Extracellular matrix promotes proliferation, migration and adhesion of airway smooth muscle cells in a rat model of chronic obstructive pulmonary disease via upregulation of the PI3K/AKT signaling pathway

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Abstract. Extracellular matrix (ECM) creates the tissue microenvironment and serves a role in airway wall remodeling in chronic obstructive pulmonary disease (COPD). However, the biological function of ECM in COPD remains to be elucidated. In the present study, 24 healthy Sprague Dawley rats were randomized to normal and COPD groups. COPD was established by intratracheal injection with lipopolysaccharide over 30 days. Subsequently, airway smooth muscle cells (ASMCs) were isolated from rats and served as a model to assess the effects of three ECM components, including collagen type I, laminin and collagen type III (COL-3). Functional analysis *in vitro,* using cell counting kit-8, flow cytometry, wound healing and cell adhesion assays indicated that the ECM components could promote cell proliferation, cell cycle progression, migration and adhesion ability, respectively. Furthermore, as demonstrated by ELISA, treatment with ECM components increased levels of C-X-C motif chemokine ligand 1 (CXCL1), CXCL8 and interleukin-6 in ASMCs. Expression of transforming growth factor β1 (TGFβ1), fibroblast growth factor-1 (FGF-1) and tissue inhibitor of metalloproteinase 1 (TIMP1) was increased, and expression of matrix metalloproteinase-9 (MMP-9) was decreased following treatment with ECM components, as demonstrated by reverse transcription-quantitative polymerase chain reaction and western blot analysis. Additionally, specific activation of phosphoinositide 3-kinase (PI3K) signaling, using insulin-like growth factor-1 (IGF-1), promoted cell proliferation and cell cycle progression, increased expression of TGFβ1, FGF-1, PI3K, AKT, phospho-AKT, serine/threonine-protein kinase mTOR (mTOR), phospho-mTOR and TIMP1, promoted cell migration capacity and reduced the expression level of MMP-9 in cells from COPD rats. Consistently, PI3K inhibitor LY294002 exerted the opposite effect to IGF-1. In conclusion, ECM proteins promoted proliferation, migration and adhesion of ASMCs form rat models of COPD through activation of the PI3K/AKT signaling pathway.

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Introduction

Chronic obstructive pulmonary disease (COPD) has become a principal international health problem with a rise in prevalence and mortality (1), as there was a 44.2% increase of the number of prevalent COPD cases between 1990 and 2015 (2). The most common symptoms of COPD include, chronic cough, expectoration, shortness of breath and difficulty in breathing (3). COPD is generally considered to be an abnormal inflammatory disorder leading to airflow limitation associated with the response of lungs to toxic and noxious particles, and gases, and is primarily caused by tobacco smoking (4). Inflammation, oxidative stress and imbalance between levels of proteinases and antiproteinases within the lungs are also the predisposing factors to COPD (5).

The pathological alterations may be identified in the large airways, small airways, lung parenchyma and pulmonary vascular of patients with COPD (6-8). Multiple alterations in the airway are closely associated with COPD, and the symptoms are similar to vascular remodeling in patients with atherosclerosis (9). Airway wall has been demonstrated to be thickened in patients with COPD, primarily as a result of hyperplasia and hypertrophy of airway smooth muscle cells (ASMCs) and accumulation of extracellular matrix (ECM) components (10). The ECM of the lung consists of a number of macromolecules, including collagens, fibronectin, laminin (LN), proteoglycans, glycosaminoglycan, elastin and tenascin, which form a complex, dynamic network (11,12). In patients with COPD, the levels of elastin (13) and proteoglycans (14) were deceased, while LN and total collagen content were elevated (15). Collagen I (COL-1) and collagen-III (COL-3) are two types of lung interstitium components that serve a role

in establishing normal lung architecture (16). LN has been reported to be the primary component of basement membranes and to regulate cell behavior in bronchiolar epithelium of patients with COPD (17). Furthermore, ECM proteins are associated with biological properties of human ASMCs including growth, survival, migration and adhesion (12). To date, a limited number of studies investigating the role of deposition of COL-1, COL-3 and LN on the biological behavior of ASMCs in patients with COPD. Airway smooth muscle (ASM) preserves the dynamic homeostasis of synthesis/degradation of ECM though secretion of various ECM proteins, including matrix metalloproteinases (MMPs) (18), tissue inhibitors of metalloproteinases (TIMPs) (19), cytokines (12), chemokines (12) and growth factors (20). Evidence accumulated from previous studies indicated that ECM proteins are implicated directly or indirectly in multiple signaling pathways to influence various biological processes (21-23). Khatiwala *et al* (24) demonstrated that ECM modulates the mitogen-activated protein kinase signaling pathway and acts downstream of transforming protein RhoA and Rho-associated protein kinase 1 for the regulation of osteogenesis. In a previous study, COL-1 was associated with morphological alterations of mouse NMuMG cells and upregulation of cadherin-2 though the phosphoinositide 3-kinase (PI3K)-Rac1-JNK pathway (24). Integrin α6β1 and FAK-MEK/ERK signaling promote mesenchymal stem cell growth induced by LN-1 under growth factors in the absence of serum and differentiation factors (25). However, few studies have evaluated the association between ECM proteins and signaling pathways in the airways of patients with COPD.

In the present study, a rat model of COPD was established to assess the impact of ECM components (COL-1, COL-3 and LN) on proliferation, migration and attachment of ASMCs. Cytokines, chemokines, growth factors, MMP-9 and its inhibitor, TIMP1, were detected. The molecular signaling that may be involved the thickening of airway wall was further elucidated.

Materials and methods

COPD animal model construction. A total of 24 healthy male Sprague Dawley (SD; 10-12 weeks; 180-220 g; five per cage) rats were purchased from Shanghai Laboratory Animal Research Center (Shanghai, China) and housed in the animal room (25±3˚C; 50% humidity; 12/12 h light and dark cycle) with free access to water and food. The rat model of COPD was established as described in previous studies (26-28). Briefly, 12 SD rats were anesthetized with 0.45% pentobarbital sodium (50 mg/kg) via intraperitoneal injection, followed by injection of lipopolysaccharide (LPS; 200 mg/200 μl) through the trachea and inhalation of fresh smoke once a day for 30 days, then kept at -20˚C for 5 min/day for the next 30 days to establish animal models of COPD. The remaining 12 rats served as the normal group and received no treatment. All experimental protocols were approved by the Animal Research Committee of Hubei University of Medicine (Suizhou, China). The care and use of animals was based on the Guidelines by the National Institutes of Health.

Isolation of rat airway smooth muscle cells (ASMCs). Rat ASMCs were isolated as described in previous studies (29-31). Briefly, entire rat tracheae were rapidly dissected from normal and COPD rats in ice-cold PBS solution. Subsequently, the mucosal and connective tissues were removed and muscle was gently separated in small bundles. Separated muscle was placed in Ham's F12 digestion solution containing 0.5% fetal bovine serum (FBS; cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with collagenase IV (2 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and papain (1 mg/ml; Sigma-Aldrich; Merck KGaA). Following centrifugation at 500 x g for 15 min at 4˚C, cell suspension was collected and isolated ASMCs were treated with 0.25% trypsin and cells from passages 3-5 were selected for the subsequent experiments.

Cell culture and treatment. All ASMCs were maintained in Ham's F12 medium (cat. no. A1526_9010; AppliChem, Inc., St. Louis, MO, USA) with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37˚C. For cell functional analysis, ASMCs were seeded in 24-well plates and each well was treated with 500 μl ECM components, including COL-1 [cat. no. CLS001654; Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China], LN (cat. no. L4544; Sigma-Aldrich; Merck KGaA) or COL-3 (cat. no. ab7535; Abcam, Cambridge, UK) at a concentration of 10 mg/l each, except for the blank control cells, which were treated with 500 μl bovine serum albumin (BSA; 10 ml/l; cat. no. A8020; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) in a humidified atmosphere containing 5% CO_2 at 37˚C for 2 h.

