

Exploration on the regulatory mechanisms of MBD2/miR-301a-5p/CXCL12/CXCR4 on acute exacerbations of chronic obstructive pulmonary disease

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Abstract

Background Chronic obstructive pulmonary disease (COPD) is characterized by irreversible expiratory airflow obstruction and its chronic course is worsened by recurrent acute exacerbations. Our previous microarray identified miR-301a-5p was associated with the progression of AE-COPD, but its regulatory mechanism underlying COPD pathogenesis remains uncovered.

Methods Serum and peripheral blood mononuclear cells (PBMCs) were isolated from healthy controls and patients with COPD in remission (R-COPD) and acute exacerbation (AE-COPD). Human HULEC-5a and HBE cells were transfected with MBD2, sh-MBD2, miR-301a-5p mimics or inhibitor, followed by stimulated with cigarette smoke extract (CSE). The co-culture assays were performed by the addition of the supernatant of medium derived from HULEC-5a cells transfected with miR-301a-5p mimics or inhibitor into si-CXCR4-transfected-lung fibroblast or THP-1 macrophages. Transwell assay was used to analyze cell migration.

Results Clinical samples showed that decreased miR-301a-5p level in AE-COPD was significantly positively correlated with the expression level of MBD2, but negatively correlated with CXCL12 expression level. MBD2 overexpression significantly promoted miR-301a-5p, while suppressed CXCL12 in HULEC-5a and HBE cells. CXCL12 was confirmed as a direct target of miR-301a-5p. CXCR4 knockdown significantly reduced the suppressive effect of miR-301a-5p mimics and attenuated the promotional effects of miR-301a-5p inhibitor on the migration of circulating fibroblasts and macrophages, as well as the expression levels of P-MEK and P-AKT.

Conclusion In summary, MBD2/miR-301a-5p/CXCL12/CXCR4 appears to be involved in such recruitments of circulating fibroblasts and macrophages during COPD exacerbations.

Introduction

Chronic obstructive pulmonary disease (COPD), characterized by chronic bronchial inflammation and irreversible expiratory airflow obstruction, is a type of quite frequent airway diseases that is predicted as the third leading cause of death by 2020 (Pauwels et al, 2004; Murray et al, 1997). Tobacco smoking is considered to be the main risk driver in COPD progression by triggering aberrant inflammation, apoptosis and oxidative stress (Fischer et al, 2011; Plataki et al, 2006). Clinically, acute exacerbation of COPD (AE-COPD) is an independent prognostic factor with higher mortality rate (Piquet et al, 2013), which has sudden aggravation of respiratory symptoms, usually accompanied by hypoxemia and worsened hypercapnia (Duan et al, 2016). Related study indicated that AE-COPD frequently worsens the chronic course of COPD and affects approximately 80% of COPD patients for more than three years (Hurst et al, 2010). In addition to the hospitalization burden, AE-COPD particularly requires more than 50% of the total direct costs of COPD in healthcare system (Halpin, 2007). Bronchial relaxation and administration glucocorticoids and antibiotics have been the current medical therapies for AE-COPD, but the clinical outcomes remains unsatisfactory with uncontrolled side effects (Woodhead et al, 2011). Therefore,

exploring the mechanisms underlying the pathogenesis of AE-COPD is urgently needed to develop powerful therapies for this disease.

MicroRNAs (miRNAs) are a class of endogenous non-coding small RNA with about 22 nucleotides in length and play a central role in regulating several aspects of inflammation as a central feature of COPD (e.g., miR-181a-2-3p (Kim et al, 2019), miR-29b (Kun et al, 2019), and miR-212-5p (Jia et al, 2018)). A previous study by Ezzie et al (Ezzie et al, 2012) showed that total 70 miRNAs were identified to be differentially expressed between lung tissues from subjects with COPD and smokers without COPD by constructing gene expression networks. Nevertheless, there have been no reports on altered miRNA profiles associated with the development of AE-COPD. Thus, our preliminary work focused on identifying the key miRNAs that may be responsible for an acute exacerbation in COPD patients. Among these differentially expressed miRNAs, miR-301a-5p was significantly down-regulated in AE-COPD, compared with COPD patients in remission phase or healthy controls. By searching reported literatures, we found altered expression of miR-301a-5p was significantly correlated with serum C-reactive protein level in patients with Crohn's disease (Zhen et al, 2016). In hepatocellular carcinoma, miR-301a-5p functions as a direct target of EPB41L4A-AS2 participating in inhibiting tumor growth and metastasis (Wang et al, 2019). It is worth noting that methyl-CpG-binding domain protein 2 (MBD2) could promote the expression of miR-301a-5p by binding to its CpG island to inhibit the methylation of the promoter sequence, indicating that miR-301a-5p is regulated by MBD2 (Wang et al, 2017). As a member of histone deacetylation complex protein family, MBD2 participates in the pathological process of a variety of human diseases by inhibiting methylation to promote the expression of multiple genes (Wood et al, 2016). In the model of severe asthma disease, MBD2 can regulate the differentiation of Th17 cells by promoting the expression of relevant important genes to ultimately affect the inflammatory response (Jia et al, 2017). Of note, reduced MBD2 expression has recently been found to enhance airway inflammation in bronchial epithelium in COPD (Zeng et al, 2018).

Our previous work additionally explored the downstream target genes of miR-301a-5p and found there was a targeted regulatory relationship between miR-301a-5p and stromal cell-derived factor-1 (SDF-1/CXCL12), a member of the chemokine protein family. CXCL12 is a ligand of the g-protein-coupled receptor or chemokine receptor 4 (c-x-c motif chemokine receptor 4, CXCR4), which is involved in a variety of cellular functions, including embryonic development, immune surveillance, inflammatory response, and tissue cell homeostasis (Puchert et al). Isles et al (Isles et al, 2019) showed that CXCR4/CXCL12 signaling may play an important role in neutrophil retention at inflammatory sites, identifying a potential new target for the therapeutic removal of neutrophils from the lung in chronic inflammatory disease. Importantly, CXCR4/CXCL12 signaling also participates in the "recruitment" of asthma cells, circulating monocytes and lung fibroblasts in AE-COPD (Dupin et al, 2016), as well as mediates the recruitment of monocytes to oral cancer spheroids (Murdoch et al, 2012). Based on these existing reports and our previous works, we made a hypothesis that MBD2/miR-301a-5p/CXCL1/CXCR4 pathway might play a crucial role in the pathogenesis of AE-COPD.

