MicroRNA-874 inhibits TNF-α-induced remodeling in human fetal airway smooth muscle cells by targeting STAT3

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Highlights

• MiR-874 overexpression inhibits TNF-α-induced remodeling in human fASM cells.
• STAT3 is a direct target of miR-874.
• STAT3 overexpression partly reverses the effects of miR-874 in human fASM cells.

ABSTRACT

Pro-inflammatory cytokines-induced airway remodeling was a significant feature of asthma disease. The aim of the present study was to explore the functional
significance of miR-874 in tumor necrosis factor (TNF)-α-treated human fetal airway smooth muscle (fASM) cells. Here, we found that TNF-α treatment significantly down-regulated the expression of miR-874 in fASM cells. MiR-874 overexpression markedly inhibited cell viability and migration, suppressed the expression of PCNA and Ki67, reduced the expression of collagen I and collagen III, decreased the expression and activity of matrix metalloproteinase (MMP)-9 and MMP-2, and induced an obvious elevation of tissue inhibitors of metalloproteinases (TIMPs). In addition, the increased production of interleukin (IL)-6, IL-8 and eotaxin induced by TNF-α were significantly inhibited by miR-874 overexpression. Signal transducers and activators of transcription (STAT) 3 was identified as a direct target of miR-874, and STAT3 overexpression partly reversed the protective effects of miR-874 against TNF-α-induced airway remodeling. Overall, these findings demonstrate that miR-874 inhibits TNF-α-induced remodeling in human fASM cells at least in part by targeting STAT3.

Keywords: MicroRNA-874; airway remodeling; human fetal airway smooth muscle cells; cell proliferation; inflammation; extracellular matrix deposition
1. Introduction

Pediatric asthma is one of the common chronic illnesses in childhood, characterized by airway obstruction and increased airway hyperresponsiveness (Saglani et al. 2005; Xepapadaki et al. 2005). Despite great advances in therapy, asthma has clinical burden, contributing to lung morbidity and children mortality (Myers 2000; Okpapi, Friend and Turner 2013). The progression of asthma is closely associated with multiple factors, such as genetics, maternal and neonatal nutritional status, and inflammation exposures (Andrew, Auinger and Weitzman 2000; Kaugars, Klinnert and Bender 2004). Increasing evidence has revealed that chronic airway inflammation induces and maintains the functional deficiencies of asthma (George and Brightling 2016). Inflammatory cytokines promote airway remodeling, which is characterized by increased airway smooth muscle (ASM) cell proliferation, increased extracellular matrix (ECM) deposition, and severe inflammatory reaction (Alagappan et al. 2005; Jr et al. 2015). Therefore, decreasing inflammatory cytokines-induced airway remodeling is now a cornerstone for pediatric asthma treatment.

MicroRNAs (miRNAs) are a class of single stranded, 18-25 nucleotides in length, non-coding RNAs. MiRNAs may partly or completely bind to the 3’-untranslated region (3’-UTR) of target mRNAs, thereby promoting their degradation or translation inhibition (Maroney, Yu and Nilsen 2006; Orang, Safaralizadeh and Kazemzadehbavili 2014), thus mediating their biological functions. MiRNAs have been shown to be actively involved in a variety of cellular processes, such as
proliferation, apoptosis, differentiation and inflammation (Bushati and Cohen 2007). In recent years, the regulation of miR-874 in various diseases has been drawing increasing attention. MiR-874 has been shown to be down-regulated and act as a tumor suppressor in several types of cancers, such as colorectal cancer (Han et al. 2016), gastric cancer (Zhang et al. 2015), breast cancer (Wang et al. 2014) and non-small cell lung cancer (Kesanakurti et al. 2013). One report has revealed that miR-874 may be a miRNA biomarker for detection of neurodegenerative diseases including Alzheimer's disease (Sheinerman et al. 2012). There are investigations reporting that miR-874 regulation may be closely associated with the development of allergic rhinitis and asthma (Suojalehto et al. 2014; Wu et al. 2015). However, the role of miR-874 in asthma development, airway remodeling, and its possible molecular mechanisms remains unknown.

