Physiological Research Pre-Press Article

1 **MiR-18a inhibits PI3K/AKT signaling pathway to regulate PDGF-BB-induced**
2 **airway smooth muscle cell proliferation and phenotypic transformation**

3 Running title: MiR-18a to regulate airway smooth muscle cell

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Abstract

Objective: The increased proliferation and migration of airway smooth muscle cells (ASMCs) is a key process in the formation of airway remodeling in asthma. In this study, we focused on the expression of mircoRNA-18a (miR-18a) in airway remodeling in bronchial asthma and its related mechanisms. **Methods:** ASMCs are induced by platelet-derived growth factor BB (PDGF-BB) for in vitro airway remodeling. The expression of miR-18a in sputum of asthmatic patients and healthy volunteers was detected by qRT-PCR. The expression of miR-18a was over-expressed or interfered with in PDGF-BB-treated ASMCs. Cell proliferation, apoptosis and migration were detected by MTT, flow cytometry and Transwell, respectively; the expression of contractile phenotype marker proteins (SM-22α, α-SM-actin, calponin) and key molecules of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (PI3K, p-PI3K, AKT and p-AKT) in ASMCs were detected by Western blot. **Results:** The expression of miR-18a was down-regulated in the sputum and PDGF-BB-treated ASMCs of asthma patients. PDGF-BB could promote the proliferation and migration of ASMCs and inhibit their apoptosis; it could also promote the phenotypic transformation of ASMCs and activate the PI3K/AKT pathway. MiR-18a could inhibit the proliferation, migration ability and phenotypic transformation of ASMCs induced by PDGF-BB to a certain extent and alleviate the effect of PDGF-BB in supressing apoptosis, while miR-18a could inhibit the activation of the PI3K/AKT pathway. **Conclusions:** MiR-18a inhibits PDGF-BB-induced proliferation, migration and phenotypic conversion of ASMCs by inhibiting the PI3K/AKT pathway, thus attenuating airway remodeling in asthma.

Keywords: miR-18a, PDGF-BB, airway smooth muscle cells (ASMCs), airway remodeling, PI3K/AKT pathway.

Introduction

Bronchial asthma is a complex respiratory syndrome caused by various pathological mechanisms. The main clinical manifestations include chronic airway

inflammation, airway hyper-reactivity, reversible airflow limitation, airway remodeling, and recurrent wheezing, cough, shortness of breath, and chest distress [1]. Airway remodeling is an important pathological feature of asthma as a repair response to persistent inflammation [2]. Most of current understanding of airway remodeling comes from studies of allergic asthma [3]. Airway remodeling in allergic bronchial asthma is thought to be the result of a chronic inflammatory response, mainly manifested by permanent airway tissue destruction and chronic tissue repair [4]. However, there is growing evidence that asthma is a syndrome composed of multiple phenotypes/endotypes [4]. Therefore, it is unknown whether the salient features of airway remodeling are associated with certain phenotypic/endotypic specificities.

Airway smooth muscle plays an important role in the lung. Hyperplastic and hypertrophic smooth muscle not only leads to airway wall thickening, causing airway stenosis but also reduces the contractility of the airway wall, increasing extracellular matrix synthesis and aggravating the fibrosis of the tracheal wall. Excessive proliferation and migration of airway smooth muscle can directly contribute to airway remodeling [5]. Airway smooth muscle cells (ASMCs), fibroblasts and myofibroblasts are the main effector cells of airway subepithelial thickening in asthma; fibroblast-to-myofibroblasttransition (FMT) is one of the main mechanisms of early airway remodeling [6]. In the pathogenesis of asthma, myofibroblasts do not initiate apoptosis immediately after completing their normal physiological functions, but rather persist in tissues, inducing contraction of surrounding cells and extracellular matrix (ECM), thus leading to secretion of growth factors and ECM components. Therefore, myofibroblasts have an impact on contractile and metabolic activities and participate in airway remodeling [7]. Previous studies on FMT in asthma have identified a variety of precipitating factors, of which humoral factors including growth factors, cytokines and chemokines, play an important role in phenotypic transformation. Among them, platelet-derived growth factor BB (PDGF-BB) has been demonstrated to induce proliferation and migration of ASMCs and exacerbate airway remodeling [8]. Due to the complex pathogenesis of asthma, factors may interact with each other and form corresponding feedback loops to further the transformation.