To validate our hypothesis, the present study will expand the collection and detection of peripheral blood samples from patients with COPD in remission and acute exacerbation, and explore the correlation of MBD2, miR-301a-5p and CXCL12 expression levels. Based on the in vitro model of circulating fibroblasts and circulating monocytes, the targeted regulation of CXCL12 by miR-301a-5p and its involvement in the migration of circulating fibroblasts and circulating monocytes through CXCR4 were further verified.

Materials And Methods

Blood sample collection

We recruited COPD patients in remission phase (R-COPD, n = 20) and acute-exacerbated phase (AE-COPD, n = 20), as well as 20 matched control subjects, who all had a history of smoking with a pack-year index of > 20 between January 2017 and September 2018 at the Second Affiliated Hospital of Kunming Medical University. All COPD patients were diagnosed according to the criteria proposed by Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017 guidelines ([Kokturk et al, 2017](#)). AE-COPD was defined as an increase of at least two lower respiratory tract symptoms, including shortness of breath, sputum purulence, cough, wheezing and chest tightness or the new onset of two or more such symptoms, with at least one symptom lasting 3 or more days. Total 4.0 ml fasting peripheral blood from each participant was collected for further analysis. All participants provided the written informed consent and this study was approved by the Ethical Committee of the Second Affiliated Hospital of Kunming Medical University.

Sample pretreatment

Total 2 ml of peripheral blood was used to isolate peripheral blood mononuclear cells (PBMCs) through gradient centrifugation with Ficoll-Hypaque (Ficoll-Paque PLUS; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and immediately preserved in liquid nitrogen. Meanwhile, serum was obtained from the remaining 2 ml of peripheral blood and stored at -80 °C.

Cell culture and treatment

Human pulmonary microvascular endothelial cell line (HULEC-5a) and human lung bronchial epithelial cell line (HBE) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HULEC-5a cells were cultured in MCDB131 medium with 10% heat-inactivated fetal bovine serum (FBS), EGF (10 µg/ml) and hydrocortisone (1 µg/ml) at 37 °C with 5% CO₂. HBE cells were grown in M199 medium supplemented with EGF (10 µg/ml), hydrocortisone (1 µg/ml) and 10% FBS at 37 °C with 5% CO₂. Both of these two cell lines were stimulated with cigarette smoke extract (CSE) for 24 h, which was prepared according to a previous protocol with minor modification ([Krimmer et al, 2012](#)).

Transfection

MBD2 expression vector pcDNA3.0-MBD2 and lentivirus-containing short hairpin-MBD2 (sh-MBD2) were synthesized by Ribobio Co., Ltd. (Guangzhou, China). MiR-301a-5p mimics and inhibitor were purchased from VipotionBio Co., Ltd. (Guangzhou, China). For the *in vitro* experiments, HULEC-5a or HBE cells were divided into the following groups: 1) Control contained only medium; 2) MBD2 group contained cells transfected with pcDNA3.0-MBD2; 3) sh-MBD2 group contained cells transfected with sh-MBD2; 4) Scramble group contained cells transfected with negative control; 5) miR-301a-5p mimics and 6) miR-301a-5p inhibitor groups containing cells transfected with miR-301a-5p mimics and inhibitor, respectively. After 48 h of transfection, cells were stimulated with CSE for the subsequent experiments.

Luciferase reporter assay

The putative binding sites of miR-301a-5p and 3'-UTR sequences of CXCL12 were predicted by TargetScan (http://www.targetscan.org/vert_71/). The 3'-UTR wild type or mutant type sequences of CXCL12 were amplified from HULEC-5a or HBE cells and cloned into pGL3 vectors (Promega, Madison, WI, USA) to generate corresponding WT or MUT luciferase reporter vectors, respectively. For luciferase reporter assay, HULEC-5a or HBE cells were transfected with luciferase reporter vectors and miR-301a-5p or NC using Lipofectamine 2000 (Invitrogen). After 48 h, the luciferase and Renilla activity was measured using a Dual Luciferase Reporter Assay Kit (Promega). The luciferase activity was normalized to *Renilla* luciferase activity.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol Reagent (Invitrogen) and complementary DNA (cDNA) was synthesized with TaqMan microRNA Reverse Transcription Kit or M-MLV Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The PCR procedure was performed using SYBR green (Applied Biosystems) following the thermal cycling conditions: 95 °C for 5 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The expression levels of MBD2, miR-301a-5p, CXCL12 and CXCR4 were quantified with $2^{-\Delta\Delta Ct}$ method. GAPDH or U6 was served as the endogenous control. The primer sequences used in this study were listed in **Table 1**. Every sample was prepared in triplicate and the experiment was repeated three times.

ELISA assay

The concentration of CXCL12 was determined in serum and cell culture supernatant with a commercially available ELISA kit (human CXCL12; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Immunofluorescence

Immunofluorescence was conducted as previously described (Zhou et al, 2015). In brief, cells from control, MBD2 or sh-MBD2 group were fixed with ice-cold methanol, blocked with 3% bovine serum

albumin and incubated with primary antibodies against MBD2 and CXCL12 for 2 h. After counterstaining with DAPI, stained cells were observed under a fluorescence microscope.

Co-culture assays

Small interfering RNA targeting CXCR4 (si-CXCR4) and si-NC were provided by RiboBio Co., Ltd. (Guangzhou, China). Human lung fibroblast cells purchased from ATCC were grown in DMEM medium supplemented with 10% FBS and fibroblast growth factors. THP-1 cells from ATCC were cultured in RMPI-1640 medium with 10% FBS and then transferred into serum-free medium containing phorbol myristate acetate (PMA, 100 ng/ml) and 0.3% BSA for 72 h to induce adherent macrophages. The co-culture assays were performed by the addition of the supernatant of blank medium or medium derived from HULEC-5a cells transfected with miR-301a-5p mimics, inhibitor or without any treatment into 2×10^5 lung fibroblast or THP-1 macrophages transfected with si-NC or si-CXCR4. After 48 h co-culture, lung fibroblast or THP-1 macrophages were collected for further analysis.

Transwell migration assay

Transwell assay was carried out to evaluate the migration ability using the 24-well transwell inserts (BD Biosciences) in human lung fibroblast and THP-1 macrophages after the above co-cultures. Briefly, cells prepared in serum-free medium were plated in the upper chamber, while the lower chamber was filled with 600 μ l medium containing 10% FBS as a chemo-attractant. After culture for 24 h, the cells that migrated into the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, China). Finally, stained cells in six random fields were captured at 200 \times magnification and calculated under a microscope.

Western blotting

Total protein samples were extracted with RIPA lysis buffer and quantified by BCA assay kit (both from Beyotime, China). The equal amounts of protein sample were separated by SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in Tris-buffer saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature. The primary antibodies, including MBD2, CXCL12, CXCR4, P-MEK, MEK, AKT, P-AKT and GAPDH were incubated with the membranes overnight at 4 °C, followed by HRP-conjugated secondary antibody for 1.5 h next day. The protein bands were visualized using enhanced chemiluminescence (ECL) detection reagent.