Immunofluorescence (IF). The morphology of the isolated ASMCs was assessed by IF staining. Briefly, ASMCs at a density of 10^6 were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were subsequently treated with pre-cooled 100% methanol for 20 min at -20˚C and subsequently blocked in PBS containing 3% BSA for 1 h at room temperature. The cells were then incubated with α-smooth muscle actin (α-SMA) mouse primary antibody (1:200; Abcam; cat. no. ab8211) at 4˚C overnight, followed by incubation with Alexa Fluor 647 (red)-conjugated secondary antibody (1:500; cat. no. Z25008; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min in the dark. DAPI (100 ng/ml; Invitrogen; Thermo Fisher Scientific, Inc.) was used to stain the nuclei (10 min at room temperature). Finally, the location of α-SMA was detected using a laser scanning confocal microscope (LSM 710; Carl Zeiss AG, Oberkochen, Germany).

Cell Counting kit-8 (CCK-8) assay. The proliferation of ASMCs was determined by CCK-8 assay (cat. no. C0037; Beyotime Institute of Biotechnology, Haimen, China). Briefly, cells were seeded into 96-well plates at a density of $3x10^4$ cells/well. Following different treatments, the supernatant was removed and 10 μl CCK-8 solution was added to each well at 24, 48 or 72 h, and the mixture was incubated for 1 h at 37˚C. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiment was repeated three times.

5-Ethynyl-2'-deoxyuridine (EdU) proliferation analysis. Following different treatments, ASMCs (10^5 cells/well) were

seeded in triplicate in 96-well plates and their proliferation was measured using the Cell-Light EdU DNA Cell Proliferation kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China) according to the manufacturer's protocol. In brief, cells were treated with 50 mM EdU for 4 h at 37˚C and fixed with 4% formaldehyde for 15 min at room temperature. Subsequently cells were incubated with 100 μl Apollo reaction cocktail for 30 min at room temperature and stained with DAPI (50 ng/ml) at room temperature for 30 min. The images of stained cells were observed using a fluorescent microscope (Olympus Corporation, Tokyo, Japan; magnification, x120). Each sample was analyzed in triplicate in three independent experiments. The experiment was repeated three times.

Cell cycle detection. ASMCs (10^5 cell/well) were treated with 0.25% trypsin and seeded into 24-well plates at a density of $5x10^5$ cells/well. Following washing with PBS, cells were fixed with 75% ethanol at room temperature for 24 h, washed again with PBS and stained with 100 ng/ml propidium iodide for 30 min at room temperature (BD Biosciences, San Jose, CA, USA). Subsequently, cell cycle analysis was performed on FACSCalibur flow cytometer (BD Biosciences). Cell Quest software 1.1 (BD Biosciences) was used to calculate the percentage of cell population in each phase, including G_0/G_1, S and G_2/M.

Wound healing analysis. The ASMCs (10^5 cells/well) were incubated overnight following treatment with different ECM components until grown to ~90% confluence. On the following day, a standard 200 μl plastic filter tip was drawn across the well to produce a wound to evaluate the directional motility of cells. The wound areas were observed and images were captured at 0 and 48 h with a fluorescent microscope under bright field illumination (Axio Observer A1; Zeiss AG, Oberkochen, Germany; magnification, x4). The wound healing effect was determined by calculating the radio of the wound area to the total area using ImageJ bundled with Java 1.8.0_112 (National Institutes of Health, Bethesda, MD, USA). The degree of wound closure was analyzed in three randomly selected fields of view. The experiment was repeated three times.

Cell adhesion assay. Following different treatments, ASMCs were trypsinized and seeded at a density of $5x10^4$ cells/well on Matrigel-precoated 96-well culture plates. The cells were incubated for 2 h at 37˚C and non-adherent cells were removed by washing with PBS. Cells were fixed with 4% paraformaldehyde at 4˚C for 24 h, followed by 0.5% toluidine blue staining for 20 min at room temperature and 1% sodium dodecyl sulfate (2 h at room temperature). The optical density values were determined using a microplate reader (Bio-Rad Laboratories, Inc.) at a wavelength of 595 nm. The experiment was repeated two times.

ELISA. The supernatants were collected from ASMCs following treatment with different ECM components. The levels of total MMP-9, C-X-C motif chemokine ligand 1 (CXCL1), C-X-C motif chemokine ligand 8 (CXCL8) and interleukin-6 (IL-6) in the supernatants were measured using MMP-9 ELISA kit (cat. no. CSB-E08008r), CXCL1 ELISA kit (cat. no. CSB-E12997r), CXCL8 ELISA kit (cat. no. ml002885) and IL-6 ELISA kit (cat. no. CSB-E04640r), according to the manufacturer's protocols (all Cusabio Technology, LLC, College Park, MD, USA). The concentrations are expressed in pg/ml. The experiment was repeated three times.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from ASMCs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. The RT-qPCR reactions were performed using ABI7500 real-time RCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). SYBR-Green Master Mix kit and the following thermocycling conditions were used for qPCR: 30 cycles of 30 sec at 94˚C, 30 sec at 55˚C and 30 sec at 72˚C. The following primer sequences were used for PCR: MMP-9 forward, 5'-TCATCC AGTTTGGTGTCGC-3' and reverse, 5'-AGTGGGCATCTC CCTGAAT-3'; TIMP1 forward, 5'-GCCTCTGGCATCCTC TTG-3' and reverse, 5'-CTGCGGTTCTGGGACTTG-3'; transforming growth factor β-1 (TGFβ1) forward, 5'-AGG CGGTGCTCGCTTTG-3' and reverse, 5'-TGCGTTGTT GCGGTCCA-3'; fibroblast growth factor 1 (FGF-1) forward, 5'-GATGGCACAGTGGATGGGAC-3' and reverse, 5'-AAG CCCGTCGGTGTCCATGG-3'; and GAPDH forward, 5'-CCT CGTCTCATAGACAAGATGGT-3' and reverse, 5'-GGGTAG AGTCATACTGGAACATG-3'. The fold-change in the expression of each target mRNA relative to GAPDH was calculated using the $2^{-\Delta\Delta Cq}$ method (32).

Western blot analysis. All ASMCs (10^6) were lysed in 120 μl radioimmunoprecipitation assay lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) with phosphatase and protease inhibitors (BIOSS, Beijing, China) for 2 h at 4˚C. Following protein quantitation using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.), proteins (30 μg/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Following blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated at 4˚C overnight with primary antibodies, including anti-TGFβ1 (1:800; cat. no. bs-0086R; BIOSS), anti-FGF-1 (1:800; cat. no. 10-P1311-1; American Research Specialty Products; Palos Verdes Estates, CA, USA), anti-MMP-9 (1:800; cat. no. 10-P1357-1; American Research Specialty Products), anti-TIMP1 (1:800; cat. no. 28-60319P; American Research Specialty Products), anti-PI3K (1:800; cat. no. 05-212-K; Merck KGaA), anti-p (phosphorylated)-RAC-α serine/threonine-protein kinase (AKT; 1:800; cat. no. 28101-025; AnaSpec; Eurogentec, Liège, Belgium), anti-p-serine/threonine-protein kinase mTOR (mTOR; 1:800; cat. no. BS4706; Bioworld Technology, Inc., St. Louis Park, MN, USA), anti-AKT (1:800; cat. no. MA514916; Thermo Fisher Scientific, Inc.), anti-mTOR (1:800; cat. no. 17-18594; American Research Specialty Products) and anti-GAPDH (1:800; cat. no. sc-47724; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2,000; cat. no. A12000-1; Epigentek Group, Inc., Farmingdale, NY, USA) for 1 h at room temperature. The protein bands were detected using a

