfection reagent according to the manufacturer’s instructions. Forty eight hours after transfection, luciferase
assay was performed using dual luciferase reporter assay kit in compliance to the manufacturer’s instructions.

4.6. H&E and Masson’s Trichrome staining

Pulmonary tissues were fixed by inflation with 4% paraformaldehyde overnight, dehydrated in 70% ethanol, and embedded in paraffin wax. Sections of 4 mm thickness were prepared and stained with H&E or Masson’s trichrome staining according to the manufacturer’s standard protocol. Degrees of microscopic interstitial fibrosis and collagen were graded as our previously described [39].

4.7. Western blot analysis

Protein concentration was quantified using a bicinchoninic acid protein assay kit and boiled with the sample buffer in a water bath for 5 min. Protein samples were separated with 15% SDS-PAGE gels for 2 hours and transferred onto a polyvinylidene difluoride membrane, which was subsequently blocked in 5% non-fat milk for 2 hours. Blots were probed using the primary antibodies. The anti-Drp1 antibody was from Santa Cruz Biotechnology. After three times washing with tris buffered saline tween, the horseradish peroxidase-conjugated secondary antibodies were added. Antigen-antibody complexes were visualized by enhanced chemiluminescence.

4.8. Statistical analysis

Data were expressed as the mean ± SD from the indicated number of independent experiments. Statistical analysis was performed with SPSS 17.0 software by one-way ANOVA and Student’s t-test. P < 0.05 was considered to indicate a statistically significant difference.
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Author Contributions: Changjun Lv and Xiaodong Song designed the experiments and wrote the initial draft. Songzi Zhang, Huizhu Liu, Yuxia Liu, Jie Zhang and Jinjin Zhang performed the experiments in vivo and in vitro. Hongbo Li, Weili Liu, Guohong Cao and Pan Xv performed the experiments in patients with IPF.

Conflicts of Interest: The authors declare no conflict of interest.

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Figure legends

(A) Circulating miRNA-30a decreased in 40 patients with IPF compared with those in normal individuals according to the qRT-PCR analysis. (B) The qRT-PCR analysis showed that TET1 increased with the time extension in the H₂O₂-treated group. A549 was treated with 120 µM of H₂O₂ and harvested at 0, 3, 6, 12, and 24 h. Data of each group are presented as means ± standard deviation (SD); n=6, *p<0.05, **p<0.01. (C) TET3 showed a disordered expression with the time extension in the H₂O₂-treated group. A549 was treated with 120 µM of H₂O₂ and harvested at 0, 3, 6, 12, and 24 h. The data of each group are presented as means ± SD; n=6, *p<0.05, **p<0.01. (D) miR-30a is inversely correlated with TET1. Statistical analysis was conducted using the Pearson correlation coefficient.

Figure 1. miR-30a expression in IPF patient and analysis of the possible target gene.
Figure 2. Experimental confirmation of TET1 as a target gene of miR-30a. (A) Binding site with miR-30a in 3′ UTR of TET1. WT: wild type. MT: mutation type. (B) Ratios of Firefly/Renilla in the dual luciferase reporter system analysis. The luciferase ratio of Firefly/Renilla represents the target gene expression. The luciferase activity of the WT 3′ UTR-TET1 significantly decreased in the cells transfected with miR-30a mimic, whereas the miR-30a mimic could not inhibit the luciferase activities of the MT 3′ UTR-TET1. (C) The miRNA-30a mimic inhibited the expression of TET1 in the A549 cells treated with H2O2 for 24 h. NC is the negative control of the miRNA-30a mimic. (D) The miRNA-30a inhibitor improved the expression of TET1 in the A549 cells treated with H2O2 for 24 hours. NC is the negative control of the miRNA-30a inhibitor. The data of each group are presented as means ± SD, *p<0.05.
**Figure 3.** Effect of TET1 siRNA on the hydroxymethylation of the Drp-1 promoter. (A) TET1 siRNA could inhibit the hydroxymethylation expression of the Drp-1 promoter compared with the H_2O_2_ group. Primer1: 5′-GGCTGGCTGTCTCCCCATCCTGA-3′, 5′-AAATGCCTGCTTTGGGAGTGATGTCG-3′;Primer2: 5′-TGGAGGCGCTAATTCCTGTCA-3′, 5′-CTCTCACCTGCGTTCCCACTAC-3′;Primer3: 5′-AGAGGAGGAAGGAGGAGGC GAACT-3′, 5′-GCTTTTATGACAGGAATTAGCG-3′. (B) The Drp-1 expression decreased significantly after TET1 siRNA. NC is the negative control of TET1 siRNA. The data of each group are presented as means ± SD,*p<0.05.
Figure 4. miR-30a as a potential therapeutic target for IPF. (A) Schematic of the miR-30a agomir action. (B) miRNA-30a agomir could improve the alveolar structure in vivo through H&E staining. (C) miRNA-30a agomir could inhibit the collagen fibers through Masson’s staining. The color blue represents collagen fibers. (D) Score of lung fibrosis on H&E staining. (E) Score of collagen content on Masson’s staining. (F) miRNA-30a expression increased in mice sprayed with agomir compared with that in the BLM group according to qRT-PCR. (G) TET1 expression decreased in mice sprayed with agomir compared with that in the BLM group according to
qRT-PCR. (H) Drp-1 and TET1 expression decreased in mice sprayed with agomir compared with that in the BLM group according to the Western blot. Each bar represents the mean±SD, n=6 and *p<0.05.

Figure 5. miR-30a agomir inhibited the indicators of pulmonary fibrosis in vivo. (A) miRNA-30a agomir could inhibit the HYP level. (B) miRNA-30a agomir could inhibit the α-SMA expression. (C) miRNA-30a agomir could promote the E-cad expression and inhibit the vimentin expression. Each bar represents the mean±SD, n=6 and *p<0.05.