Statistical analysis

All experiments were repeated at least three times and data were expressed as the mean \pm standard deviation (SD). All differential comparisons were performed using student's t-test between two groups and one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple groups. The analysis of correlation between factors was performed by Spearman's correlation coefficient rank test. All *p* values less than 0.05 were considered to be statistically significant.

Results

The expression levels of MBD2, miR-301a-5p, CXCL12 and CXCR4 in blood samples derived from COPD patients

Based on our preliminary results and database predictive analysis, we speculated that MBD2/miR-301a-5p/CXCL12/CXCR4 pathway might play an important role in COPD pathogenesis. Thus, we analyzed their expression levels in isolated PBMCs from control, R-COPD and AE-COPD group using qRT-PCR analysis. The results showed that the expression level of MBD2 was significantly lower in the R-COPD and AE-COPD groups than in the control group, but there was no significant difference between R-COPD and AE-COPD (**Figure 1A**). Similarly, miR-301a-5p expression levels were also significantly lower in the R-COPD and AE-COPD groups than in the control group, and AE-COPD was significantly lower than R-COPD (**Figure 1B**). On contrast, Both CXCL12 (**Figure 1C**) and CXCR4 (**Figure 1D**) expression levels were obviously higher in the R-COPD and AE-COPD groups, compared with control group, and AE-COPD was significantly higher than R-COPD. In addition, we analyzed the expression of MBD2, miR-301a-5p, CXCL12 and CXCR4 in serum samples. As shown in **Figure 1E**, the expression levels of miR-301a-5p were consistent with that in PBMCs. Western blotting further confirmed that MBD2 was down-regulated, while CXCL12 and CXCR4 were up-regulated in R-COPD and AE-COPD groups compared with control group, and AE-COPD presented more obvious trend (**Figure 1F**). Given that CXCL12 was an intracellular and extracellular protein, ELISA assay was performed to analyze its expression levels in serum sample. As presented in **Figure 1G**, the concentration of serum CXCL12 in the R-COPD and AE-COPD groups was significantly higher than that in the control group, and that in the AE-COPD group was significantly lower than that in the R-COPD group. Furthermore, Spearman's correlation demonstrated that miR-301a-5p was significantly positively correlated with the expression level of MBD2 (**Figure 1H**), but negatively correlated with CXCL12 expression level (**Figure 1I**).

The effects of CSE on the expression levels of MBD2, miR-301a-5p, CXCL12 and CXCR4 in vitro

As smoking is the leading cause of COPD, we next stimulated HULEC-5a or HBE cells with CSE and determined the expression levels of MBD2, miR-301a-5p, CXCL12 and CXCR4. As expected, CSE treatment significantly suppressed the expression of MBD2 and miR-301a-5p and promoted CXCL12 expression, but did not obviously affect the expression of CXCR4, as determined by qRT-PCR analysis and ELISA assay (**Figure 2A-2B**).

The regulatory effects of MBD2 on the expression levels of miR-301a-5p and CXCL12 in vitro

To investigate the role of MBD2 in CSE-induced expression levels of miR-301a-5p and CXCL12, HULEC-5a or HBE cells were transfected with MBD2 or sh-MBD2, followed by CSE treatment. The results from qRT-PCR (**Figure 3A-3B**) and western blotting (**Figure 3C**) analysis indicated that MBD2 transfection

significantly up-regulated MBD2 and miR-301a-5p expression levels, while down-regulated CXCL12 expression level. Consistently, sh-MBD2 transfection obtained the opposite results. ELISA results showed that extracellular CXCL12 concentration was consistent with the above qRT-PCR and western blotting results, which also proved that MBD2 could affect CXCL12 expression level (**Figure 3D**). Moreover, immunofluorescence staining of MBD2 and CXCL12 further intuitively displayed the increased MBD2 and inhibited CXCL12 expression after MBD2 overexpression, while decreased MBD2 and enhanced CXCL12 expression after sh-MBD2 transfection in both HULEC-5a and HBE cells (**Figure 3E-3F**). These results demonstrated that there was a positive correlation between MBD2 and miR-301a-5p and a negative correlation between miR-301a-5p and CXCL12 in vitro.

CXCL12 was a direct target of miR-301a-5p

From the above results, we predicted the target genes of miR-301a-5p by using online bioinformatics tool TargetScan. Interestingly, CXCL2 was the putative target of miR-301a-5p. The binding sites of CXCL12 3'UTR (UCAGAG) with miR-301a-5p were presented in **Figure 4A**. Luciferase reporter assay was then used to validate whether miR-301a-5p directly targeted CXCL12. As shown in **Figure 4B**, the relative luciferase activities repressed significantly by miR-301a-5p transfection in WT group, while was unchanged with co-transfection of the MUT and miR-301a-5p as compared with NC in both HULEC-5a and HBE cells. In addition, CXCL12 mRNA (**Figure 4C-4D**) and protein (**Figure 4E**) expression were negatively regulated by miR-301a-5p in HULEC-5a and HBE cells. Consistently, the concentration of CXCL12 in cell culture supernatant was significantly reduced in miR-301a-5p mimics-transfected cells and elevated in miR-301a-5p inhibitor-transfected cells in both HULEC-5a and HBE cells (**Figure 4F**).

Effects of miR-301a-5p/CXCL12/CXCR4 on the migration of circulating fibroblasts and macrophages

Next, we evaluated the effects of miR-301a-5p/CXCL12/CXCR4 on the migration of circulating fibroblasts and monocytes, as main recruited circulating cells in lung tissues. Here, human lung fibroblasts were used as circulating fibroblasts and monocytes THP-1 was applied to adherent macrophages in vitro. After co-culture assays, we found the number of migrated human lung fibroblasts was significantly decreased after miR-301a-5p overexpression, but increased after miR-301a-5p knockdown, compared with control group. By comparing si-NC and si-CXCR4 group, we observed that CXCR4 knockdown strongly suppressed the cell migration ability in cell culture supernatant derived from human lung fibroblasts transfection with miR-301a-5p mimics or inhibitor (**Figure 5A**). Similar results were also validated in THP-1 macrophages (**Figure 5B**). What's more, we explored whether miR-301a-5p regulated circulating fibroblasts and macrophages migration was associated with CXCR4-activated MEK/AKT pathway. Western blotting indicated that miR-301a-5p mimics obviously suppressed, while miR-301a-5p inhibitor promoted the expression of CXCR4, P-MEK and P-AKT. We further found CXCR4 knockdown enhanced the suppressive effect of miR-301a-5p mimics and attenuated the promotional

effects of miR-301a-5p inhibitor on P-MEK and P-AKT expression in both human lung fibroblasts (**Figure 6A**) and THP-1 macrophages (**Figure 6B**). These findings suggested that the activated-MEK/AKT pathway might be the downstream mechanism through which CXCL12 induces the migration of circulating fibroblasts and macrophages.