Signal transducers and activators of transcription (STATs) have been reported to be a family of cytokine-inducible transcription factors that play a crucial role in immune and inflammatory responses (Pernis and Rothman 2002; Simeone-Penney et al. 2007). STATs become activated by phosphorylation of tyrosine residues in response to cytokine stimulation. STAT3 is critical for cytokine signaling, and many reports have revealed that STAT3 is required for the airway inflammation in asthma (Liu, Nong and Shu-Quan 2011; Simeone-Penney et al. 2007). However, the regulation of STAT3 in asthma progression still needs to be expounded clearly. We had predicted that 3'-UTR of STAT3 mRNA contain the binding sequences of miR-874 by bioinformatics analysis. In this study, we hypothesized that STAT3 is a
target gene of miR-874, and the effects of miR-874 on the airway remodeling may partly mediated by STAT3.

The aim of the present study was to investigate the functional significance of miR-874 in inflammatory cytokines-induced airway remodeling. We evaluated the effects of miR-874 on cell proliferation, ECM deposition and inflammatory factors production in tumor necrosis factor (TNF)-α-treated human fetal airway smooth muscle (fASM) cells, and investigated its underlying regulation mechanisms.
2. Materials and methods

2.1. Culture of human fASM cells

Human fASM cells were enzymatically dissociated from fetal tracheobronchial tissue as described previously (Faksh et al. 2016; Pandya et al. 2002). Human fetal trachealis tissue was obtained from 10 normal fetuses (18-20 week gestational age) after voluntary termination of pregnancy, through the First Hospital, Jilin University. Human fASM cells were cultured and passaged in DMEM/F-12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen), maintained in a humidified incubator with 5% CO₂ at 37 °C. Cells from passages 2 to 10 were utilized for experiments. Cells were serum deprived in 0.5% FBS for 24 h prior to treatment of 20 ng/mL TNF-α (R&D Systems, Minneapolis, USA).

2.2. Cell transfection

MiR-874 mimic and corresponding negative control (miR-NC) were purchased from GenePharma (Shanghai, China). 5×10⁴ or 2×10⁶ cells were seeded in 96- or 6-well plates 24 h before transfection. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions at a final concentration of 100 nM. Analyses were carried out 48 h after transfection. Full-length STAT3 obtained by polymerase chain reaction (PCR) was cloned into pcDNA3.1 vector (NotI/XbaI, insert: vector=4:1), and named as pcDNA3.1-STAT3. The forward primer sequences were 5’-CCGGCTAGCATGGCCCAATGGAATCAG-3’, and the reverse primer sequences
were 5'-CCCAAGCTTTCACATGGGGGAGGTAGC-3’. The empty pcDNA3.1 vector (containing an enhanced green fluorescent protein (EGFP)) was used as negative control, and named as pcDNA3.1-empty. For the experiments performed to check the effect of STAT3 overexpression in TNF-α-treated fASM cells, co-transfection with miR-874 mimic and either pcDNA3.1-STAT3 or pcDNA3.1-empty vectors (0.8 µg/well/24-well) were performed.

2.3. Quantitative real time PCR (qRT-PCR)

Total RNA was extracted from the cells by using 1 ml Trizol Reagent (Invitrogen) according to the manufacturer’s instructions, and then reverse transcribed into cDNA by using Primerscript RT Reagent (Takara, Dalian, China). The expression levels of miR-874 were detected by TaqMan microRNA assays (Applied Biosystems) with specific primers. qRT-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Shanghai, China). PCR programs were carried out as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, and a final extension for 5 min at 72°C. The expression of U6 small nuclear RNA was used as an endogenous control. The relative expression level was calculated using 2^(-ΔΔCt) method.

2.4. MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were seeded into 96-well plates, and then performed cell transfection. 48 h after transfection, 20 µl MTT solution (5 mg/mL) was added to each well, and
incubated for 4 h at 37°C. Then 200 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the crystals. The absorption at 570 nm was measured with a microplate reader (Molecular Devices Corp., CA, USA).

2.5. Transwell assay

Following 24 h transfection, the cells were harvested and washed twice with PBS, and then 200 ml cell suspension in serum-free medium (1 × 10^5 cells) were seeded in the upper compartment of transwell plates (8 mm pore size, Corning, NY). In the lower chamber, 600 ml medium containing 20% FBS was added. After 24 h incubation (48 h after transfection), cells that did not migrate through the pores were removed by cotton swabs, while the cells on the filter surface were fixed with 4% formaldehyde, stained with 0.4% crystal violet, and counted in five high power fields.

