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Original Article

miR-425 reduction causes aberrant proliferation and collagen synthesis through modulating TGF-β/Smad signaling in acute respiratory distress syndrome

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Abstract: *Background*: Acute respiratory distress syndrome (ARDS) is a severe form of acute lung injury which may trigger persistent fibrosis. Exosomes are small extracellular vesicles that reflecthost cell conditions and contain functional molecules including miRNAs. Methods: In this study, we isolated plasma exosomes from 53 ARDS patients and 53 controls. Six candidate miRNAs levels were determined by qRT-PCR. The H3K27me3 level on the promoter region of Smad2 was detected by ChIP assay followed by qPCR. Dual luciferase assay and immunoblotting were employed to verify the interaction between miRNA and target genes. The cells proliferation was determined by MTT dependent cell viability assay. *Results*: miR-425 was reduced in the ARDS patient exosomes. Cytokine treatment also reduced the miR-425 level in A549 and HFL-1 cells. miR-425 inhibition induced Smad2 overexpression by increasing KDM6A level and demethylated H3K27me3 in the Smad2 promoter region. miR-425 reduction induced collagen expression after TGF-β1 treatment and promoted fibroblast proliferation. *Conclusion*: We identified miR-425 reduction in the exosomes from ARDS patients' peripheral blood, which has the potential to be used as a biomarker for ARDS diagnosis. We demonstrated that miR-425 reduction in lung fibroblasts contributes to the fibrosis through upregulating KDM6A and then activates the TGF-β signaling pathway. This sheds light on the mechanism of lung fibrosis during ARDS.

Keywords: miRNA, histone methylation, ARDS, lung fibrosis

Introduction

Acute respiratory distress syndrome (ARDS), a severe form of acute lung injury that occurring in critically ill or wounded patients, is characterized by widespread inflammation in the lungs, which reduces the oxygen uptake [1, 2]. About a third of the patients die, and many survivors suffer from complications such as breathing problems caused by pulmonary fibrosis and oxygen deficit [3, 4]. Interstitial and intra-alveolar fibrosis are the main features a few weeks after the onset of ARDS which involves excessive synthesis and deposition of extracellular matrix proteins, in particular collagen [5]. Prediction of outcome in patients with ARDS is of major importance for appropriate treatment decisions and resource allocation. However, the complex etiology has complicated ARDS diagnosis and treatment. Although many protein-based biomarkers have been identified from patients with ARDS, none of them have been translated for ARDS clinical diagnosis [6].

MicroRNAs (miRNAs) are a group of gene expression negative regulators which repress gene expression at the post-transcriptional level by targeting the 3-untranslated region (UTR) region of target mRNA [7]. miRNAs are involved in gene function during many different biologic processes, such as proliferation, apoptosis, and differentiation. Abnormal miRNA expressions has been related to human diseases including ARDS. In the LPS-induced ARDS mouse model, researchers found certain miRNAs were up-regulated and others were down-regulated [8, 9]. In human, blood miR-181a and miR-92a level are risk markers, and miR-424 level is a protective marker for ARDS [10].

Exosomes are small extracellular vesicles derived from endosomal compartment vesicles

Table 1. Clinical characteristics of ARDS patients

Phenotype	n	Age	Pathogenic factors	Serum IL-1β (pg/mL)	Lung Ly6G+ neutrophil (%)	PaO_2/FiO_2 (mm Hg)
Healthy controls	53	42.1	NA	12.1 ± 4.3	4.6 ± 0.6	450.8 ± 31.6
ARDS	32	43.2	Primary pneumonia	48.3 ± 13.2	10.1 ± 1.2	88.6 ± 32.8
	18	40.5	Trauma	49.6 ± 13.3	10.5 ± 1.8	130.2 ± 54.6
	3	38.6	Blood transfusion	37.5 ± 16.7	9.6 ± 3.7	245.7 ± 89.4

Values represent mean ± SD.

budding from the plasma membrane. Importantly, exosomes can be produced by almost all types of cells in culture and in various human body fluids including blood, saliva, urine, and breast milk [11]. As an important part of cell-cell communication, exosomes protect miRNAs from degradation and deliver specific miRNAs from supplier cells to receiver cells. Recently, researchers found that exosomes derived from endothelial progenitor cells ameliorate acute lung injury by transferring miR-126 to target endothelial cells [12].