Discussion

In the present study, we found the expression of miR-301a-5p was down-regulated in peripheral blood samples from R-COPD and AE-COPD group, compared with control group. Moreover, reduced miR-301a-5p expression level was positively correlated with MBD2. During recent years, the role of miRNAs by serving as therapeutic targets in COPD has attracted research interests ([Bracke et al](#)). Compared to miRNA in lung tissue, expression changes of circulating miRNAs were much easier to detect and used as screening tests. As reported by Jia et al ([Jia, 2018](#)), the ectopic expression of several miRNAs, including miR-34a, miR-223 and miR-212-5p was identified in serum samples of COPD patients. As tobacco smoking can initiate and aggravate the inflammation process in COPD, we thus stimulated HULEC-5a and HBE cells with CSE. As expected, we found CSE stimulation significantly down-regulated the expression of MBD2 and miR-301a-5p in both HULEC-5a and HBE cells. Zeng et al ([Zeng, 2018](#)) consistently demonstrated that MBD2 expression was decreased in patients with COPD and HBE after CSE stimulation in vitro. These results indicated that the decreased expression of MBD2 and miR-301a-5p induced by CSE was involved in COPD, especially AE-COPD.

By performing gain-of-function and loss-of-function assays, we analyzed the regulatory role of MBD2 on the expression of miR-301a-5p and CXCL12 in HULEC-5a and HBE cells. Our data showed that MBD2 positively regulated miR-301a-5p, while negatively regulated CXCL12 expression level. In fact, MBD2, as a methylation-dependent reader, actively participates in DNA methylation-mediated transcriptional repression and/or heterochromatin formation ([Rao et al, 2011](#)). Accumulating evidence suggests that MBD2 is associated with several immunological disorders, such as systemic lupus erythematosus ([Balada et al, 2007](#)) and autoimmune encephalomyelitis ([Zhong et al, 2014](#)). Of note, MBD2 was confirmed to bind to the methylated CpG elements of miR-301a-5p promoter and knockdown of MBD2 was associated with the suppression of miR-301a-5p in vancomycin-induced acute kidney injury ([Wang, 2017](#)). Multiple studies have now established a pivotal role for chemokines in COPD. For example, CCL2 levels have been found to be increased in COPD patients compared to healthy controls in various organs, such as whole blood, induced sputum, and *in situ* lung tissues ([Henrot et al, 2019](#)). Serum CXCL8 levels were elevated from controls to stable, and exacerbation stage ([Zhang et al, 2018](#)). Another study showed CXCL10 was elevated in the sputum of COPD patients during an exacerbation compare to values after recovery ([Warwick et al, 2013](#)). In line with these facts, the mRNA and concentration of CXCL12 were significantly increased in the serum samples of AE-COPD and CSE-stimulated HULEC-5a and HBE cells. By contrast, the number of CXCR4 positive circulating fibrocytes and the blood level of CXCL12 are unchanged at the stable state in COPD patients ([Dupin, 2016](#)), suggesting that their chemotactic properties are altered in different sources of cells. Through online bioinformatics prediction, we confirmed CXCL12 was a target gene of miR-301a-5p, which subsequently underwent validation by

luciferase reporter assay. Here, the expression of CXCL12 was down-regulated after MBD2 overexpression, which indicated that MBD2 negatively regulated the expression of CXCL12 through positively regulating miR-301a-5p in CSE-stimulated HULEC-5a and HBE cells (**Figure 7**).

Another interesting finding of our study is that miR-301a-5p could effectively suppress the migration of circulating fibroblasts and macrophages by down-regulating CXCL12/CXCR4. It has been reported that increased number of circulating fibrocytes can be observed in peripheral blood of patients with chronic obstructive asthma and idiopathic pulmonary fibrosis, which was significantly increased in acute exacerbation of these patients ([Wang et al, 2008](#); [Moeller et al, 2009](#); [Wang et al, 2015](#)). In addition, the recruitment of circulating monocytes into the airway and lungs is another important process of promote pulmonary pathological damage in COPD, in which CXCL12/CXCR4 is an important regulator ([Murdoch, 2012](#); [Tourkina et al, 2010](#)). Moreover, CXCL12-CXCR4 axis could participate to the recruitment of fibrocytes to the lungs during COPD exacerbations ([Dupin et al, 2019](#)). During acute exacerbations of COPD, blood fibrocytes are recruited through a CXCR4-dependent pathway ([Dupin, 2016](#)). Our further analysis showed that miR-301a-5p regulated circulating fibroblasts and macrophages migration was associated with CXCR4-activated MEK/AKT pathway. As demonstrated by Tian et al ([Tian et al, 2018](#)), knockdown of CXCR4, receptor of CXCL12 could reverse the CXCL12-induced migration of oligodendrocyte precursor cells and block the MEK/ERK and PI3K/AKT pathways. According to these data, we thus speculated that miR-301a-5p regulated by MBD2 further affected the expression of CXCL12/CXCR4 in peripheral blood of COPD patients, and eventually recruited circulating fibroblasts and circulating mononuclear cells into surrounding tissues, ultimately promoting the course of COPD.

Conclusion

In conclusion, this study first demonstrated miR-301a-5p was significantly down-regulated during COPD exacerbations. From the in vitro experiments, MBD2/miR-301a-5p/CXCL12/CXCR4 appears to be involved in such recruitments of circulating fibroblasts and macrophages. Thus, identified miR-301a-5p played a protective role in limiting the recruitment of circulating fibroblasts and macrophages, which might be a potential therapeutic target for AE-COPD.

Declarations

Abbreviations

COPD: chronic obstructive pulmonary disease; CSE: cigarette smoke extract; PBMCs: peripheral blood mononuclear cells; AE-COPD: acute exacerbation of COPD; R-COPD: remission of COPD.

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None.

Authors' contributions

WS, ZYW and KFY conceived and designed the work that led to the submission. MJF, SKW, and RW acquired data. YZ, HT, XWa, XWu, XLY, WW contributed reagents/materials/analysis tools. All authors approved the final version.

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Data Availability Statement

Data can be provided upon a reasonable request.

Ethics approval and consent to participate

All participants provided the written informed consent and this study was approved by the Ethical Committee of the Second Affiliated Hospital of Kunming Medical University.

Consent for publication

All authors gave consent for publication.