2.6. Western blot

Total protein was extracted from the cells by using 1 ml cell lysis buffer (Cell Signaling Technology, Shanghai, China). The concentrations of protein were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, USA). Equal protein were separated by SDS-polyacrylamide gels (SDS-PAGE), and then transferred to polyvinylidene difluoide (PVDF) membranes (Millipore, Massachusetts, USA). After blocking with 5% skim milk, the membranes were incubated with primary antibodies: rabbit monoclonal to PCNA (Cell Signaling Technology, 1:1000), mouse monoclonal to Ki67 (Santa Cruz biotechnology, 1:1000), rabbit polyclonal to collagen I (Abcam, 1:1000), rabbit polyclonal to collagen III (Abcam, 1:1000), rabbit polyclonal to MMP-9 (Abcam, 1:1000), rabbit polyclonal to
MMP-2 (Abcam, 1:1000), rabbit polyclonal to TIMP-1 (Abcam, 1:1000), rabbit monoclonal to IL-6 (Cell Signaling Technology, 1:1000), mouse monoclonal to IL-8 (Abcam, 1:1000), rabbit polyclonal to eotaxin (Abcam, 1:1000), rabbit monoclonal to STAT3 (Abcam, 1:1000) and rabbit monoclonal to STAT3 (phospho Y705) (p-STAT3, Abcam, 1:1000) overnight at 4˚C. After washing thrice with TBS buffer, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz biotechnology, Inc.) at room temperature for 1 h. The protein band was detected using ECL reagent (Millipore). The relative intensities of protein bands were quantified using Image J software. GAPDH was used as the internal control.

2.7. Gelatin Zymography

The activity of MMP-9 was measured by gelatin zymography as described previously (Jr et al. 2015). Briefly, Cell media was collected from each group and concentrated using centrifugal filter units (Millipore). Samples were separated by using 10% SDS-PAGE gelatin gel (Bio-Rad Criterion). The gel was rinsed with deionized water, and then incubated with gentle rocking in 1× zymogram renaturation buffer (Bio-Rad) at room temperature. The gel was then rinsed again with deionized water before being incubated in 1× zymogram developing buffer (Bio-Rad) for 36 h at 37°C. Gels were stained in Coomassie blue, destained in a solution of 50% methanol, 40 % DI water, and 10% acetic acid, and then quantified by densitometry using a Li-Cor Odyssey XL system. Activity was standardized to cell lysate protein concentration.
2.8. **ELISA assay**

Concentrated cell culture supernatants from each group were assayed for levels of IL-6, IL-8 and eotaxin using human IL-6 QuantiGlo ELISA Kit, human IL-8/CXCL8 QuantiGlo ELISA Kit and human CCL11/Eotaxin Quantikine ELISA Kit (R&D Systems), according to manufacturer's instructions.

2.9. **Luciferase assay**

Prediction of potential miR-874 target genes was performed using the publicly available program microRNA.org (http://www.microrna.org/). The human STAT3 3'-UTR sequences containing the wild-type or mutant miR-874 binding site were synthesized by Sangon Biotech Co. Ltd (Shanghai, China), and then were inserted into the pGL3-basic vector (Promega, USA) (KpnI/XhoI, insert: vector=4:1), named as STAT3 3'-UTR or STAT3 3'-UTR mut vector. For the luciferase assay, the cells were seeded in 96-well plates for 24 h, and then co-transfected with luciferase reporter vectors and miR-874 mimic or miR-NC using Lipofectamine 2000. Renilla luciferase expression of pRL-TK plasmids (Promega, Madison, WI, USA) was used for normalization. The activities of firefly and Renilla luciferase were measured using the Dual-Luciferase Reporter assay (Promega) at 48 h after transfection. The relative luciferase activity was calculated as firefly fluorescence/Renilla fluorescence.

2.10. **Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5 software. Data are expressed as means ± standard error (means ± SEM) from at least three independent experiments. Statistical analysis between two groups was performed by using
Student's t-test, and analysis of more than two groups was performed by using one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.
3. Results