In this study, we detected 6 candidate miRNAs in the exosomes extracted from the peripheral blood of 53 ARDS patients and 53 healthy controls. We found the level of miR-425 was significantly reduced. After functional study, we found that miR-425 can regulate TGF-β/Smad signaling through targeting histone demethylation.

Materials and methods

Study population

53 ARDS patients and 53 healthy volunteers matched by age (± 5 years) and sex were obtained from Department of Critical Care Medicine, Renmin Hospital of Wuhan University. The clinical features are listed in **Table 1**. All ARDS subjects met the Berlin diagnostic definition[4]: timing of ARDS was within 1 week of a known clinical insult or new or worsening respiratory symptoms; chest imaging showed bilateral opacities (not fully explained by effusions, lobar/lung collapse, or nodules); respiratory failure was not fully explained by cardiac failure or fluid overload; and ARDS severity was based on PaO_2/FiO_2 ratio. Patients with diffuse alveolar hemorrhage or chronic lung disease were excluded. Treatment with granulocyte colony-stimulating factor or inhibitors of tumor necrosis factor were exclusion criteria [13]. Patients were enrolled in the study immediately after meeting all inclusion criteria [4]. Institutional review board of Department of Critical Care Medicine, Renmin Hospital of Wuhan University approved this study.

Exosome isolation from plasma samples

EVs were extracted from plasma samples using Puro Exo exosome isolation kit (101Bio, USA) following the manufacturer's instruction. Briefly, plasma sample was centrifuge at 3000 × g for 10 minutes at 20°C to remove cells and debris, followed by centrifugation at 10,000 × g for 20 minutes. The supernatant was filtered with a 0.22 μm filter and then mixed with 0.2 volume exosome precipitation reagent. After 2 hours' incubation at 4°C, the sample was centrifuged at 10,000 × g for 30 minutes at 20°C. The pellet was resuspended in PBS for further study.

RNA extraction

Total RNA was extracted from exosomes using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Before RNA extraction, cel-miR-39 was added to EV samples as an external reference. RNA concentration and purity were determined using a model ND-2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Only samples with absorbance ratios 260 nm/280 nm of ~2.0, and 260 nm/230 nm of 1.9-2.2 were considered for inclusion in the study.

Quantitative RT-PCR

Quantitative RT-PCR analysis was used to determine the relative level of selected miRNAs. The levels were detected by TaqMan miRNA RT-Real Time PCR. Single-stranded cDNA was synthesized using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and then subjected to qPCR using miRNA-specific TaqMan MGB probes (Applied Biosystems). Cel-miR-39 was

used for normalization. Each sample in each group was measured in triplicate and the experiment was repeated at least three times.

Cell culture

Human lung fibroblasts HFL-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Human alveolar epithelial A549 cells were purchased from Chinese National Infrastructure of Cell Line Resources. All these cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 100 IU/ml streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO_2.

Cell treatment

We exposed the HFL-1 and A549 cells to a mixture of three different cytokines referred to as cytomix (IL-1β, TNF-α, and IFN-γ, 50 ng/ml each) (R&D systems, Minneapolis, MN, USA). It is reported that these are the major proinflammatory cytokines in pulmonary edema fluid from patients with ARDS [14].

Dual luciferase assay

To generate 3'-UTR luciferase reporter, full length of Smad2 3'UTR or a 342 bp segment of 3'UTR from KDM6A was cloned downstream of the firefly luciferase gene in the pmirGLO plasmid (Promega, Madison, WI USA). miRNA mimics and inhibitors were purchased from GenePharma Co., Ltd (Shanghai, China). For the luciferase reporter assay, A549 cells were seeded in 48-well plates. Luciferase reporter vectors were co-transfected with miRNA mimic or inhibitor using lipofectamine 2000 (Invitrogen). 48 hours after transfection, cells were lysed and the cell lysates were subjected to dual luciferase assay with the Dual-Luciferase Assay kit (Promega, Madison, WI USA). Each treatment was performed in triplicate in three independent experiments. The results are expressed as relative luciferase activity (Firefly Luciferase/Renilla Luciferase).