Competing interests

The authors declare that they have no competing interests.

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Table 1

Table 1 Primers used for qRT-PCR analysis

Target ID	Primer sequence 5'-3'
MBD2	F: AAGTGTACCGAAAATCTGGGC R: TGCCAAGTGAGGCTTGCTTC
hsa-miR-301a-5p	RT: CTCAACTGGTGTGGAGTCGGCAATTGAGAGTAGTGC F: ACACTCCAGCTGGGCTCTGACTTTATTGCAC R: CTCAACTGGTGTGGGA
CXCL12	F: ATTCTAACACTCCAAACTGTGC R: ACTTTAGCTTCGGGTCAATGC
CXCR4	F: ACTACACCGAGGAAATGGGCT R: CCCACAATGCCAGTTAAGAAGA
GAPDH	F: TGTCGTCATGGGTGTGAAC R: ATGGCATGGACTGTGGTCAT
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTCGCT

F: forward; R: reverse; RT: Reverse transcription;

Figures

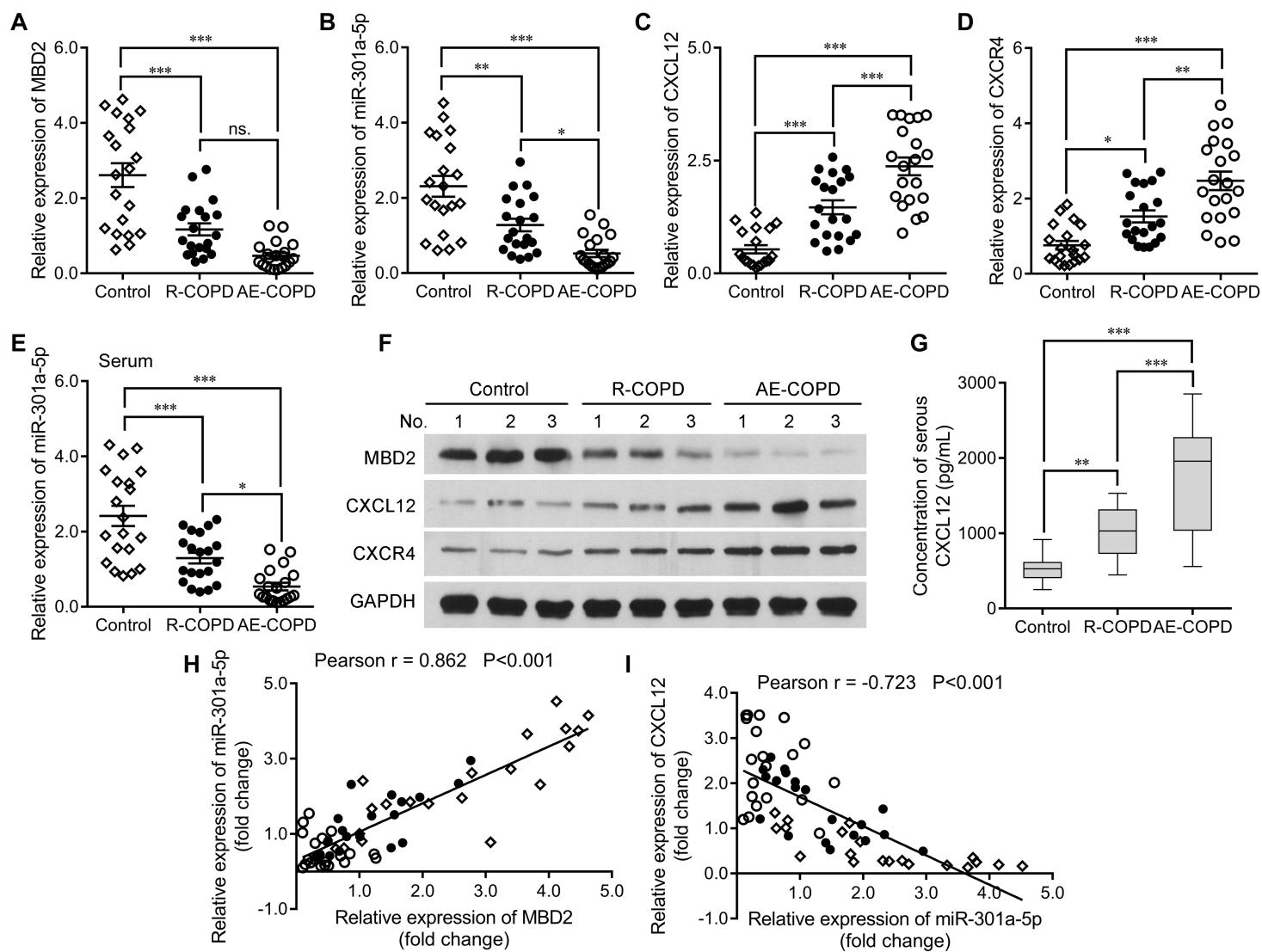


Figure 1

The expression levels of MBD2, miR-301a-5p, CXCL12 and CXCR4 in blood samples derived from COPD patients. The expression levels of (A) MBD2, (B) miR-301a-5p, (C) CXCL12 and (D) CXCR4 were determined in isolated PBMCs from control ($n = 20$), R-COPD ($n = 20$) and AE-COPD ($n = 20$) group using qRT-PCR analysis. (E) Serum miR-301a-5p expression levels from the above three groups were analyzed using qRT-PCR. (F) The protein expression levels of MBD2, CXCL12 and CXCR4 were measured in serum samples from the above three groups were analyzed using western blotting. (G) ELISA assay was conducted to analyze the serum CXCL12 concentration levels. Data were expressed as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Correlation analysis between MBD2 and miR-301a-5p (H), as well as between miR-301a-5p and CXCL12 (I) was performed by Spearman's correlation coefficient rank test.