90 Therefore, studying the interaction of various regulatory factors of FMT, thus
91 identifying specific therapeutic targets, is an important direction in the treatment of
92 irreversible airway remodeling.

93 The role of microRNA (miRNA) in regulating human biological functions and
94 diseases has been a hot topic in research. MiRNAs are single-stranded non-coding
95 RNAs containing only 19-22 bases of single-stranded small ribonucleic acid
96 molecules. MiRNAs inhibit gene translation by binding to the mRNA3-untranslated
97 region (3'-UTR), which in turn negatively regulates gene expression [9-10]. Studies
98 have reported that miRNAs are involved in many biological processes, including
99 stress and inflammatory regulation of host immune cells [11]. An increasing number
100 of studies have confirmed that miRNAs also play a regulatory role in asthma airway
101 remodeling. Shao et al found that niRNA-133a can target and regulate insulin-like
102 growth factor 1 receptors (IGF1R) and alleviate airway remodeling in asthma by
103 inhibiting the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of
104 rapamycin (mTOR) signaling pathway activation [12]. Lou et al found that
105 miR-192-5p attenuates airway remodeling and autophagy in asthma by targeting
106 matrix metalloproteinase-16 (MMP-16) and autophagy-related gene 7 (ATG7) [13].
107 Some studies have found that miR-18a is down-regulated in nasal biopsies of
108 asthmatic patients [14]. MiR-18a is also involved in the regulation of multiple
109 signaling pathways [15-18] and regulates multiple downstream target genes to exert
110 effects. However, the specific mechanism of action of miR-18a regulation in early
111 airway remodeling in asthma is unknown. Therefore, in the present study, we
112 measured the expression of miR-18a after PDGF-BB-induced ASMCs. We also
113 explored the role of PI3K/AKT signaling pathway and miR-18a in early airway
114 remodeling in cell model, laying a foundation for identifying a key therapeutic target
115 for early intervention of irreversible airway remodeling.

116

117 Methods and Materials

118 Subjects

119 This study recruited 20 patients with bronchial asthma and 20 healthy controls.

In the asthma group, there were 12 males and 8 females, aged 14-80 years, all of whom visited the Outpatient and Inpatient Department of Respiratory and Critical Care of the Second Affiliated Hospital of Shenzhen University between 2018 and 2021. Inclusion criteria included medical history, physical examination, and physiological examination consistent with the national asthma control guidelines for asthma diagnosis. Exclusion criteria included history of smoking, chronic lung disease other than asthma within the past year; other serious chronic diseases, including congestive heart failure, chronic kidney disease, liver disease, and viral infections.

A total of 20 patients were enrolled in the control group (Normal group). All healthy controls (8 males and 12 females, aged 18-58 years) underwent a physical examination at our hospital during the same period as above, with good health and no history of asthma, allergic rhinitis or other allergic diseases. All subjects were non-smokers. This study was approved by the Ethics Committee of the People's Hospital of Shenzhen Baoan District, and all participants provided written informed consent (Approval Number: 2021042112015753).

Sputum sample

Subjects' first sputum in the morning was collected. The subjects were asked to gargle with water for 10 min before sputum collection and deep sputum was collected. Each sample must contain over 3 mL of sputum to avoid saliva and nasal secretions. When transfer sputum specimen, the sample should be kept at a low temperature on ice. The collected sputum samples were transferred into a 50 mL centrifuge tube, added with cell washings (30 mL/subject) and Dithiothreitol (DTT, 6 mM, 1 mL), and shaken on a shaker until the sputum was completely digested. The sample was then centrifuged at 2000 rpm for 10 min; the supernatant was discarded, and the cells at the bottom were transferred to the cell preservative for future use.

Cell culture and processing

ASMCs were purchased from the American type culture collection (ATCC; Virginia, USA). Cell cultures were maintained in high-glucose dulbecco's modified

150 eagle medium (DMEM; ScienCell, California, USA) containing 10 % Fetal Bovine
151 Serum (FBS; ScienCell, California, USA) at 37 ℃ in a humidified 5 % CO_2
152 incubator.

153 MiR-18a mimics, inhibitor and negative control (NC, 100 nM) were purchased
154 from Jima Gene Company (Shanghai, China). Transfection was performed using
155 Lipofectamine2000 reagent (Invitrogen, Carlsbad, CA, USA), and cells were
156 transfected according to the instructions when they grew to 70-80 % confluence in
157 six-well plates. The corresponding expression levels and functional assays were
158 performed 24 hours after transfection.