Immunoblotting

Proteins were extracted from cell samples and then separate by electrophoresis after boiling in sodium dodecyl sulfate/β-mercaptoethanol sample buffe. The proteins in the gels were blotted onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. After blocking by 5% nonfat milk for 1 hour at room temperature, the membrane was incubated with rabbit anti-KDM6A polyclonal antibody (Abcam, Cambridge, MA, USA) or mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After three times washes, the membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit or rabbit anti-mouse secondary antibody for another 2 hours at room temperature. Detection by the chemiluminescence reaction was carried out using an ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control.

Chromatin immunoprecipitation (ChIP) assay

miR-425 mimic or inhibitor treated cells were collected and the cell nuclei were isolated. Nuclear lysates were sonicated to make small DNA fragments ranging from 100 to 1000 base pairs and then incubated with ChIP grade anti-H3K27me3 or H3K4me3 or H3K9me3 rabbit antibody (Abcam). Isotype-matched control Ab was used as negative control. Immune complexes containing DNA fragments were precipitated using Magna A/G beads supplied within Zymo-Spin ChIP kit (Zymo Research). Relative enrichment of the target regions in the pellets were detected by qPCR using Fast SYBER Green Master Mix (Applied Biosystems). All data were normalized to input controls.

Cell proliferation assay

Cell proliferation was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A549 and HFL-1 cells were seeded in wells of 96-well plates at low density (2×10^3) in DMEM medium and allowed to attach overnight. The cells were then transfected with miRNA mimic or inhibitor, with scrambled sequence RNA as control. There were 3 wells in each group. Twenty microliters of MTT (5 mg/mL) (Sigma-Aldrich) were added to each well 48 h after transfection, and the cells were incubated for 4 h. The absorbance was recorded at 570 nm with a 96-well plate reader after addition of dimethyl sulfoxide (DMSO).

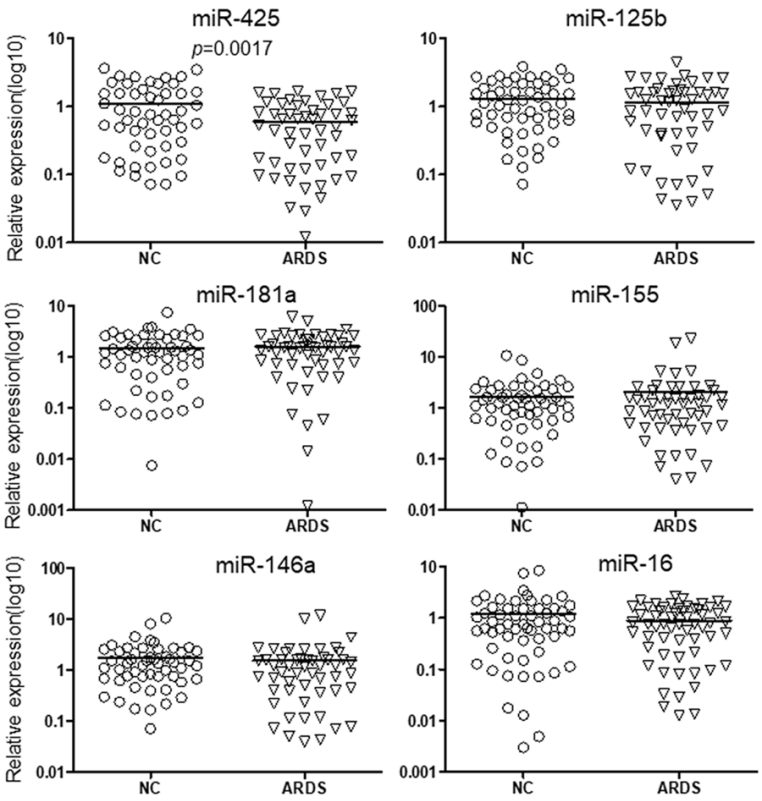

Figure 1. Exosomal miR-425 was reduced in the plasma from ARDS patients. Exosomes were isolated from ARDS patients' plasma samples followed by RNA extraction. Six candidate miRNAs were quantified by qRT-PCR. Results were analyzed by t-test and P < 0.05 was considered significant.