3.1. MiR-874 overexpression inhibits TNF-α-induced cell proliferation and migration in human fASM cells

We performed qRT-PCR assay to assess the expression of miR-874, and found that TNF-α treatment induced significant reduction of miR-874 expression, implying that miR-874 was involved in the regulation of TNF-α-treated fASM cells. To investigate the biological effects of miR-874, we transfected the cells with miR-874 mimic to elevate its expression, and mimic-NC was used as a negative control (Fig. 1A). MTT assay was performed to measure cell viability, which was markedly increased after TNF-α treatment. As compared with TNF-α group or mimic-NC+TNF-α group, the cell viability was significantly inhibited in miR-874 mimic +TNF-α group (Fig. 1B). Compared with control cells, the cell viability had no significant changes in the cells transfected with miR-874 alone, suggesting that miR-874 overexpression may have no marked effects in untreated fASM cells (Fig. 1C). We further detected the expression of two proliferation markers, PCNA and Ki67, by Western blot assay. As shown in Fig. 1D and E, the increased expression of PCNA and Ki67 induced by TNF-α was significantly suppressed by the transfection of miR-874 mimic. Transwell assay showed that miR-874 overexpression significantly inhibited TNF-α-induced cell migration in fASM cells (Fig. 1F). These data suggested that miR-874 expression was reduced in TNF-α-treated fASM cells, and miR-874 overexpression could inhibit TNF-α-induced cell proliferation and migration in fASM cells.
3.2. MiR-874 overexpression inhibits TNF-α-induced extracellular matrix (ECM) deposition in human fASM cells

To investigate the effects of miR-874 on ECM deposition, we performed Western blot assay to measure the expression of collagen I, collagen III, matrix metalloproteinase (MMP)-9, MMP-2 and tissue inhibitors of metalloproteinases (TIMPs). As shown in Fig. 2A, C and D, the expression of collagen I, collagen III, MMP-9 and MMP-2 were significantly increased in TNF-α group as compared with control group. However, as compared with TNF-α group or mimic-NC+TNF-α group, miR-874 mimic transfection markedly decreased the expression of these three proteins. TNF-α treatment did not affect the expression of TIMP-1, while its expression was significantly elevated in miR-874 mimic +TNF-α group. Furthermore, we found that miR-874 mimic transfection significantly suppressed TNF-α-induced enhanced activities of MMP-9 and MMP-2 (Fig. 2B). Taken together, these data suggested that miR-874 overexpression inhibited TNF-α-induced production of collagen I, collagen III, MMP-9 and MMP-2, promoted the expression of TIMP-1, and thus suppressed the ECM deposition in human fASM cells.

3.3. MiR-874 overexpression inhibits TNF-α-induced inflammatory cytokines production in human fASM cells

To investigate the effects of miR-874 on inflammatory cytokines production, we performed ELISA and Western blot assay to measure the levels of IL-6, IL-8 and eotaxin. As shown in Fig. 3, the production of IL-6, IL-8 and eotaxin in TNF-α group was significantly elevated as compared with control group, which showed an obvious
reduction by the transfection of miR-874 mimic. These data suggested that miR-874 overexpression inhibited TNF-α-induced inflammation in human fASM cells.

3.4. **STAT3 is a direct target of miR-874**

We next examined the expression of STAT3 by Western blot assay, and found that TNF-α treatment significantly up-regulated the protein level of STAT3 and p-STAT3 in a time-dependent manner (Fig. 4A and B), which was markedly suppressed by the overexpression of miR-874 (Fig. 4C and D), implying that there may be a regulation between miR-874 and STAT3. We predicted that the 3′-UTR of STAT3 contain two binding sites of miR-874 by bioinformatics software. To further confirm the target reaction of miR-874 on the 3′-UTR of STAT3 mRNA, a luciferase activity assay was performed. As shown in Fig. 4E and F, the result indicated that the miR-874 overexpression significantly inhibited the luciferase activity of the plasmid containing wild-type STAT3 3′-UTR, but not the plasmid containing mutating STAT3 3′-UTR. The results suggested that miR-874 was able to directly bind to the 3′-UTR of STAT3 mRNA and suppress its expression.

3.5. **STAT3 overexpression partly reverses the effects of miR-874 in human fASM cells**

To determine whether STAT3 mediated the effects of miR-874 in TNF-α-treated fASM cells, we co-transfected the cells with miR-874 mimic and pcDNA3.1-STAT3 or pcDNA3.1-empty vector. The transfection efficiency of pcDNA3.1-STAT3 was shown in Fig. 5A. The cell viability showed no significant changes between cells transfected with pcDNA3.1-STAT3 alone and control cells (Fig. 5B), suggesting that STAT3 overexpression may have no marked effects in untreated fASM cells. Western
blot assay was performed to measure the expression of STAT3. The results demonstrated that co-transfection of miR-874 mimic and pcDNA3.1-STAT3 significantly elevated STAT3 expression as compared with cells transfected with miR-874 mimic alone (Fig. 5C). We then detected the cell viability, cell migration, MMP-9 activity and IL-8 production, and the results showed that STAT3 overexpression partly reversed the inhibition of cell viability and cell migration, the reduction of MMP-9 activity and the decrease of IL-8 induced by miR-874 mimic transfection in TNF-α-treated fASM cells (Fig. 5D-G). These data suggested that the protective role of miR-874 overexpression in TNF-α-treated fASM cells was in part mediated by inhibiting STAT3 signaling.
4. Discussion