159 Proliferation and migration of ASMCs were induced using PDGF-BB
160 (PeproTech, Cranbury, NJ, USA). Cell grouping were named as follows: sham group:
161 without any treatment; PDGF-BB group: 20 ng/mL PDGF-BB-induced cells;
162 PDGF-BB + mi-NC group: PDGF-BB-induced cells and transfected mimic NC;
163 PDGF-BB + mi-miR-18a group: PDGF-BB-treated cells and transfected miR-18a
164 mimic; PDGF-BB + in-NC group: PDGF-BB-treated cells and transfected inhibitor
165 NC; PDGF-BB + in-miR-18a group: PDGF-BB-treated cells and transfected miR-18a
166 inhibitor.

167 The PI3K/AKT pathway agonist IGF-1 (insulin-like growth factors-1, O2O,
168 Israel) was used at a concentration of 10 μg/mL. In the PDGF-BB + mi-miR-18a +
169 IGF-1 group, cells were treated with PDGF-BB and IGF-1 and transfected with
170 miR-18a mimic.

171

172 **qRT-PCR**

173 Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA) from
174 collected cells. Reverse transcription from RNA to cDNA was performed using a
175 reverse transcription kit (TaKaRa, Tokyo, Japan), according to the instructions of the
176 kit. Expression of genes was detected using a LightCycler480 (Roche, Indianapolis,
177 IN, USA) quantitative fluorescence PCR instrument, and reaction conditions were set
178 according to the operating instructions of the quantitative fluorescence PCR kit
179 (SYBRGreenMix, RocheDiagnostics, Indianapolis, IN). U6 was adopted as the

180 internal reference. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method. The miR-18a
181 and U6 primers used for qRT-PCR were purchased from GeneCopoeia Company
182 (MD, USA).
183
184 **MTT assay**
185 The cell suspension was inoculated into a 96-well plate (100 μL/well, 2500
186 cells/well), and the plate was pre-cultured with 5 % CO_2 at 37 °C for 24 h, 48 h and
187 72 h respectively. 10 μL MTT solution was added to each well. The culture plate was
188 returned to the incubator for another 2 h. After that, the opticaldensity (OD) at 570 nm
189 was measured with a microplate reader. The test was repeated three times.
190
191 **Annexin V-FITC/PI**
192 Cells with a confluence of 80 - 90 % were digested and neutralized with EDTA-free
193 trypsin, centrifuged at 2000 rpm for 5 min, and washed twice with precooled PBS; the
194 supernatant was discarded. Prepared according to the instructions of the kit; 5 μL of
195 PI and FITC-labeled Annexin V was added to the cells which was then mixed well
196 and reacted at room temperature in the shade for 15 min, finally tested.
197
198 **Transwell**
199 Sterile transwell inserts were placed into 24-well plate wells; 100 μl of equal cell
200 concentration was added to the upper chamber of the inserts, and 800 μl of complete
201 medium containing 10 % FBS was added to the lower chamber, then placed in the
202 incubator for another 12 h. After incubation, the transwell inserts were gently washed
203 three times with PBS and subsequently adding in 4% paraformaldehyde and fixed for
204 30 min. Non-migrated cells in the upper chamber were gently wiped away with a
205 cotton swab and washed three times with PBS. After 0.4 % crystal violet staining, five
206 randomly selected fields were photographed under a microscope; the number of cells
207 in each field was counted. ImageJ was used for statistical analysis.
208
209 **Western blot**