Statistical analysis

Data were analyzed using SPSS Statistical Package version 19.0 (SPSS Inc., Chicago, IL, USA). The luciferase activities, cell apoptosis, and proliferation assay were analyzed using paired t-test. A two-tailed *P* value < 0.05 was considered significant.

Results

To identify new biomarkers for ARDS, we first isolated exosomes from ARDS patients' plasma samples. Total RNA was extracted from plasma exosomes and the level of six candidate miRNAs (miR-425, miR-125b, miR-181a, miR-155, miR-146a and miR-16) were determined by qRT-PCR. We chose these miRNAs because these miRNAs' levels were altered in ARDS patients or mouse models, and these miRNAs were found to exist in the exosomes [15-19]. We found miR-425 level was significantly reduced ($P = 0.017$) in the exosome samples of ARDS patients (**Figure 1**). To find the miR-425 level in the lungs of ARDS patients, we treated the A549 and HFL-1 cells with a cytokine mixture containing IL-1β, TNF-α, and IFN-γ to mimic the conditions in ARDS patients. These cytokines are the major proinflammatory cytokines in pulmonary edema fluid from patients with ARDS. After 48 hours' treatment, we detected the miR-425 level in the cells and in the cell culture medium. We found miR-425 level was reduced in both cells and media (**Figure 2A**).

TGF-β/Smad signaling plays important roles in tissue fibrosis, including lung fibrosis during ARDS [20-22]. Thus, we treated A549 cells using miR-425 inhibitor or control RNA oligos, and detected the level of Smad2, Smad3, Smad4 and phosphorylated Smad2 (P-Smad2). We found that Smad2 protein and mRNA levels were significantly increased in the miR-425 inhibitor-treated cells (**Figure 2B**). P-Smad2 level was detectable after TGF-β treatment and significantly increased in the miR-425 inhibitor-transfected cells (**Figure 2B**). Since miRNAs regulate target genes through targeting the 3' untranslated regions (3'UTR), we constructed Smad2 3'UTR reporter vector. A549 cells were transfected with Smad2 reporter vector and miR-425 mimic or inhibitor for 48 hours. The cells were lysed and luciferase activity was detected. As shown in **Figure 2C**, miR-425 did not target Smad2 3'UTR directly.

Histone methylation is a powerful system regulating gene transcription, especially the methylation of histone H3. To investigate whether miR-425 involved in the histone methylation system, we did ChIP assay using H3K4me3, H3K9me3 and H3K27me3 antibodies separately. Three pairs of primers were designed to amplify three segments that locate at -2 kb, -0.3 kb and +0.5 kb relative to the first code of Smad2. As shown in **Figure 2D**, the H3K27me3 antibody recruited more Smad2 promoter region segments in the cells treated by miR-425 inhibitor, suggesting that miR-425 reduction modulates Smad2 expression through pro-