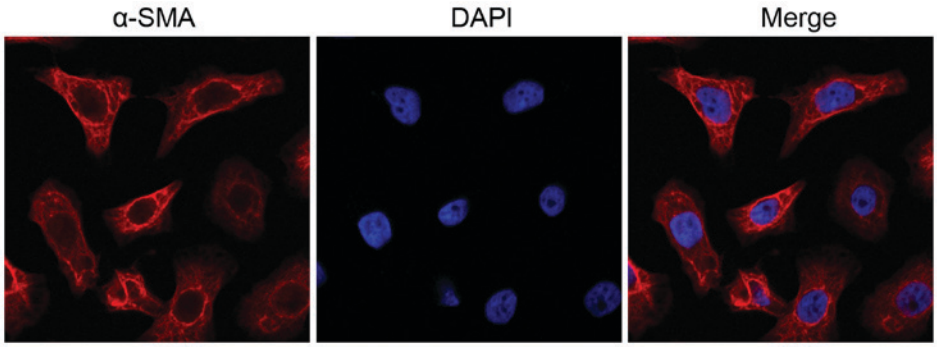

Figure 1. *In vitro* culture and identification of normal ASMCs. Immunofluorescence ASMCs were spindle-shaped and grew vigorously. The expression of α-SMA was demonstrated by red fluorescence within the ASMCs. DAPI was used to stain the nuclei of the cells (blue fluorescence). Magnification, x120. ASMCs, airway smooth muscle cells; α-SMA, α-smooth muscle actin.

Chemiluminescence Exposure Screen kit (cat. no. 62242; Pierce; Thermo Fisher Scientific, Inc.). The experiment was repeated three times.

Signal transduction pathways. COL-3-mediated adhesion and migration of ASMC was studied using specific pharmacological antagonists of PI3K. The PI3K inhibitor LY294002 (500 nM; cat. no. S1105; Selleck Chemicals, Houston, TX, USA) and activator insulin-like growth factor-1 (50 mM; IGF-1; Sigma-Aldrich; Merck KGaA) were added to the COPD ASMCs 30 min prior to the addition of COL-3 and remained in contact with the cells for the duration of the present study. The experiment was repeated three times.

Statistical analysis. All data are presented as the mean ± standard deviation. The differences between groups were analyzed using GraphPad Prism (version 7; GraphPad Software, Inc., La Jolla, CA, USA) using one-way analysis of variance with Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ECM components promote cell proliferation and cell cycle progression of ASMCs. ASMC were successfully isolated from normal and COPD rats. Following inoculation of ASMC into culture plates at 37˚C for 3 days, the cells presented spindle shape (round centered nuclei). Subsequently, the expression of α-SMA in normal and COPD rat ASMCs was determined by IF. The ASMCs exhibited abundant expression of α-SMA (Fig. 1) indicating that ASMCs were isolated in high purity. To determine the role of ECM components in proliferative kinetics of ASMCs, a CCK-8 assay was performed to determine cell proliferation ability of ASMCs. COL-1 and COL-3 significantly promoted proliferation of control and COPD ASMCs ($P<0.05$; Fig. 2A), while LN exhibited no significant effect on cell proliferation ability. The percentage of cells in S phase was significantly increased following treatment of normal and COPD ASMCs with COL-1 and COL-3 compared with the respective control groups ($P<0.05$ and $P<0.01$; Fig. 2B). Representative histograms of cell cycle distribution are presented in Fig. 2C. Promotion of cell cycle progression induced by ECM components was more significant in ASMCs derived from COPD rats compared with normal ASMCs.

ECM components promote migration and adhesion of ASMCs. Wound-healing assay was performed to determine the effect of ECM components on cell migration ability. Deceleration in the wound closure rate was observed in ASMCs derived from normal rats following treatment with ECM components compared with the control group ($P<0.01$, $P<0.001$; Fig. 3A and B). Treatment with COL-1 and COL-3 significantly elevated cell migration ability of ASMCs derived from rats with COPD. Furthermore, adhesion ability of ASMCs was assessed by cell adhesion assay. The results indicated that the adhesive ability of ASMCs was stronger in groups treated with ECM components compared with the control cells in normal ASMCs and COPD ASMCs ($P<0.05$ and $P<0.01$; Fig. 3C).

Effect of ECM components on factors associated with inflammation, growth and migration of ASMCs. It is widely accepted that inflammation serves a critical role in the pathogenesis of COPD (33,34). Expression levels of factors associated with inflammation were determined using ELISA. ECM components, including COL-1, LN and COL-3, altered the levels of CXCL1, CXCL8 and IL-6 in ASMCs derived from normal rats (Fig. 4A) and rat models of COPD (Fig. 4B). Expression of factors associated with growth and migration in ASMCs was also determined by RT-qPCR and western blotting assays. The results indicated that expression levels of TGFβ1 and FGF-1, both associated with cell growth, were significantly elevated following treatment with ECM components in ASMCs from normal rats and COPD model rats (Fig. 5). Consistent with the promoted cell migration ability, treatment with ECM components markedly decreased expression of MMP-9 and increased expression of TIMP1 in ASMCs derived from normal rats and rat models of COPD. The above data suggests that ECM may promote inflammation, proliferation and migration ability of ASMCs.

Effects of ECM components of ASMCs in rat models of COPD are mediated by the PI3K/AKT signaling pathway. PI3K is a signaling pathway associated with cell proliferation and migration (35,36). Based on the above results, which indicated that ECM components promoted migration and adhesion of ASMCs, the subsequent experiments aimed to determine whether PI3K signaling pathway mediated the effects of the ECM components. The above results indicated that COL-3 was more efficient at promoting cell proliferation and migration,