210 Cell lysis performed with RIPA lysis solution (Beyotime, Shanghai, China) to
211 obtain cellular protein. The protein concentration was measured with the BCA kit
212 (Beyotime). After membrane transfer, they were blocked in blocking solution for 60
213 min at room temperature and incubated with primary antibodies β-actin (4970S, Cell
214 Signaling Technology, Boston, USA), SM-22α (ab14106, Abcam, Mass., USA),
215 α-SM-actin (A5288, Sigma, St.Louis, Missouri, USA), calponin (17819S, Cell
216 Signaling, Boston, USA), PI3K (4249S, Cell Signaling Technology), p-PI3K (17366s,
217 Cell Signaling Technology), AKT (9272S, Cell Signaling Technology), p-AKT
218 (4060S, Cell Signaling Technology) at 4 ℃ overnight. The next day, secondary
219 antibody (horseradish peroxidase-labelled goat anti-rabbit IgG, Beijing Kangwei
220 Century Biotechnology Co., Ltd., China, Beijing) and incubated for 1 hour at room
221 temperature and finished with three 10 min washes. After adding the developer to the
222 membrane, the detection was performed using a chemiluminescence imaging system
223 (Bio-rad).
224
225 **Statistical analysis**
226 Statistical analysis was performed by SPSS 24.0 software. Statistical data were
227 expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA)
228 or Student's t-test was used to compare more than two groups or two groups. Each
229 experiment was triplicated. $P < 0.05$ indicated a significant difference.
230
231 **Results**
232 **Down-regulation of miR-18a expression in the sputum of an asthma patient and**
233 **PDGF-BB-induced ASMCs**
234 MiR-18a expressions in the sputum of asthmatic patients examined by qRT-PCR
235 ($P<0.001$) was noticeably lower than that of healthy volunteers (Figure 1A). Further,
236 PDGF-BB was used to treat ASMCs and intervene the expression of miR-18a. As
237 presented by Figure 1B, miR-18a expression in PDGF-BB group was drastically
238 down-regulated relative to the sham group ($P<0.001$); miR-18a expression in
239 PDGF-BB + mi-miR-18a group was up-regulated comparing to PDGF-BB + mi-NC

(P<0.01); miR-18a expression in PDGF-BB + in-miR-18a group was down-regulated in comparison with PDGF-BB + in-NC (P<0.01). These results demonstrated that miR-18a expression was reduced in sputum from asthmatic patients and in ASMCs after PDGF-BB treatment.

MiR-18a inhibits PDGF-BB-induced proliferation and migration of ASMCs and promotes their apoptosis.

To investigate the role of miR-18a in ASMCs, we transfected miR-18a mimics and miR-18a inhibitor in ASMCs cells to over-express or disrupt miR-18a expression. MTT, flow cytometry and transwell were applied to examine the proliferation, apoptosis and migration ability of cells in each treatment group. The results showed (Figure 2A–E) that PDGF-BB could promote the proliferation and migration of ASMCs and inhibit their apoptosis compared with the sham group. However, overexpression of miR-18a significantly alleviated the effects of PDGF-BB treatment on the proliferation, apoptosis and migration of ASMCs. Interference with miR-18a expression significantly indorsed the promoting effect of PDGF-BB treatment on the proliferation and migration of ASMCs and the inhibitory effect on apoptosis. These outcomes confirmed that PDGF-BB promoted PDGF-BB cell proliferation and migration as well as suppressed apoptosis, while miR-18a suppressed cell proliferation and migration as well as promoted apoptosis.

MiR-18a attenuates the inhibitory effect of PDGF-BB-induced vasoconstrictor phenotype proteins in ASMCs

We then assessed the effect of miR-18a on PDGF-BB-induced phenotypic changes in ASMCs by detecting the expression of vasoconstrictor phenotype marker proteins by Western blot. Figure 3 showed that the expressions of SM-22α, α-SM-actin, and calponin were significantly up-regulated in the PDGF-BB group relative to the sham group; SM-22α, α-SM-actin, and calponin were significantly down-regulated in the PDGF-BB + mi-miR-18a group in comparison to the PDGF-BB + mi-NC; and SM-22α, α-SM-actin, and calponin were up-regulated in the

PDGF-BB + in-miR-18a group comparing to the PDGF-BB + in-NC.

MiR-18a inhibits PDGF-BB-induced activation of the PI3K/AKT signaling pathway in ASMCs

To investigate the mechanism of miR-18a in ASMCs, the protein expression of key molecules of the PI3K/AKT signaling pathway was detected by western blot. Figure 4 illustrated that compared with the sham group, the ratios of p-PI3K/PI3K and p-AKT/AKT were significantly up-regulated in the PDGF-BB group; in comparison to PDGF-BB + mi-NC, the ratios were significantly down-regulated in the PDGF-BB + mi-miR-18a group; and comparing to PDGF-BB + in-NC, the ratios were up-regulated in the PDGF-BB + in-miR-18a group. These outcomes suggest that PDGF-BB can promote proliferation and migration, as well as inhibit apoptosis by activating the PI3K/AKT pathway, while miR-18a can block the process.