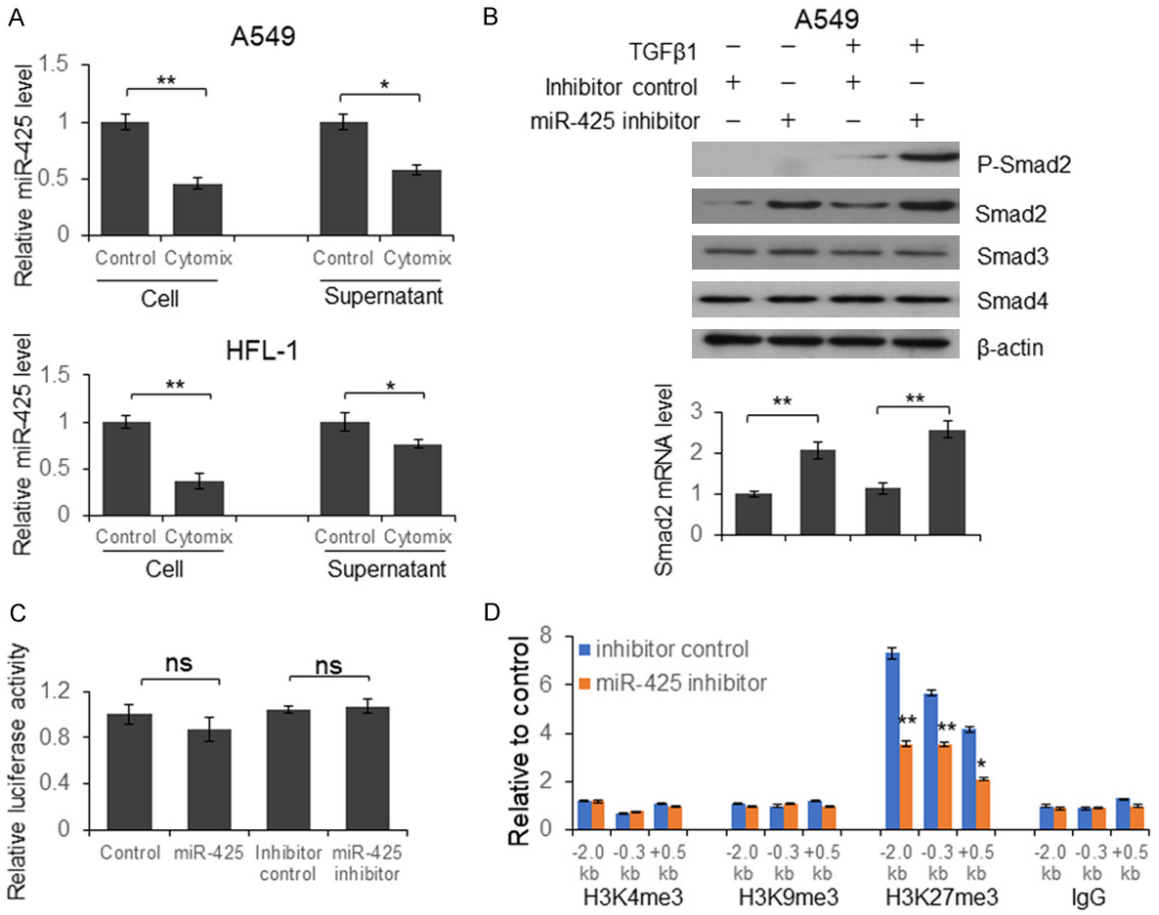

Figure 2. miR-425 reduction increases Smad2 expression by demethylating the promoter region of Smad2. A. A549 and HFL-1 cells were treated by cytokine mix and the miR-425 levels in the cells and the medium were detected by qRT-PCR. B. A549 cells were transfected by miR-425 inhibitor or control oligo, with or without TGF-β treatment. The levels of Smad2, Smad3, Smad4 and phosphorylated Smad2 were determined by immunoblotting. C. Smad2 3'UTR reporter vector co-transfected with miR-425 mimic or inhibitor for 48 hours. Luciferase activities were detected using cell lysates. D. A549 cells were transfected with miR-425 inhibitor for 48 hours and the promoter region of Smad2 gene was quantified after a ChIP assay. Results were analyzed by t-test and $P < 0.05$ was considered significant. *$P < 0.05$, **$P < 0.01$.

moting the demethylation of H3K27me3 in the Smad2 promoter region.

To investigate how miR-425 regulates histone methylation, we predicted the miR-425 targets using online bioinformatics tools TargetScan (http://www.targetscan.org/vert_70/) and RNAhybrid (https://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid). We found lysine demethylase 6A (KDM6A) is a potential target of miR-425 (**Figure 3A**). Subsequently, we cloned a 342 bp segment of KDM6A 3'UTR containing the predicted miR-425 site into pmirGLO, following the coding region of firefly luciferase to generate the reporter vector. The KDM6A reporter vector was transiently transfected into A549 cells with one of the oligos (miR-425 mimic, Control oligo, miR-425 inhibitor and inhibitor control oligo) for 48 hours. The cells were lysed and luciferase activities were detected. We found the relative luciferase activity was significantly repressed by miR-425 mimic and up-regulated by miR-425 inhibitor (**Figure 3B**). Also, when three nucleotides were mutated in the predicted miR-425 target region, the luciferase activity was not repressed by miR-425, indicating that miR-425 repressed firefly luciferase expression by targeting KDM6A 3'UTR.