Asthma is a common chronic respiratory disease in children, and its pathogenesis has not yet been fully elucidated. Airway inflammation has been showed to act as one crucial event in the progression of asthma (Pelaia et al. 2015). Emerging studies have shown that ASM cell has the ability to produce various inflammatory factors within a specific stimulus condition, thereby participating in the maintenance and aggravation of airway inflammation (Tliba and Jr 2008; Tliba, Amrani and Panettieri 2008). Inflammatory cytokines-induced airway remodeling, characterized by increased ASM cell proliferation and increased ECM deposition, is a key feature of asthma disease. TNF-α is a potent pro-inflammatory cytokine that functions as an important regulator of airway inflammation (Cho et al. 2011). Accumulating evidence revealed that TNF-α could promote the secretion of inflammatory factors by activating multiple signaling pathways including STAT3 (He et al. 2017). In the present study, we treated the fASM cells with TNF-α to induce the injury leading to airway remodeling.

MiRNAs are small RNAs that function as suppressors of gene expression at the posttranscriptional level, and thus modulate a variety of biological processes including cell proliferation, inflammation and survival (Bartel 2004; Guo et al. 2010). Asthma and allergic rhinitis (AR) commonly coexist and can be taken as manifestations of one syndrome. Wu et al have reported that miR-874 was significantly down-regulated in extracellular vesicles from nasal mucus of AR patients (Wu et al. 2015). By evaluating the miRNA profiles of the nasal mucosa of patients with long-term asthma, Suojalehto et al have reported that many miRNAs including
miR-874 were downregulated in asthmatic patients (Suojalehto et al. 2014). Consistent with the previous studies, we found that the expression of miR-874 was significantly suppressed by the effect of TNF-α in fASM cells, suggesting that miR-874 regulation may be deeply involved in the airway remodeling induced by TNF-α. We further found that miR-874 overexpression could inhibit TNF-α-induced cell proliferation and cell migration in fASM cells, and might protect fASM cells against TNF-α-induced airway damage.

Altered ECM deposition contributing to airway remodeling is a crucial feature of asthma (Johnson 2001). In the present study, miR-874 overexpression markedly inhibited the up-regulation of collagen I and collagen III and suppressed the increased expression and activity of MMP-9 and MMP-2 induced by TNF-α. Moreover, the expression of TIMP1, one endogenous MMP inhibitor, had an obvious elevation by the overexpression of miR-874. These results demonstrate that miR-874 overexpression inhibited TNF-α-induced ECM deposition by decreasing collagen expression, and by altering the balance between MMPs and TIMPs. We further investigated the effects of miR-874 on the production of inflammatory cytokines induced by TNF-α. IL-6 is a multipotent pro-inflammatory factor that participates in the regulation of immunological and inflammatory responses (Oliver et al. 2006). IL-8 and eotaxin, act as important chemokines, play an extremely important role in the recruitment and activation of eosinophils and neutrophil cells (Peng et al. 2005). Here, we found that the elevated production of IL-6, IL-8 and eotaxin induced by TNF-α showed an obvious reduction by miR-874 overexpression, suggesting that
miR-874 overexpression inhibited TNF-α-induced inflammation in fASM cells.

However, the mechanisms underlying how miR-874 protected fASM cells against TNF-α-induced airway remodeling were not defined. Therefore, we searched for potential target of miR-874 using bioinformatics methods. And we focused on the candidate target gene STAT3. Zhang et al have reported that miR-874 functions as a tumor suppressor by inhibiting angiogenesis through targeting STAT3 in gastric cancer (Zhang et al. 2015). In the present study, we showed that miR-874 negatively regulated STAT3 expression by binding to a specific target site within its 3’-UTR. Moreover, overexpression of miR-874 significantly suppressed the increased protein level of STAT3 and p-STAT3 induced by TNF-α. The results suggested that miR-874 could directly bind to the 3’-UTR of STAT3 mRNA to suppress its expression.