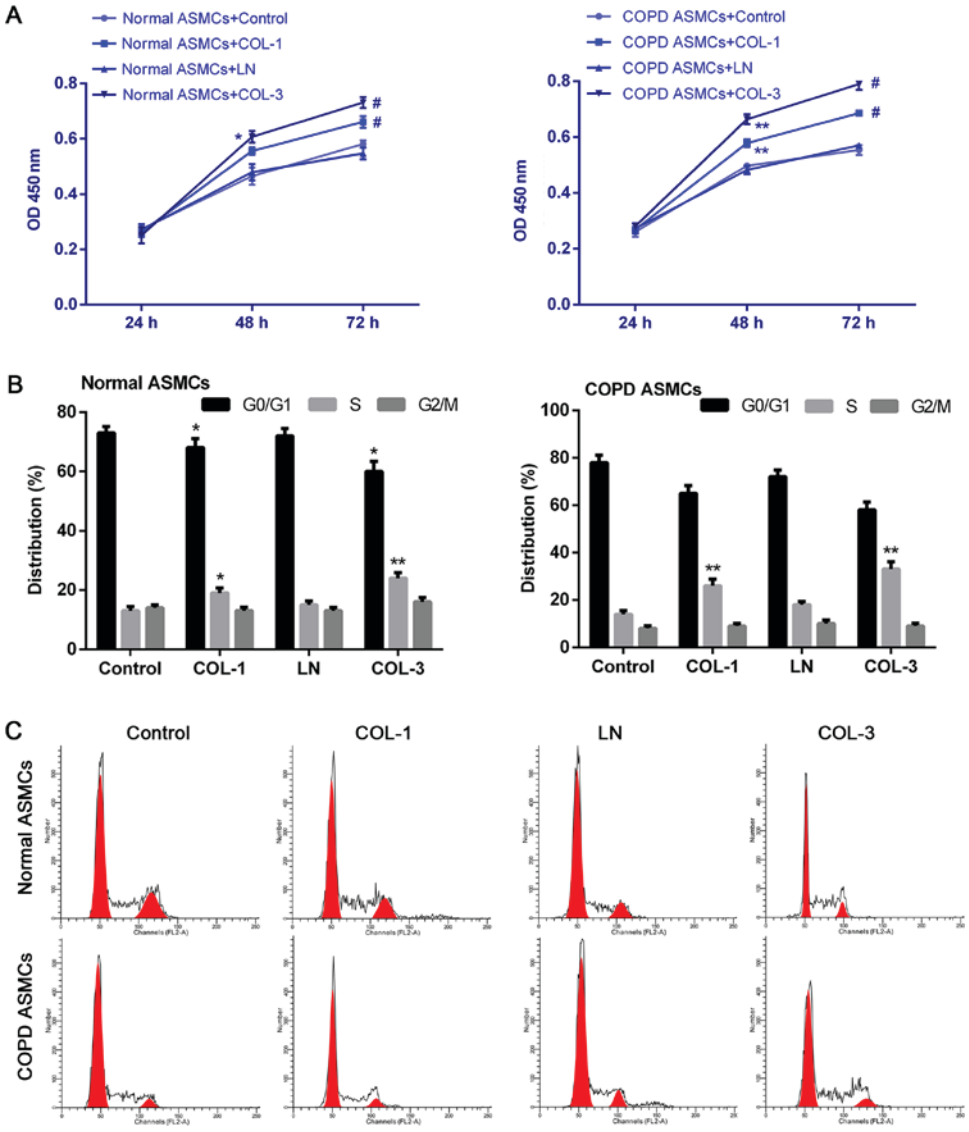

Figure 2. Effects of ECM components on proliferation and cell cycle progression of ASMCs. (A) Cell proliferation was determined in ASMCs from normal rats and rat models of COPD using Cell Counting kit-8 assay following treatment with ECM components, including COL-1, LN and COL-3. (B) Statistical analysis of cell cycle distribution of ASMCs from normal rats and rat models of COPD, following treatment with ECM components. (C) Flow cytometry was used to analyze cell cycle in ASMCs form normal rats and from rat models of COPD. Data are presented as the mean ± standard deviation in triplicate. *P<0.05 and **P<0.01 vs. the control group. $^{\#}$P<0.05 vs. the control group at 72 h time point. ECM, extracellular matrix; ASMCs, airway smooth muscle cells; COL, collagen; LN, laminin; OD, optical density; COPD, chronic obstructive pulmonary disease.

compared with COL-1 and LN. Therefore, COL-3-mediated promotion of adhesion and migration of ASMCs was studied using specific PI3K inhibitor LY294002 or activator IGF-1. As demonstrated in Fig. 6A, incubation of ASMCs form rat models of COPD with COL-3 and IGF-1 increased the proportion of EdU-positive cells, compared with cells incubated with COL-3 only, indicating enhanced cell proliferation. By contrast, incubation with COL-3 and LY294002 resulted in the opposite effect. In the cell cycle assay, treatment with IGF1 significantly reduced the percentage of cells in G_0/G_1 phase and increased the percentage of cells in S phase. The opposite was the case following incubation with LY294002 (Fig. 6B). Furthermore, cell migration ability was enhanced by incubation with IGF-1 and impaired following treatment with LY294002 in ASMCs derived from rat models of COPD (Fig. 7A and B; P<0.01). At the molecular level, western blot analysis confirmed that treatment with IGF1 markedly elevated the level of TGFβ1, FGF-1, TIMP1, PI3K, p-AKT and p-mTOR, and decreased the expression of MMP-9 (Fig. 7C). Consistently, treatment with LY294002 induced the opposite effects on these molecules. These results further demonstrated that the effects of ECM components on ASMCs are associated with the activation of the PI3K signaling pathway.

Discussion

COPD is a disorder typically caused by inhalation of toxic gases and particles, which leads to impairments of lung function, including emphysema, chronic bronchitis, mucus production and irreversible airway obstruction (37). Alteration of ECM deposition is a characteristic of COPD and provides a suitable environment for airway wall remodeling, especially airway wall thickening (18). The present study aimed to determine the response of ASMCs to the accumulation of

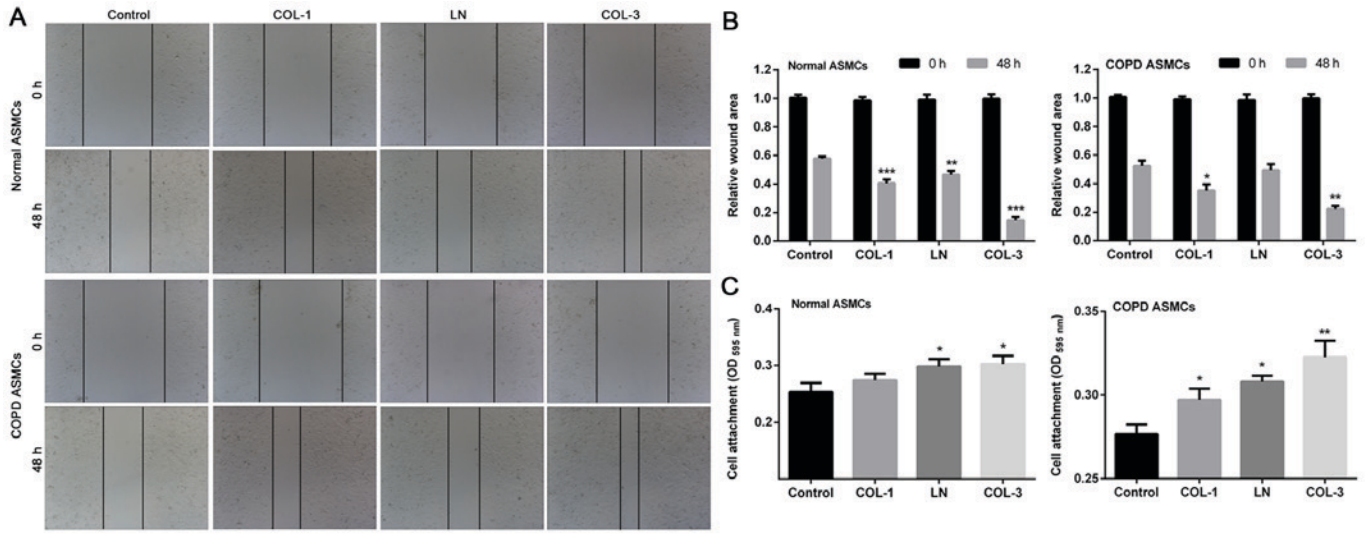

Figure 3. Effects of ECM components on migration and adhesion of ASMCs. Wound-healing assay was used to evaluate motility of ASMCs following treatments ECM components, including COL-1, LN and COL-3 in normal rat ASMCs and ASMCs form rat models of COPD. The wound area was (A) visualized and (B) calculated at 0 and 48 h. (C) Cell adhesion assay was performed in ASMCs following treatment with COL-1, LN and COL-3. Data are presented as the mean ± standard deviation in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. the control group. ECM, extracellular matrix; COL, collagen; LN, laminin; ASMCs, airway smooth muscle cells; COPD, chronic obstructive pulmonary disease; OD, optical density.