To further verify endogenous KDM6A was repressed by miR-425, A549 cells were transfected with miR-425 mimic or inhibitor for 48 hours and the level of KDM6A was determined by western blotting. The H3K27me3 level in the

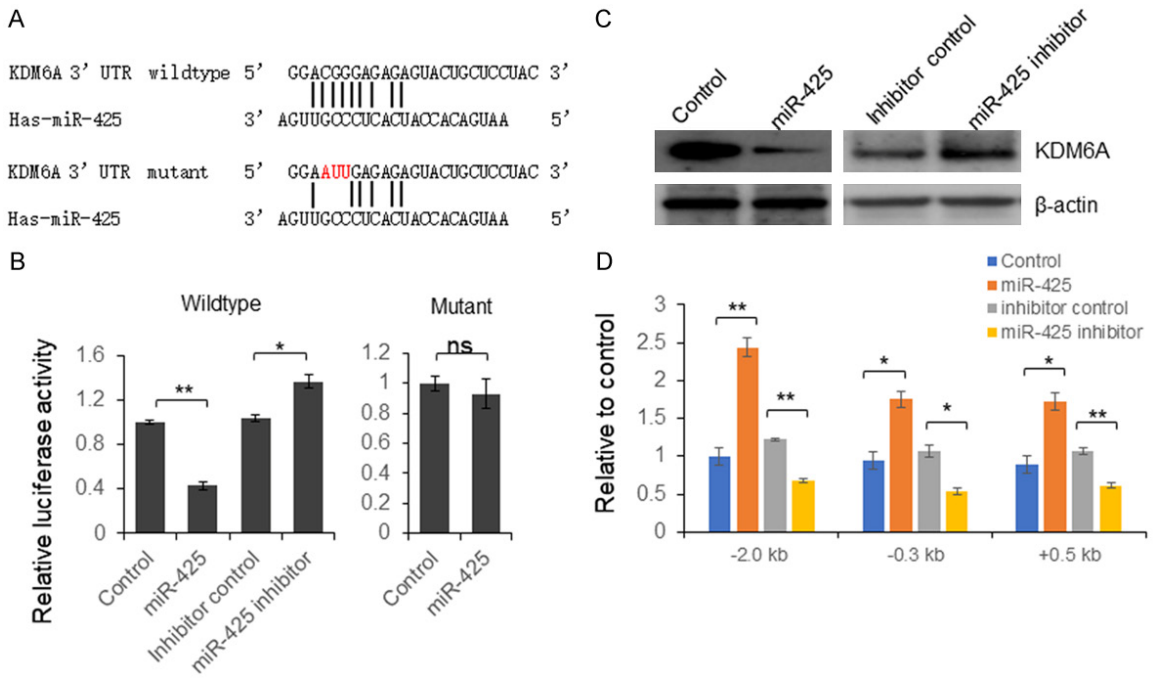

Figure 3. miR-425 represses KDM6A expression through targeting 3'UTR. A. Predicted interaction between miR-425 and KDM6A 3'UTR. Red letters represent the mutant nucleotides. B. Wildtype or mutant Smad2 reporter vector transfected with miR-425 mimic or inhibitor into A549 cells for 48 hours. The luciferase activities were examined using cell lysates. C. miR-425 mimic or inhibitor transiently transfected into A549 cells for 48 hours. The protein level of endogenous KDM6A was detected by immunoblotting, with the level of β-actin as a loading control. D. The segments of -2.0 kb, -0.3 kb and +0.5 kb region relative to the first code of Smad2 were quantified after ChIP assay by qPCR, 48 hours post-transfection by miR-425 mimic or inhibitor. Results were analyzed by t-test and $P < 0.05$ was considered significant. *$P < 0.05$, **$P < 0.01$.

promoter region of Smad2 gene was detected by ChIP assay followed by qRT-PCR. We found that the endogenous KDM6A protein level was reduced by miR-425 mimic and increased by miR-425 inhibitor, indicating that KDM6A is a direct target of miR-425 (**Figure 3C**). The H3K27me3 level of Smad2 promoter region was increased after miR-425 mimic treatment and reduced in the miR-425 inhibitor-treated cells (**Figure 3D**).