To further validate that miR-18a is inhibiting airway remodeling by preventing PI3K/AKT signaling pathway activation, the PI3K/AKT signaling pathway agonist IGF-1 for reversion experiments was used. The experimental results showed (Figure 5A-C) that IGF-1 reversibly over-expressed miR-18a on PDGF-BB-induced proliferation, apoptosis and migration of ASMCs. Figure 5D showed that miR-18a increased the expression of contractile phenotype marker proteins (SM-22α, α-SM-actin, calponin) in PDGF-BB-induced ASMCs, while IGF-1 reversed the expression of the above proteins. These outcomes together confirmed that miR-18a exerts an inhibitory effect on airway remodeling by restraining the PI3K/AKT signaling pathway.

Discussion

Airway remodeling in bronchial asthma is a series of chronic airway structural changes including airway wall thickening, mucosal epithelial injury, subepithelial fibrosis, increased extracellular matrix deposition, goblet cell metaplasia, smooth muscle cell proliferation, and revascularization [19-21]. Studies have shown that airway remodeling occurs in the early stage of asthma, without effective clinical

300 prevention and treatment in the irreversible late stage. Therefore, the early and
301 effective intervention of irreversible airway lesions is an urgent challenge. Airway
302 smooth muscle can proliferate under the activation of cytokines (such as PDGF,
303 VEGF, EGF and TGF-β, etc.), accompanied by increased cell migration [22]. It has
304 been confirmed that the proliferation and migration ability of airway smooth muscle
305 of asthmatic patients in vitro is comparable to or higher than that of smooth muscle in
306 response to cytokines such as PDGF, and significantly higher than the level in normal
307 airway smooth muscle [23]. Therefore, in this study, PDGF-BB was used to stimulate
308 ASMCs and to investigate their phenotypic changes upon cytokine stimulation and
309 the regulation of cell proliferation and migration by miR-18a. It has been reported that
310 miR-18a expression decreased in nasal biopsies of asthma patients [24], and the
311 results of this study are consistent with those reported in the articles. We have found
312 that the miR-18a expression decreased in the sputum of asthma patients. The cellular
313 miR-18a expression drastically decreased after PDGF-BB induction of ASMCs. The
314 elevation in proliferation and migration of ASMCs is a key process in asthma-related
315 airway remodeling [25]. PDGF-BB can induce proliferation and migration of ASMCs,
316 and the apoptotic ability is thus attenuated. Further over-expression of miR-18a
317 inhibited the effect of PDGF-BB on the proliferation and migration of ASMCs. This
318 shows that miR-18a is involved in regulating the role of ASMCs in airway
319 remodeling.

320 ASMCs phenotype modulation is mainly reversible conversion between
321 contractile and proliferative phenotypes [26]. ASMCs express a variety of proteins,
322 such as α-SM-actin, SM-22α, calponin, in the contractile phenotype. When ASMCs
323 cells switch to a synthetic phenotype, contractile phenotype proteins α-SM-actin,
324 SM-22α, and calponin expression decrease, and cells begin to proliferate and migrate.
325 This study showed that α-SM-actin, SM-22α, and calponin protein expression was
326 significantly lower in ASMCs under the induction of PDGF-BB. However,
327 overexpression of miR-18a promoted the expression of contractile proteins.

328 In recent years, the role of the PI3K/AKT signaling pathway on airway
329 remodeling has attracted much attention. Studies have shown that the activation of the

PI3K/AKT signaling pathway is closely related to the proliferation of AMSCs [24, 27-28], that is, the activation of the PI3K signaling pathway can cause airway remodeling. It has been shown that targeting PI3K signaling significantly inhibits fibroblast expansion and fibrotic remodeling [29]. In this study, PDGF-BB induced PI3K/AKT pathway activation in ASMCs, while miR-18a inhibited the PI3K/AKT pathway. Recovery experiments confirmed that miR-18a inhibited the proliferation and migration of AMSCs and ECM protein production by inhibiting the PI3K/AKT pathway through IGF-1, an activator of the PI3K/AKT pathway. These results were consistent with those reported in the literature.

In summary, miR-18a expression was significantly down-regulated in proliferating ASMCs as proven by this study. Its high expression was able to suppress the activation of PI3K/AKT pathway, thereby alleviating PDGF-BB stimulation-induced proliferation and migration of ASMCs. Our study provides important new insights into the study of the molecular mechanism of miR-18a in asthma. The pathogenesis of asthma is closely related to airway remodeling. The targeted over-expression of miR-18a in ASMCs may provide a potential novel therapeutic approach for inhibiting airway remodeling associated with asthma pathogenesis.

Declaration of Interest: The authors report no conflicts of interest.