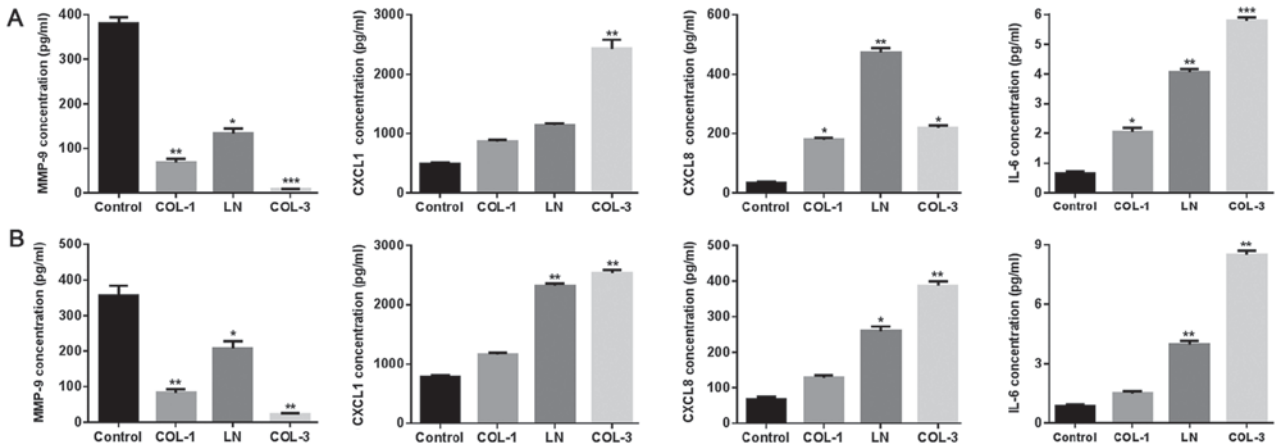

Figure 4. Effects of ECM components on cytokines in ASMCs. The expression of MMP-9, CXCL1, CXCL8 and IL-6 was determined following treatment with ECM components, including COL-1, LN and COL-3 in (A) normal ASMCs and (B) ASMCs from rat model of chronic obstructive pulmonary disease using ELISA assay. Data are presented as the mean ± standard deviation in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. the control group. ECM, extracellular matrix; ASMCs, airway smooth muscle cells; MMP-9, matrix metalloproteinase-9; COL, collagen; LN, laminin; CXCL, C-X-C motif chemokine ligand; IL-6, interleukin-6.

components of ECM, including COL-1, COL-3 or LN. COL-1 and COL-3 markedly increased proliferation of ASMCs, whereas no alterations were detected in cells treated with LN, indicating that the proliferative response of ASMCs varied depending exposure to different components of ECM. Consistently, the present study revealed similar results to previous studies; components of ECM differentially affected proliferation of vascular smooth muscle cells in cell culture studies (38,39). Following treatment with COL-1 and COL-3, there was an increased percentage of ASMCs in the S phase. Migration of cells is controlled by multiple stages, including extension of the lamellipodium, formation of new adhesions and retraction of protrusions (40). Correct modulation of adhesion and de-adhesion are necessary for cell migration (40). In the present study, migratory responses to treatment with ECM substrates were estimated using a wound-healing assay. Culture dishes treated with COL-1, COL-3 or LN exhibited relatively decreased wound area in ASMCs from normal rats and rat models of COPD, compared with the respective controls. The data suggests that these three ECM components promote migration of ASMCs in culture. Compared with the control, ASMCs from normal rats and the COPD model exhibited enhanced attachment following stimulation with COL-1, LN or COL-3, compared with the control. Increased cell adhesion may contribute to migration of ASMCs induced by treatment with COL-1, COL-3 or LN.

COPD results in aberrant thickening of generalized airway walls, particularly in smokers, with airway walls thickened throughout the lung (41). Hyperplasia and hypertrophy of the smooth muscle are associated with the pathology of COPD (42). Induction of proliferation, migration and attachment by ECM components in the present study lead to investigation of the

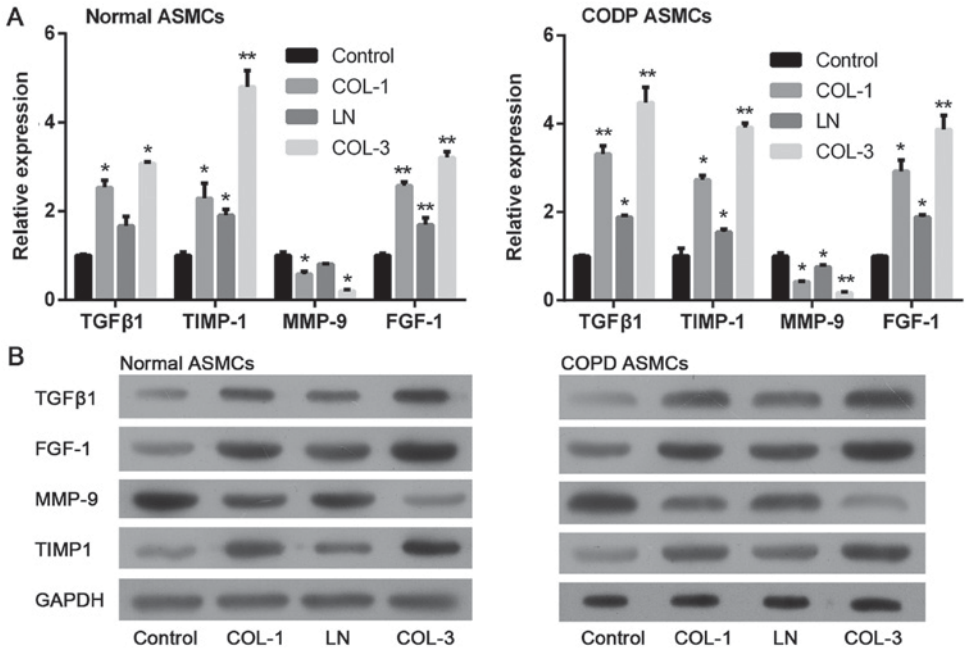

Figure 5. Effects of ECM components on the downstream molecules in ASMCs. Relative expression of TGFβ1, FGF-1, MMP-9 and TIMP1 was determined in ASMCs following treatment with ECM components, including COL-1, LN and COL-3 by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blotting. Data are presented as the mean ± standard deviation in triplicate. *P<0.05 and **P<0.01 vs. the control group. ECM, extracellular matrix; ASMCs, airway smooth muscle cells; COL, collagen; LN, laminin; TGFβ1, transforming growth factor β-1; FGF-1, fibroblast growth factor-1; MMP-9, matrix metalloproteinase-9; TIMP1, metalloproteinase inhibitor 1; COPD, chronic obstructive pulmonary disease.

effects on cytokines, growth factors, MMPs, TIMPs and chemokines synthesized by ASMCs. The level of MMP-9 was downregulated, while the concentration of its inhibitor TIMP1 was upregulated in ASMCs from normal rats and the COPD model rats following treatment with the ECM molecules. Previous studies demonstrated that the activated and precursor forms of MMP-9 may bind to its inhibitor TIMP-1 to form a complex, thus the ratio of MMP-9 and TIMP-1 is closely associated with the degradation and accumulation ECM (43). An increased TIMP-1/MMP-9 ratio facilitates TGFβ1 induced synthesis of ECM substrates, including COL-1, COL-2, COL-5 and fibronectin in human ASMCs. Furthermore, TGFβ has been demonstrated to promote expression of certain other ECM proteins, including perlecan, elastin, LN, COL-3, COL-4 and thrombospondin (18,44). In the present study, mRNA and protein expression levels of TGFβ1 were evaluated following treatment with COL-1, COL-3 or LN, thus it was suggested that TGFβ1 induced over production of multiple ECM proteins in ASMCs in COPD rats. The results of the present study suggest that there may be an association between ECM and TGFβ1 in rats. Cell proliferation and migration are promoted by ECM proteins (12). In the cells treated with COL-1, COL-3 and LN, an upregulation of TGFβ1 and alteration of ECM was identified, which may result in ASMC hyperplasia and migration.