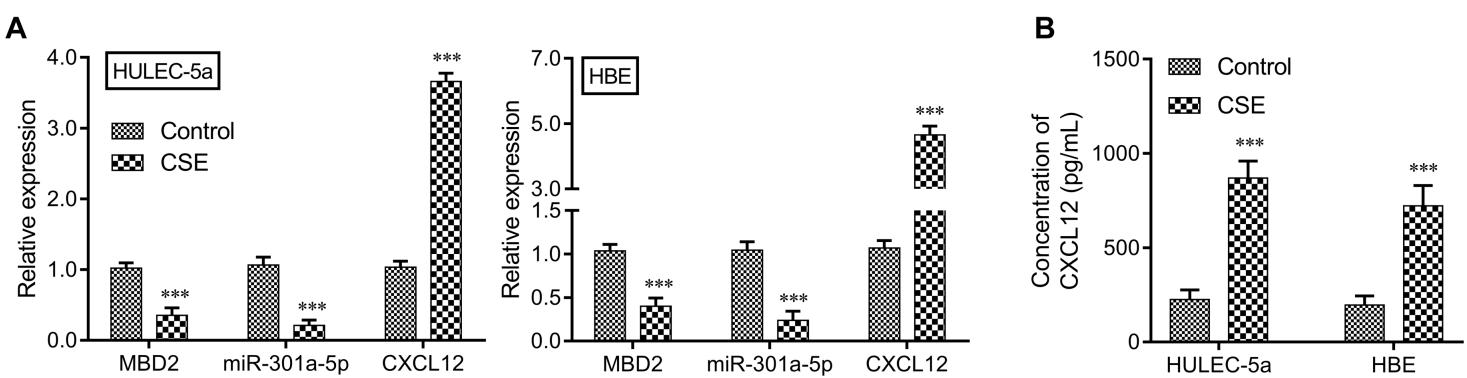


Figure 2

The effects of CSE on the expression levels of MBD2, miR-301a-5p, CXCL12 and CXCR4 in vitro. HULEC-5a or HBE cells were treated by CSE. The expression levels of MBD2, miR-301a-5p and CXCL12 were measured using qRT-PCR in (A) HULEC-5a and HBE cells. (B) The extracellular CXCL12 concentration was determined with ELISA assay. Data were expressed as the mean \pm SD. *** $P < 0.001$, compared with control.

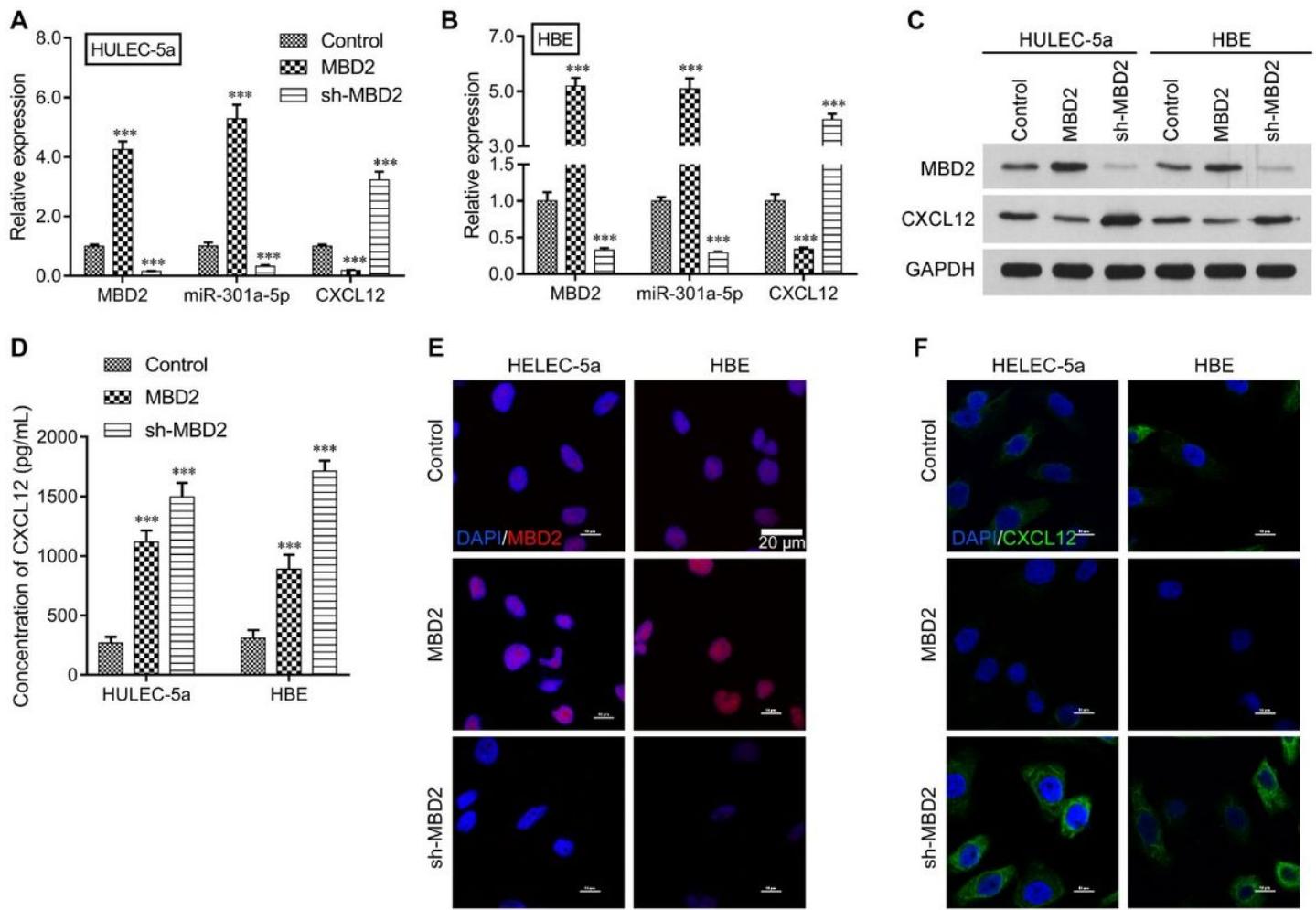
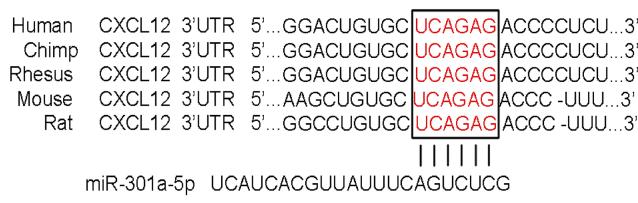
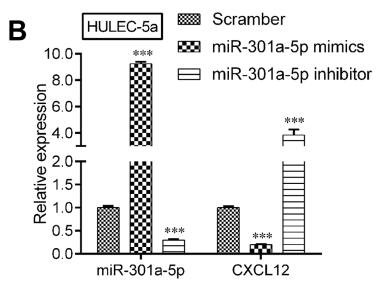
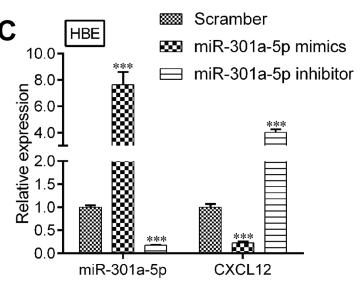
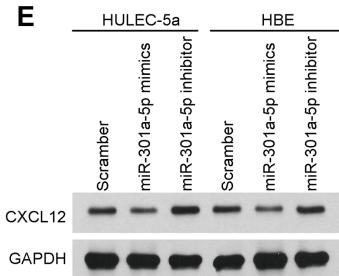
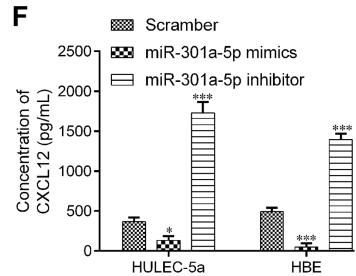