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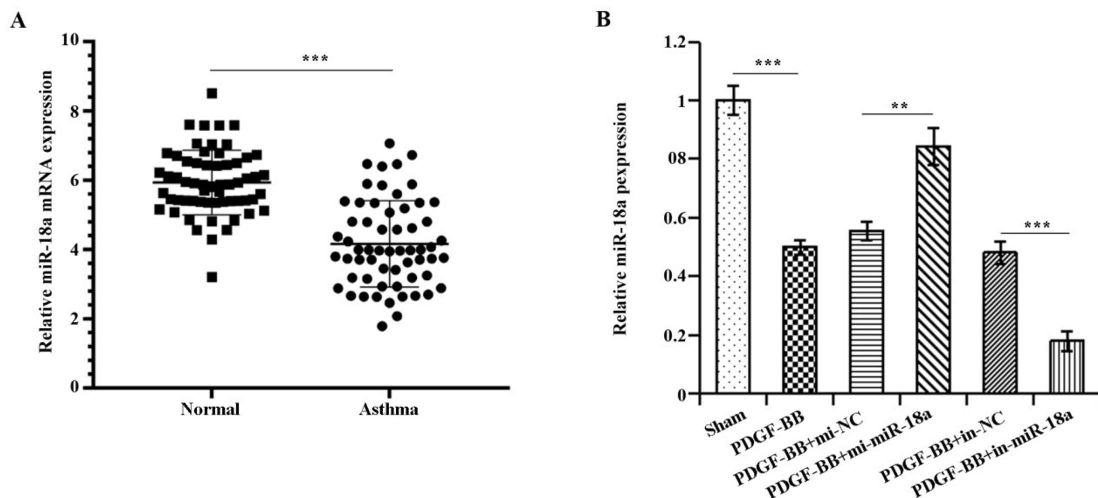

Figure 1 MiR-18a expression was down-regulated in the sputum of an asthma patient and PDGF-BB-interfered ASMCs.

A. qRT-PCR results on miR-18a expression in normal and asthma subjects; B. qRT-PCR results on miR-18a expression in the experimental group. **P<0.01, ***P<0.001.

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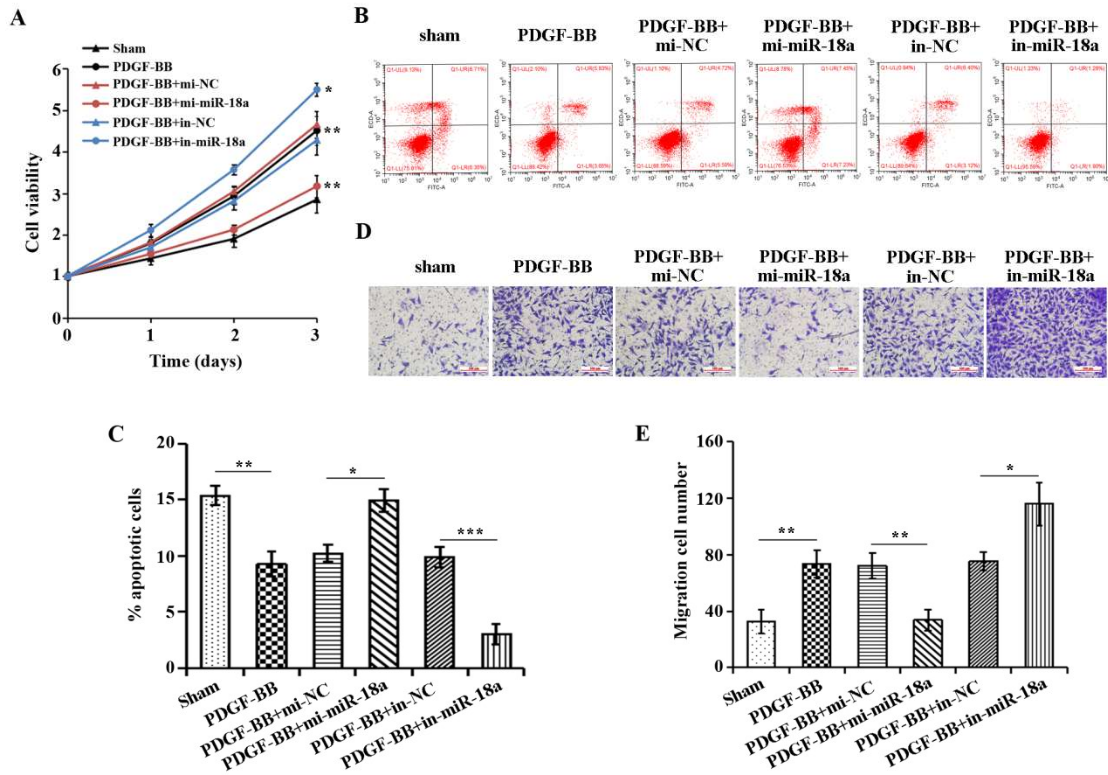