It has been previously reported that cytokines, chemokines and growth factors may influence the secretion of ECM proteins by ASMCs and therefore impact biological behavior of ASMCs (12). ECM proteins are considered to promote production of cytokines and chemokines by ASMCs, which are associated with the development of COPD pathophysiology (12). In the present study, the pro-inflammatory chemokines CXCL1 and CXCL8, cytokine IL-6, and FGF-1 were increased in ASMCs by treatment with ECM molecules. The imbalance in ECM homeostasis causes hyper proliferative and migratory phenotypes in ASMCs.

PI3K is a signaling pathway associated with proliferation and migration of ASMCs (45,46). Incubation of rat ASMCs with PI3K agonist IGF-1 induced progression to S-phase and enhanced migration, whereas the opposite effect was observed following treatment with PI3K inhibitor LY294002. Protein expression levels of PI3K, p-AKT and p-mTOR, which have roles in PI3K signaling, were upregulated by treatment with IGF-1 and decreased following treatment with LY294002. These results indicate that activation of the PI3K signaling promotes proliferation and migration of ASMCs. Additionally, protein expression levels of TGFβ1, FGF-1 and TIMP1 were upregulated by treatment of IGF-1, while the effect was reversed by treatment with LY294002. Stewart *et al* (47) demonstrated that FGFs can stimulate the PI3K signaling pathway to induce proliferation of ASMCs in COPD. In addition, TGF and collagen proteins also regulate the PI3K signaling pathway in human ASMCs (48). Following treatment with IGF-1, alterations in the levels of TGFβ1, FGF-1, MMP-9 and TIMP1 may result in ECM imbalance, and the PI3K signaling may be activated by TGFβ1 and FGF-1, leading to induction of proliferation and migration.

In conclusion, the present study demonstrated that COL-1, COL-3 and LN are effective stimulators of proliferation, migration and attachment of rat AMSCs. Treatment with ECM components enhanced the expression of factors associated with inflammation, growth and migration. COL-3 induced proliferation, migration and progression to S-phase in ASMCs, partially though activation of the PI3K signaling pathway. The present study may contribute to further understanding of the molecular mechanisms underlying the COPD airway wall thickening.

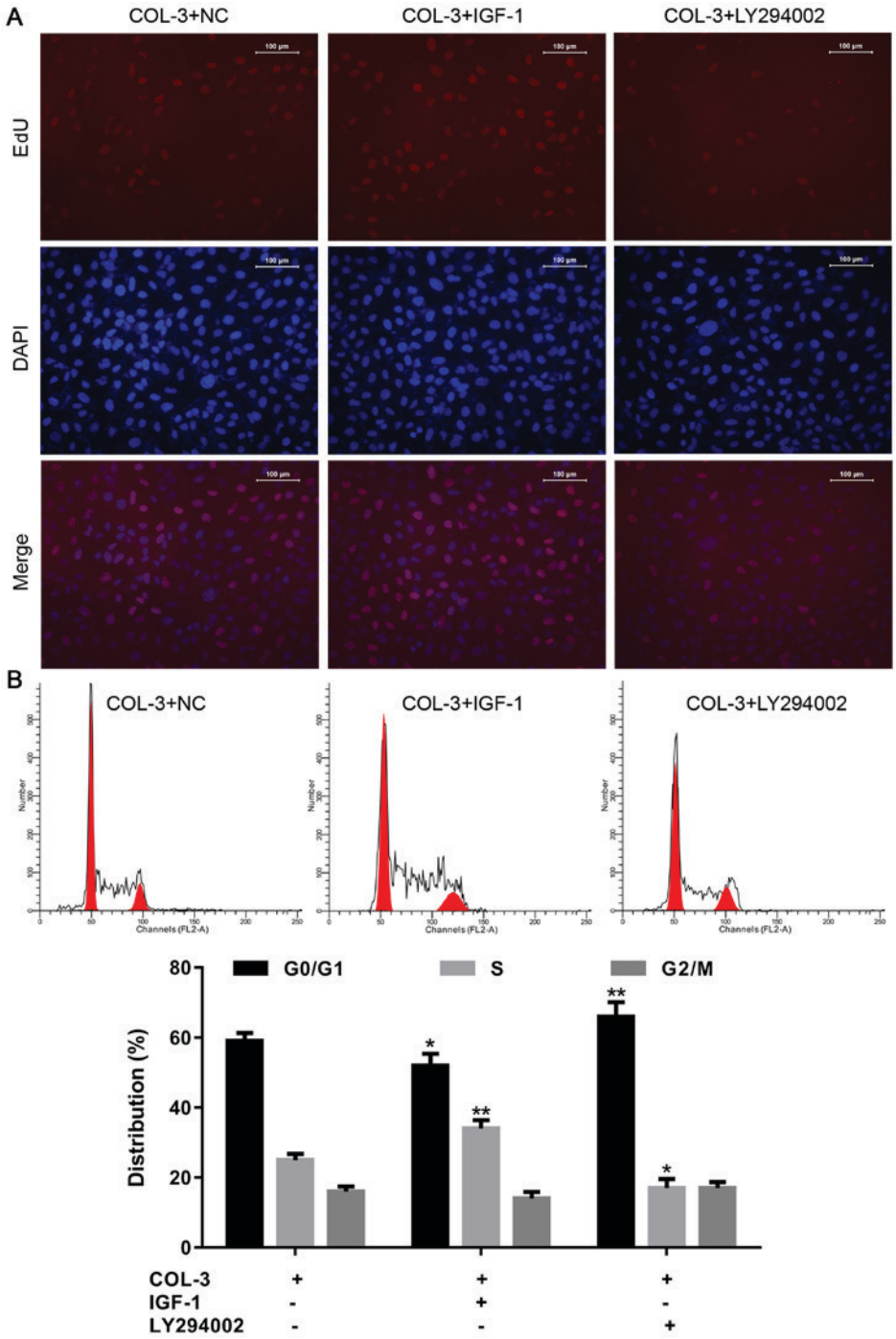

Figure 6. Effects of PI3K/AKT signaling pathway on proliferation and cell cycle progression of ASMCs from rat models of chronic obstructive pulmonary disease. (A) Representative images of ASMCs following treatment with COL-3 and PI3K inhibitor LY294002 or activator IGF1 following double staining with EdU (red) and DAPI (blue) under a fluorescent microscope. (B) Cell cycle distribution analysis was performed in ASMCs following treatment with COL-3 and PI3K inhibitor LY294002 or activator IGF-1. Data are presented as the mean ± standard deviation in triplicate. *P<0.05 and **P<0.01 vs. the control group. PI3K, phosphoinositide 3-kinase; ASMCs, airway smooth muscle cells; COL, collagen; NC, negative control (PBS); IGF-1, insulin-like growth factor I; EdU, 5-ethynyl-2'-deoxyuridine.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