Figure 3

The regulatory effects of MBD2 on the expression levels of miR-301a-5p and CXCL12 in vitro. HULEC-5a or HBE cells were transfected with MBD2 or sh-MBD2, followed by CSE treatment. The expression levels of MBD2, miR-301a-5p and CXCL12 were measured using qRT-PCR in transfected (A) HULEC-5a and (B) HBE cells. (C) The protein expression levels of MBD2 and CXCL12 were measured using western blotting in transfected HULEC-5a and HBE cells. (D) The extracellular CXCL12 concentration was determined with ELISA assay in transfected HULEC-5a and HBE cells. Data were expressed as the mean \pm SD. *** P < 0.001, compared with control; (E, F) Immunofluorescence staining of MBD2 and CXCL12 was performed in transfected HULEC-5a and HBE cells.

A**B****C****E****F****Figure 4**

MiR-301a-5p repressed CXCL12 by targeting 3'UTR. (A) Predicted miR-301a-5p target sequence in the 3'UTR of CXCL12. (B) Luciferase reporter assay was performed in HULEC-5a and HBE cells after co-transfection with miR-301a-5p or NC together with WT or MUT CXCL12. Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC) and analyzed by student's t-test. HULEC-5a and HBE cells were transfected with miR-301a-5p mimics, inhibitor or scramble, which were used for analysis of miR-301a-5p and CXCL12 expression using qRT-PCR analysis (C-D), as well as CXCL12 protein expression using western blotting (E) and ELISA assay (F). Data were expressed as the mean \pm SD. *** P < 0.001, compared with NC or scramble.

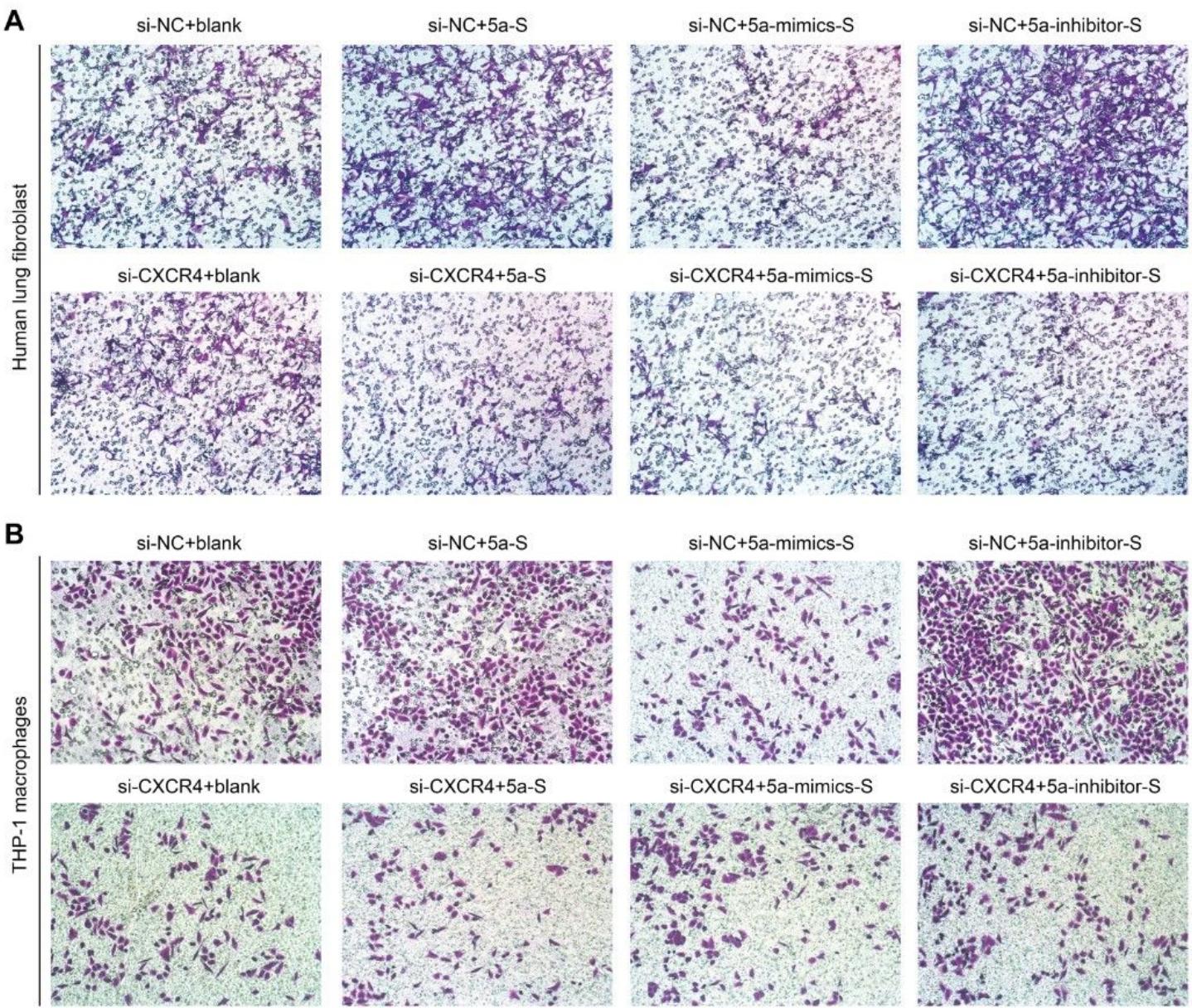


Figure 5

Effects of miR-301a-5p/CXCL12/CXCR4 on the migration of circulating fibroblasts and macrophages. The co-culture assays were performed by the addition of the supernatant of blank medium or medium derived from HULEC-5a cells transfected with miR-301a-5p mimics, inhibitor or without any treatment into 2×10^5 lung fibroblast or THP-1 macrophages transfected with si-NC or si-CXCR4. The migration ability was assessed using transwell assay in lung fibroblast (A) and THP-1 macrophages (B).