477 **Figure 2 Effect of over-expression or knockdown of miR-18a on proliferation,**
478 **apoptosis and migration of ASMC intervened by PDGF-BB**

479 A. MTT assay outcome on cell proliferation in each group; B. Flow cytometry
480 detection of apoptosis in each treatment group; C: Quantitative analysis by flow
481 cytometry. D. Transwell detection of cell migration in each treatment group. E:
482 Quantitative analysis of transwell detection. *P<0.05, **P<0.01, ***P<0.001.

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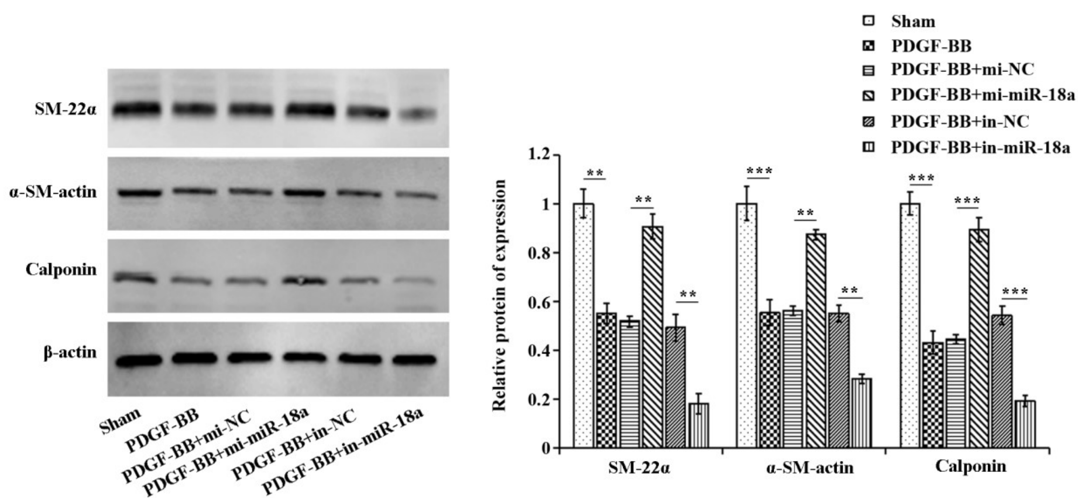

485

486 **Figure 3 MiR-18a elevates the expression of vasoconstrictor phenotypic markers**
487 **in PDGF-BB-induced ASMCs**