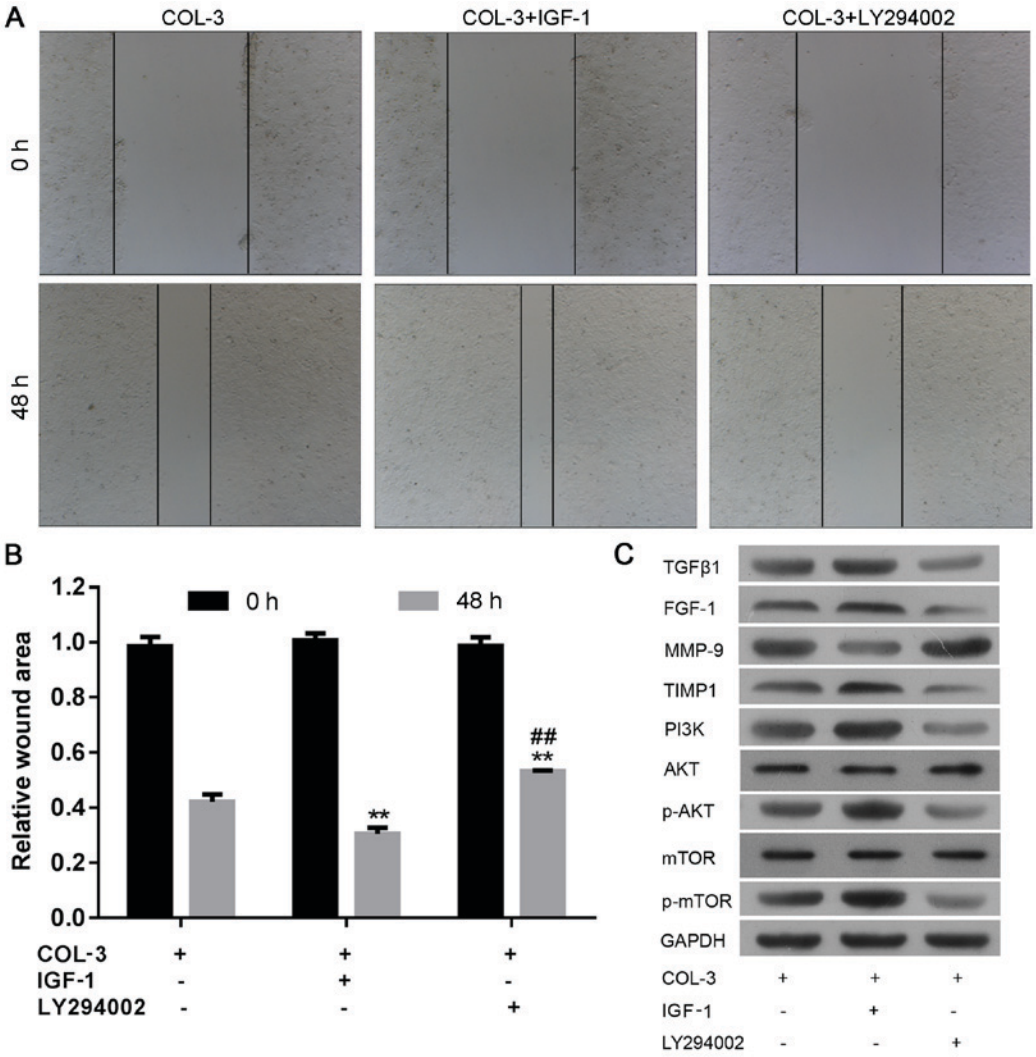

Figure 7. Effects of extracellular matrix components of ASMCs in rat models of chronic obstructive pulmonary disease are mediated by the PI3K/AKT signaling pathway. The wound area was (A) visualized and (B) calculated to evaluate motility of ASMCs following combined treatment with COL-3 and PI3K inhibitor LY294002 or activator IGF1. (C) Western blot analysis of components of the PI3K/AKT signaling pathway in ASMCs following combined treatment with COL-3 and PI3K inhibitor LY294002 or activator IGF1. Data are presented as the mean ± standard deviation in triplicate. **P<0.01 vs. the control group and $^{\#\#}$P<0.01 vs. the IGF1 treatment group. ASMCs, airway smooth muscle cells; COL, collagen; IGF-1, insulin-like growth factor-1; TGFβ1, transforming growth factor β-1; FGF-1, fibroblast growth factor-1; MMP-9, matrix metalloproteinase-9; TIMP1, tissue inhibitors of metalloproteinase-1; PI3K, phosphoinositide 3-kinase; p-, phospho-; mTOR, serine/threonine-protein kinase mTOR.

Authors' contributions

ZW designed the experiment, performed the statistical analysis and interpretation of data and wrote the manuscript. RL and RZ conducted the experiments and made substantial contributions to conception and design, and acquisition of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Research Committee of Hubei University of Medicine (Suizhou, China). The care and use of animals was based on the Guidelines of the National Institutes of Health.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Asia Pacific CRG: Global Initiative for Chronic Obstructive Lung Disease strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease: An Asia-Pacific perspective. Respirology 10: 9-17, 2005.
2. GBD 2015 Chronic Respiratory Disease Collaborators: Global, regional, and national deaths, prevalence, disability-adjusted life years, and years lived with disability for chronic obstructive pulmonary disease and asthma, 1990-2015: A systematic analysis for the Global Burden of Disease Study 2015. Lancet. Respir Med 5: 691-706, 2017.
3. Celli BR, MacNee W and Force AET: Standards for the diagnosis and treatment of patients with COPD: A summary of the ATS/ERS position paper. Eur Respir J 23: 932-946, 2004.
4. Agusti A, MacNee W, Donaldson K and Cosio M: Hypothesis: Does COPD have an autoimmune component? Thorax 58: 832-834, 2003.