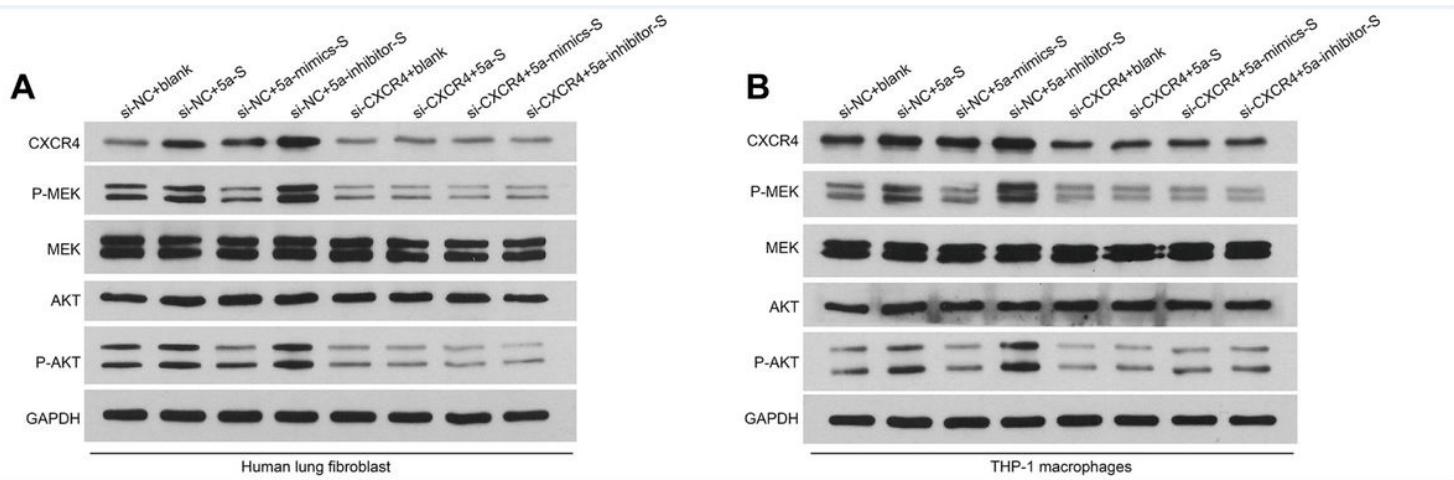


Figure 6

Effects of miR-301a-5p/CXCL12/CXCR4 on the MEK/AKT pathway. The co-culture assays were performed by the addition of the supernatant of blank medium or medium derived from HULEC-5a cells transfected with miR-301a-5p mimics, inhibitor or without any treatment into 2×10^5 lung fibroblast or THP-1 macrophages transfected with si-NC or si-CXCR4. Western blotting was performed to detect the protein expression of CXCR4, P-MEK, MEK, AKT and P-AKT in lung fibroblast (A) and THP-1 macrophages (B).

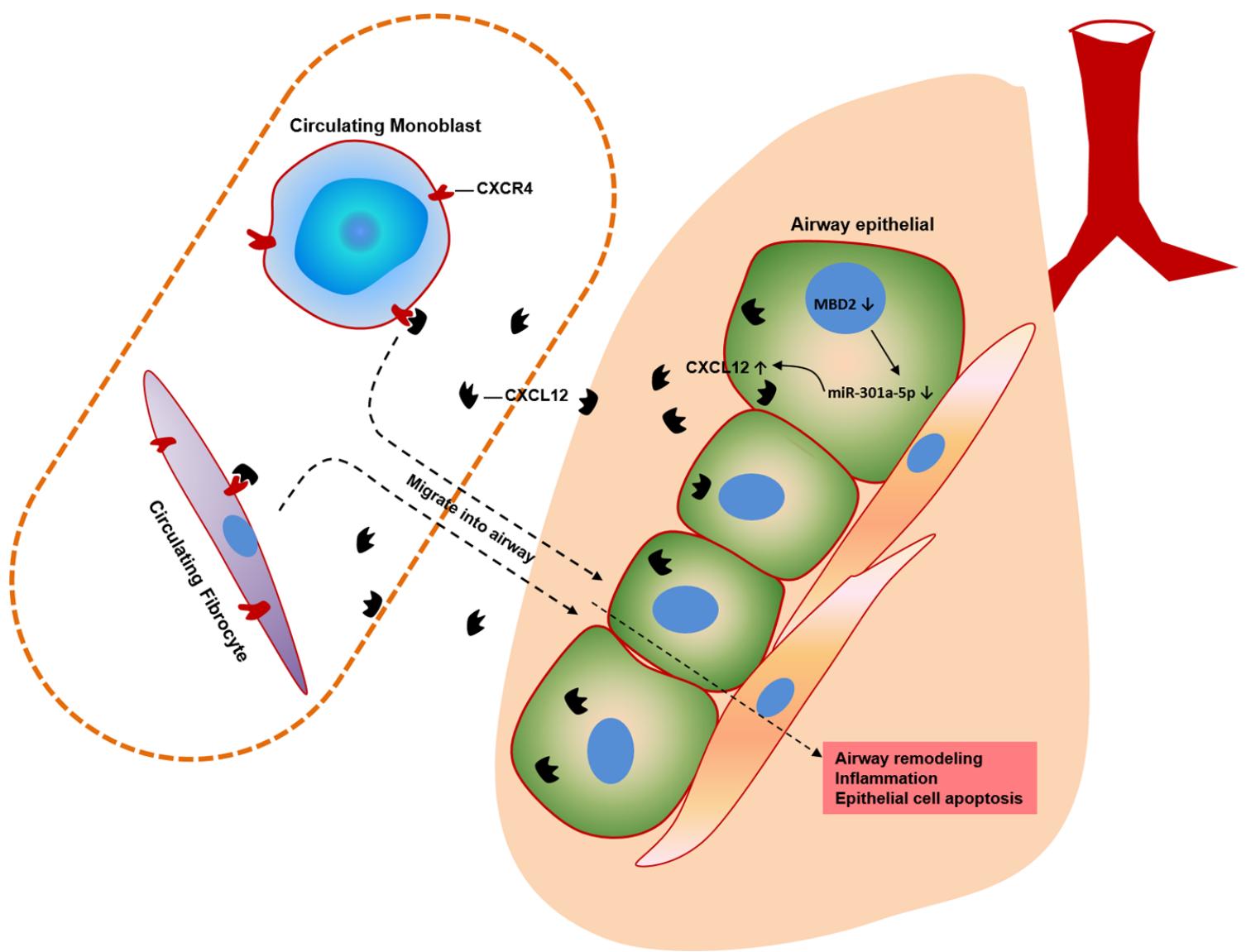


Figure 7

Regulatory mechanisms of MBD2/miR-301a-5p/CXCL12/CXCR4 on acute exacerbations of chronic obstructive pulmonary disease.