488 Western blot was used to examine the protein expression of SM-22α, α-SM-actin, and
489 calponin in cells of each treatment group. **P<0.01, ***P<0.001.
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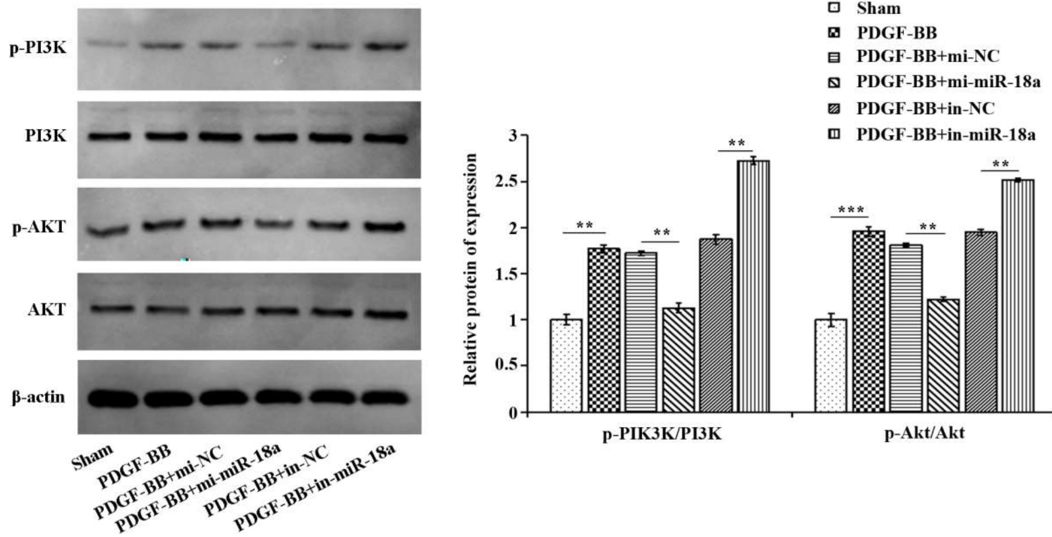
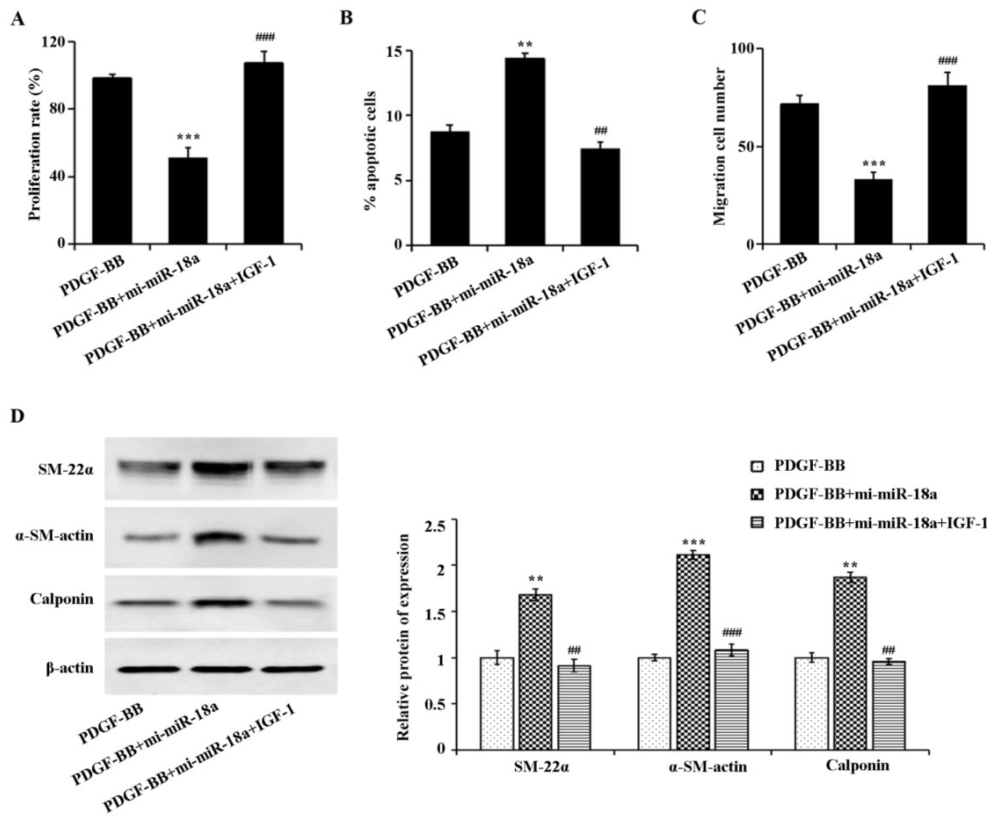


492
493 **Figure 4 MiR-18a inhibits PDGF-BB-induced activation of PI3K/AKT signaling**
494 **pathway in ASMCs**
495 A. Western blot for investigating the expression of PI3K, p-PI3K, AKT and p-AKT in
496 each treatment group. **P<0.01, ***P<0.001.



497

498 **Figure 5 PI3K/AKT signaling pathway agonist inhibited miR-18a-induced**
499 **interference on PDGF-BB-related proliferation, apoptosis, migration and**
500 **phenotypic transformation of ASMCs**

501 A.MTT assay examination on the changes in cell proliferation ability of each
502 treatment group; B: Flow cytometry outcome on the apoptosis of each treatment
503 group; C: Transwell detection of the changes in cell migration ability of each
504 treatment group; D: Western blot results on the expression levels of SM-22α,
505 α-SM-actin, and calponin in cells of each treatment group; D: Quantitative analysis of
506 gray values of SM-22α, α-SM-actin, and calponin bands, respectively. **$P<0.01$,
507 ***$P<0.001$ vs. PDGF-BB group. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. PDGF-BB+mi-miR-18a
508 group.

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