5. van Eeden SF and Sin DD: Oxidative stress in chronic obstructive pulmonary disease: A lung and systemic process. Can Respir J 20: 27-29, 2013.
6. Saetta M, Di Stefano A, Turato G, Facchini FM, Corbino L, Mapp CE, Maestrelli P, Ciaccia A and Fabbri LM: CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 157: 822-826, 1998.
7. Peinado VI, Barbera JA, Abate P, Ramírez J, Roca J, Santos S and Rodriguez-Roisin R: Inflammatory reaction in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease. Am J Respir Crit Care Med 159: 1605-1611, 1999.
8. Celli BR: Pathophysiology of chronic obstructive pulmonary disease. Respir Care Clin N Am 4: 359-370, 1998.
9. Aoshiba K and Nagai A: Differences in airway remodeling between asthma and chronic obstructive pulmonary disease. Clin Rev Allergy Immunol 27: 35-43, 2004.
10. Chung KF: The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease. Proc Am Thorac Soc 2: 347-354, 2005.
11. Ge Q, Chen L, Jaffar J, Argraves WS, Twal WO, Hansbro P, Black JL, Burgess JK and Oliver B: Fibulin1C peptide induces cell attachment and extracellular matrix deposition in lung fibroblasts. Sci Rep 5: 9496, 2015.
12. Parameswaran K, Willems-Widyastuti A, Alagappan VK, Radford K, Kranenburg AR and Sharma HS: Role of extracellular matrix and its regulators in human airway smooth muscle biology. Cell Biochem Biophys 44: 139-146, 2006.
13. Merrilees MJ, Ching PS, Beaumont B, Hinek A, Wight TN and Black PN: Changes in elastin, elastin binding protein and versican in alveoli in chronic obstructive pulmonary disease. Respir Res 9: 41, 2008.
14. van Straaten JF, Coers W, Noordhoek JA, Huitema S, Flipsen JT, Kauffman HF, Timens W and Postma DS: Proteoglycan changes in the extracellular matrix of lung tissue from patients with pulmonary emphysema. Modern Pathol 12: 697-705, 1999.
15. Vlahovic G, Russell ML, Mercer RR and Crapo JD: Cellular and connective tissue changes in alveolar septal walls in emphysema. Am J Respir Crit Care Med 160: 2086-2092, 1999.
16. Annoni R, Lancas T, Yukimatsu Tanigawa R, de Medeiros Matsushita M, de Morais Fernezlian S, Bruno A, Fernando Ferraz da Silva L, Roughley PJ, Battaglia S, Dolhnikoff M, *et al*: Extracellular matrix composition in COPD. Eur Respir J 40: 1362-1373, 2012.
17. Johansson O, Erjefält J, Bjermer L, Löfdahl C-G, Westergren-Thorsson G and Hallgren O: Aberrant intracellular expression of laminin α-2 and -5 in bronchiolar epithelium of COPD patients. Eur Respir J 14: 3955, 2014.
18. Johnson PR, Black JL, Carlin S, Ge Q and Underwood PA: The production of extracellular matrix proteins by human passively sensitized airway smooth-muscle cells in culture: The effect of beclomethasone. Am J Respir Crit Care Med 162: 2145-2151, 2000.
19. Elshaw SR, Henderson N, Knox AJ, Watson SA, Buttle DJ and Johnson SR: Matrix metalloproteinase expression and activity in human airway smooth muscle cells. Br J Pharmacol 142: 1318-1324, 2004.
20. McKay S, de Jongste JC, Saxena PR and Sharma HS: Angiotensin II induces hypertrophy of human airway smooth muscle cells: Expression of transcription factors and transforming growth factor-beta1. Am J Respir Cell Mol Biol 18: 823-833, 1998.
21. Berezin V, Walmod PS, Filippov M and Dityatev A: Targeting of ECM molecules and their metabolizing enzymes and receptors for the treatment of CNS diseases. Prog Brain Res 214: 353-388, 2014.
22. Hinderer S, Layland SL and Schenke-Layland K: ECM and ECM-like materials-Biomaterials for applications in regenerative medicine and cancer therapy. Adv Drug Deliv Rev 97: 260-269, 2016.
23. Trapani V, Bonaldo P and Corallo D: Role of the ECM in notochord formation, function and disease. J Cell Sci 130: 3203-3211, 2017.
24. Khatiwala CB, Kim PD, Peyton SR and Putnam AJ: ECM compliance regulates osteogenesis by influencing MAPK signaling downstream of RhoA and ROCK. J Bone Miner Res 24: 886-898, 2009.
25. Mruthyunjaya S, Manchanda R, Godbole R, Pujari R, Shiras A and Shastry P: Laminin-1 induces neurite outgrowth in human mesenchymal stem cells in serum/differentiation factors-free conditions through activation of FAK-MEK/ERK signaling pathways. Biochem Biophys Res Commun 391: 43-48, 2010.
26. Nie YC, Wu H, Li PB, Luo YL, Zhang CC, Shen JG and Su WW: Characteristic comparison of three rat models induced by cigarette smoke or combined with LPS: To establish a suitable model for study of airway mucus hypersecretion in chronic obstructive pulmonary disease. Pulm Pharmacol Ther 25: 349-356, 2012.
27. Geng L, Chen Z, Ren H, Niu X, Yu X and Yan H: Effects of an early intervention using human amniotic epithelial cells in a COPD rat model. Pathol Res Pract 212: 1027-1033, 2016.
28. Zhang C, Feng L, Li M, Dong C and Zhang W: Effects of Xiaoqinglong decoction on gene expression profiles in a rat chronic obstructive pulmonary disease model. Biosci Trends 6: 262-269, 2012.
29. Placeres-Uray FA, Febres-Aldana CA, Fernandez-Ruiz R, Gonzalez de Alfonzo R, Lippo de Becemberg IA and Alfonzo MJ: M2 muscarinic acetylcholine receptor modulates rat airway smooth muscle cell proliferation. World Allergy Organ J 6: 22, 2013.
30. Durand-Arczynska W, Marmy N and Durand J: Caldesmon, calponin and alpha-smooth muscle actin expression in subcultured smooth muscle cells from human airways. Histochemistry 100: 465-471, 1993.
31. He F, Li B, Zhao Z, Zhou Y, Hu G, Zou W, Hong W, Zou Y, Jiang C, Zhao D and Ran P: The pro-proliferative effects of nicotine and its underlying mechanism on rat airway smooth muscle cells. PloS One 9: e93508, 2014.
32. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
33. Hakansson K, Konge L, Thomsen SF, Backer V and von Buchwald C: Sinonasal inflammation in COPD: A systematic review. Eur Res J 42: 1402-1411, 2013.
34. Loukides S, Bartziokas K, Vestbo J and Singh D: Novel anti-inflammatory agents in COPD: Targeting lung and systemic inflammation. Curr Drug Targets 14: 235-245, 2013.
35. Wang Y, Wan D, Zhou R, Zhong W, Lu S and Chai Y: Geraniin inhibits migration and invasion of human osteosarcoma cancer cells through regulation of PI3K/Akt and ERK1/2 signaling pathways. Anticancer dsrugs 2017.
36. Li M, Cheng W, Luo J, Hu X, Nie T, Lai H, Zheng X, Li F and Li H: Loss of selenocysteine insertion sequence binding protein 2 suppresses the proliferation, migration/invasion and hormone secretion of human trophoblast cells via the PI3K/Akt and ERK signaling pathway. Placenta 55: 81-89, 2017.
37. Wood AM and Stockley RA: The genetics of chronic obstructive pulmonary disease. Respir Res 7: 130, 2006.
38. Yamamoto M, Yamamoto K and Noumura T: Type I collagen promotes modulation of cultured rabbit arterial smooth muscle cells from a contractile to a synthetic phenotype. Exp Cell Res 204: 121-129, 1993.
39. Thyberg J and Hultgardh-Nilsson A: Fibronectin and the basement membrane components laminin and collagen type IV influence the phenotypic properties of subcultured rat aortic smooth muscle cells differently. Cell Tissue Res 276: 263-271, 1994.
40. Huynh-Do U, Vindis C, Liu H, Cerretti DP, McGrew JT, Enriquez M, Chen J and Daniel TO: Ephrin-B1 transduces signals to activate integrin-mediated migration, attachment and angiogenesis. J Cell Sci 115: 3073-3081, 2002.
41. Hashimoto M, Tanaka H and Abe S: Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. Chest 127: 965-972, 2005.
42. Hirst SJ, Twort CH and Lee TH: Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. Am J Respir Cell Mol Biol 23: 335-344, 2000.
43. Roderfeld M, Graf J, Giese B, Salguero-Palacios R, Tschuschner A, Müller-Newen G and Roeb E: Latent MMP-9 is bound to TIMP-1 before secretion. Biol Chem 388: 1227-1234, 2007.
44. Black PN, Young PG and Skinner SJ: Response of airway smooth muscle cells to TGF-beta 1: Effects on growth and synthesis of glycosaminoglycans. Am J Physiol 271: L910-917, 1996.
45. Liu Y, Yang K, Sun X, Fang P, Shi H, Xu J, Xie M and Li M.: MiR-138 suppresses airway smooth muscle cell proliferation through the PI3K/AKT signaling pathway by targeting PDK1. Exp Lung Res 41: 363-369, 2015.
46. Goueffic Y, Guilluy C, Guerin P, Patra P, Pacaud P and Loirand G: Hyaluronan induces vascular smooth muscle cell migration through RHAMM-mediated PI3K-dependent Rac activation. Cardiovascular Res 72: 339-348, 2006.
47. Stewart DM, Tian L and Nelson DL: Linking cellular activation to cytoskeletal reorganization: Wiskott-Aldrich syndrome as a model. Curr Opin Allergy Clin Immunol 1: 525-533, 2001.
48. Stamatiou R, Paraskeva E, Gourgoulianis K, Molyvdas PA and Hatziefthimiou A: Cytokines and growth factors promote airway smooth muscle cell proliferation. ISRN Inflamm 2012: 731472, 2012.