To determine the mechanism of how miR-425 involved in the fibrosis, A549 and HFL-1 cells were transiently transfected by miR-425 inhibitor or inhibitor control for 24 hours and then treated or non-treated with TGF-β for another 24 hours. The levels of Smad2, P-Smad2, Collagen I and Collagen III were detected by western blotting. We found the Smad2 and P-Smad2 levels were significantly increased in the miR-425 inhibitor treated cells (**Figure 4A** and **4B**). The level of Collagen I and Collagen III was increased after TGF-β treatment, and, the miR-425 inhibitor and TGF-β dual-treated A549 and HFL-1 cells had the highest levels of Collagen I and Collagen III. We also detected the function of miR-425 inhibitor on cell proliferation. We found A549 and HFL-1 cells treated by miR-425 inhibitor grew faster than the control cells 48 hours after treatment (**Figure 4C** and **4D**). In the HFL-1 cells, KDM6A specific siRNA partially restored the function of miR-425 inhibitor. However, in the A549 cells, siKDM6A did not significantly restore the function of miR-425 inhibitor regarding cell proliferation (**Figure 4C** and **4D**).

Discussion

Exosomes are small extracellular vesicles containing proteins and RNAs, and represent some characteristics of the host cells. Exosomes are easily obtained from biologic fluids like blood and urine, and relatively stable. Thus, we screened the exosomal miRNAs from ARDS patients to find a clue for ARDS diagnosis and treatment. We found that the miR-425 level was significantly reduced in the exosomes from