STAT3 has been reported to play an important role in a wide range of cellular processes, including cell proliferation, survival and inflammation (Simeonepenney 2008). Besides, several reports revealed that the biological role of IL-6 could be mediated by STAT3 signaling (Lin et al. 2009; Mcloughlin et al. 2005). Therefore, targeting the inhibition of STAT3 activation might represent an effective therapeutic strategy to prevent many diseases including asthma. In the present study, overexpression of STAT3, by the transfection of overexpression plasmid pcDNA3.1-STAT3, effectively reversed the protective effects of miR-874 overexpression against TNF-α-induced airway modeling, including increased cell proliferation, increased ECM deposition and elevated inflammation. These results suggested that the protective effects of miR-874 overexpression against
TNF-α-induced airway modeling were partly depended on STAT3.

In summary, we demonstrate that miR-874 overexpression plays a protective role in TNF-α-induced fASM cells by regulating airway remodeling, including decreasing cell proliferation and migration, reducing ECM deposition and down-regulating inflammatory factors production. And the effects of miR-874 were closely associated with the regulation of STAT3. These findings indicate that miR-874 overexpression and/or STAT3 signaling inhibition may be effective methods for the treatment of asthma.

Disclosures

The authors have indicated no financial conflicts of interest.

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Figure legends

**Fig. 1.** MiR-874 overexpression inhibits TNF-α-induced cell proliferation and migration in human fASM cells. fASM cells were transfected with miR-874 mimic or mimic-NC for 24 h, followed by TNF-α treatment for another 24 h. (A) The expression of miR-874 was detected by qRT-PCR assay and normalized to U6 expression. (B and C) Cell viability was measured by MTT assay. (D) The protein expression of PCNA and Ki67 was measured by Western blot assay, and normalized to GAPDH expression. (E) The relative protein level was shown in bar graph. (F) Cell migration was detected by Transwell assay. *P < 0.05 vs control group; # P < 0.05 vs TNF-α group.

**Fig. 2.** MiR-874 overexpression inhibits TNF-α-induced ECM deposition in human fASM cells. (A) The expression of collagen I, collagen III, MMP-9, MMP-2 and TIMP-1 was detected by Western blot assay and normalized to GAPDH expression. (B) The activity of MMP-9 and MMP-2 was measured by gelatin zymography. (C) The relative protein expression of collagen I and collagen III was shown in bar graph. (D) The relative protein expression of MMP-9, MMP-2 and TIMP-1 was shown in bar graph. *P < 0.05 vs control group; # P < 0.05 vs TNF-α group.

**Fig. 3.** MiR-874 overexpression inhibits TNF-α-induced inflammatory cytokines production in human fASM cells. (A) The production of IL-6, IL-8 and eotaxin was detected by ELISA assay. (B) The expression of IL-6, IL-8 and eotaxin was measured by Western blot assay. (C) The relative protein expression of IL-6, IL-8 and eotaxin was shown in bar graph. *P < 0.05 vs control group; # P < 0.05 vs TNF-α group.
**Fig. 4.** STAT3 is a direct target of miR-874. (A and C) The expression of STAT3 and p-STAT3 was measured by Western blot assay. (B and D) The relative expression of STAT3 and p-STAT3 was shown in bar graph. *$P < 0.05$ versus the control group; # $P < 0.05$ vs TNF-α group. (E and F) The predicted target relationship between miR-874 and STAT3 3′-UTR. MiR-874 mimic or mimic-NC was co-transfected with STAT3 3′-UTR or STAT3 3′-UTR mut reporter plasmids. The relative luciferase activity was analyzed 48 h later by Dual-Luciferase Reporter assay. *$P < 0.05$ versus mimic-NC group.

**Fig. 5.** STAT3 overexpression partly reversed the protective effects of miR-874 in TNF-α-treated fASM cells. (A) The transfection efficiency of pcDNA3.1-STAT3 plasmids (containing enhanced green fluorescent protein (EGFP)). (B) Cell viability was measured by MTT assay. (C) The expression of STAT3 was measured by western blot assay and the relative expression of STAT3 was shown in bar graph. (D) The cell viability was detected by MTT assay. (E) Cell migration was detected by Transwell assay. (F) The activity of MMP-9 was measured by gelatin zymography. (G) The level of IL-8 was assessed by ELISA assay. *$P < 0.05$ versus the control group; # $P < 0.05$ vs TNF-α group; &$P < 0.05$ vs miR-874 mimic +TNF-α group.