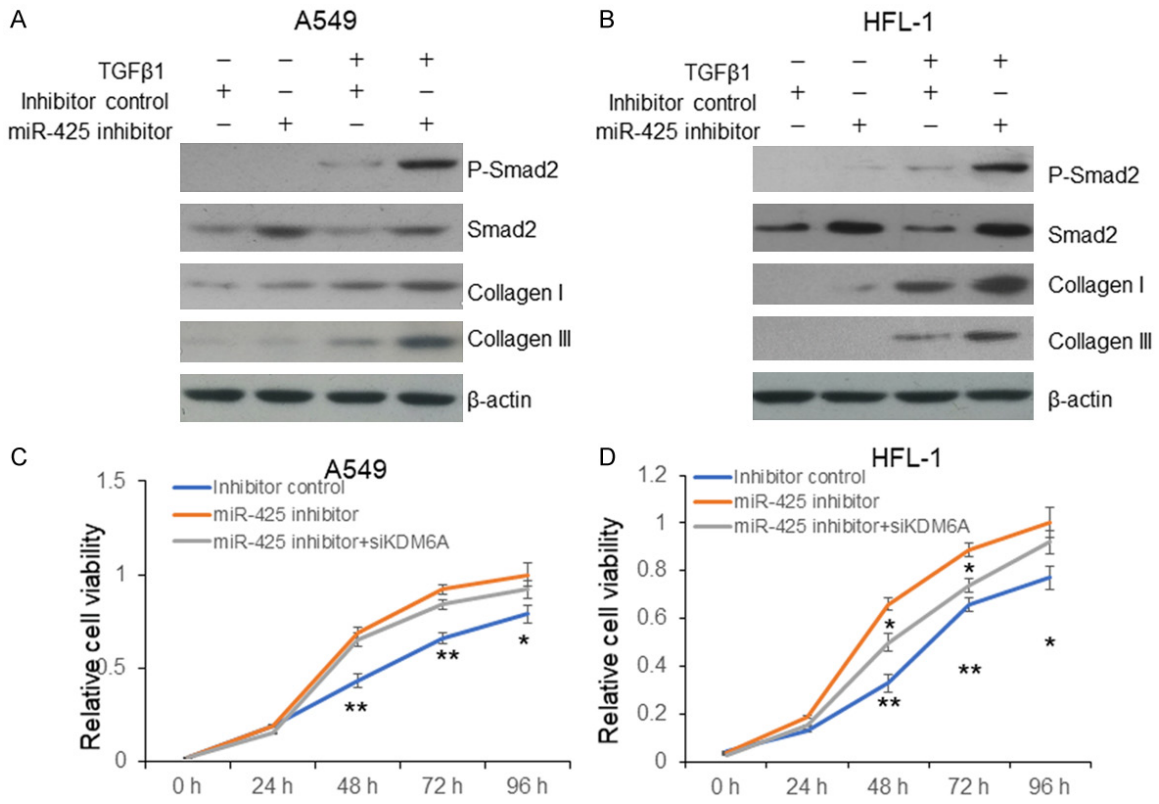

Figure 4. miR-425 reduction relates to fibrosis through increasing collagen synthesis and fibroblast proliferation. A549 (A) or HFL-1 (B) cells were transfected with miR-425 inhibitor followed by treatment with or without TGF-β. The levels of Smad2, P-Smad2, Collagen I and Collagen III were detected by immunoblotting. A549 (C) or HFL-1 (D) cells were transfected with miR-425 inhibitor or combined with KDM6A siRNA mixture. The cell viabilities were quantified by MTT assay. Results were analyzed by t-test and $P < 0.05$ was considered significant. *$P < 0.05$, **$P < 0.01$.

ARDS patients when compared with controls. We treated A549 and HFL-1 cells by a cytokine mix which had the major proinflammatory cytokines found in pulmonary edema fluid from patients with ARDS. We observed a reduced miR-425 level in the cells and the medium after treatment, suggesting the exosomal miR-425 level in blood may represent the conditions in the lung.

TGF-β signaling pathways have been considered the master regulator of fibrosis which contributes to the activation of myofibroblasts, and excessive production of extracellular matrix (ECM) [23]. Herein, we found Smad2, a key component of the canonical TGF-β signaling pathway, is regulated by miR-425. However, Smad2 is not the direct target of miR-425. We verified that miR-425 level closely relates to H3K27me3 level in the promoter region of Smad2 through regulating KDM6A expression directly. KDM6A also known as UTX, is a H3K27me3 demethylase. Since somatic mutation of KDM6A was found in human tumors, KDM6A was considered as a tumor repressor [24]. KDM6A was also found to modulate cell fate which is necessary for muscle regeneration and ESC differentiation [25, 26]. In this study, we found miR-425 repressed KDM6A expression and promoted Smad2 expression by upregulating H3K27me3 level in the Smad2 promoter region. On the contrary, miR-425 inhibited cells had more P-Smad2 level as well as increased collagen synthesis after TGF-β1 treatment. These results indicated that miR-425 inhibitor increased KDM6A expression, which removed the H3K27me3 repression marker of the Smad2 promoter and promoted fibrosis.

Aberrant proliferation of lung fibroblasts is involved in the process of lung fibrosis, so we finally detected the function of miR-425 inhibitor on cell proliferation using human lung fibroblasts HFL-1 and human alveolar epithelial A549 cells. Our results showed that miR-425

inhibitor promoted these two cell lines' proliferation. The co-transfection of KDM6A specific siRNA partially rescued the function of miR-425 inhibitor in HFL-1 cells, indicating miR-425 inhibitor promoted HFL-1 cell proliferation partially through up-regulating KDM6A. However, in A549 cells, siKDM6A did not rescue the miR-425 inhibitor's function, suggesting the function of miR-425 is cell type-specific.

In conclusion, we identified miR-425 reduction in the exosomes from ARDS patients' peripheral blood. This has potential to be used as a biomarker for ARDS diagnosis. We demonstrated that miR-425 reduction in lung fibroblasts contributes to the fibrosis through upregulating KDM6A and then activates the TGF-β signaling pathway, which sheds light on the mechanism of lung fibrosis during ARDS.

Disclosure of conflict of interest

None.

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