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MSCs Relieve Lung Injury of COPD Mice through Promoting Proliferation of Endogenous Lung Stem Cells*

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Summary: Bone marrow mesenchymal stem cells (MSCs) transplantation could repair injury tissue, but no study confirms whether MSCs can promote the proliferation of endogenous lung stem cells to repair alveolar epithelial cells of mice with chronic obstructive pulmonary disease (COPD). This study was designed to investigate the effect of MSCs on the proliferation of endogenous lung stem cells in COPD mice to confirm the repair mechanism of MSCs. The mice were divided into control group, COPD group, and COPD+MSCs group. The following indexes were detected: HE staining of lung tissue, the mean linear intercept (MLI) and alveolar destructive index (DI), the total cell number in bronchoalveolar lavage fluid (BALF), pulmonary function, alveolar wall apoptosis index (AI) and proliferation index (PI), the number of $CD45^-/CD31^-/Sca\text{-}1^+$ cells by flow cytometry (FCM), and the number of bronchoalveolar stem cells (BASCs) in bronchoalveolar duct junction (BADJ) by immunofluorescence. As compared with control group, the number of inflammatory cells in lung tissue was increased, alveolar septa was destroyed and the emphysema-like changes were seen, and the changes of lung function were in line with COPD in COPD group; AI of alveolar wall was significantly increased and PI significantly decreased in COPD group. There was no significant difference in the number of $CD45^-/CD31^-/Sca\text{-}1^+$ cells and BASCs between control group and COPD group. As compared with COPD group, the number of inflammatory cells in BALF was decreased, the number of $CD45^-/CD31^-/Sca\text{-}1^+$ cells and BASCs was increased, AI of alveolar wall was decreased and PI was increased, and emphysema-like changes were relieved in COPD+MSCs group. These findings suggested that MSCs transplantation can relieve lung injury by promoting proliferation of endogenous lung stem cells in the cigarette smoke-induced COPD mice.
Key words: mesenchymal stem cells; chronic obstructive pulmonary disease; bronchoalveolar stem cells

Chronic obstructive pulmonary disease (COPD) involves chronic inflammation of the lung, particularly in peripheral airways and parenchyma, characterized by airflow limitation. COPD is a common chronic respiratory disease and currently ranked the sixth cause of morbidity and mortality in the worldwide, and is expected to be ranked the third in 2020[1]. COPD is caused by exposure to harmful gases or particles, especially cigarette smoke. Pathogenic mechanisms include inflammation/anti-inflammation, protease/anti-protease, oxidant/antioxidant and apoptosis/proliferation imbalance. The initial treatment for COPD is to stop smoking, but the inflammation and oxidative stress persist in lung tissue[2, 3]. Current major drug therapies of COPD are using bronchodilator, corticosteroids, and theophylline to reduce airway obstruction, limit acute exacerbations of COPD and improve the quality of life[4]. However, the current treatment could not prevent the progress of COPD and reduce mortality. Therefore it is important to establish an alternative for treating COPD[5].

Many researches have shown mesenchymal stem cells (MSCs) transplantation could repair tissue injury[6]. After MSCs transplantation through the intravenous manner or airway, MSCs are deposited more significantly in the lung tissue than in other tissues. However, the MSCs in the injured tissue are re-differentiated poorly, and they functions mainly through the paracrine mechanism, immunomodulation and anti-apoptosis[6]. Currently, MSCs have been widely used in various tissue injuries, such as acute lung injury, bacterial pneumonia, asthma, bronchiolitis obliterans, bronchopulmonary dysplasia, COPD, pulmonary hypertension, pulmonary ischemia-reperfusion injury, obstructive sleep apnea syndrome, radiation-induced lung injury, sepsis and burns, autoimmune lung injury. The transplantation of allograft MSCs in clinical trials is verified to be safe and feasible[7–10].

Various types of endogenous lung stem/progenitor cells (LSPCs) exist in the airway and lung, and have self-renewal capability and can differentiate into mature cells of lung tissue under certain conditions. After lung injury, endogenous lung stem cells supplement damage cells locally, but their proliferative potential is low and the repair efficiency is insufficient. Subsequently, exogenous stem cells go into the lung tissue and further repair the injured tissue. For different lung damage by different causes at different locations, the repair types

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and mechanisms by endogenous lung stem cells are different[11−13]. COPD is a chronic inflammation disease of the airway and lung parenchyma induced by many causes, and alveolar structure is progressively injured. Cigarette smoke-induced COPD model has revealed no proliferation of endogenous lung stem cells to repair the lung injury. Recent study has shown that MSCs transplantation in myocardial injury model can stimulate the proliferation of endogenous cardiomyocyte stem cells and differentiation into cardiomyocytes, thereby improving cardiac function[14]. The study of bronchopulmonary dysplasia model also has shown the ability of MSCs to promote the proliferation of bronchoalveolar stem cells (BASCs)[15]. MSCs have roles in anti-inflammation, anti-oxidation, and anti-apoptosis[16] in COPD, but no study confirms whether MSCs can promote the proliferation of endogenous lung stem cells to repair alveolar epithelial cells of COPD.

There are many endogenous stem cells in the terminal airways, including variation Clara cells distributed in the bronchioles, BASCs in bronchoalveolar duct junction (BADJ), and type 2 alveolar epithelial cells (AT Ⅱ) in alveoli[17]. BASCs are considered pluripotent stem cells, and capable of withstanding the damage from bronchioles and alveoli and proliferation in the epithelial regeneration process[18], and differentiating into Clara cells, AT Ⅱ and AT Ⅰ. Kim found that 85% of $CD45^-CD31^-/Sca\text{-}1^+$ cells in the lung detected by flow cytometry were BASCs, containing small number of AT Ⅱ and Clara cells at the same time[19]. BASCs express Clara cell surface marker CC10 and AT Ⅱ surface marker SPC, simultaneously. This study measured the number of $CD45^-CD31^-/Sca\text{-}1^+$ cells and BASCs in lung of COPD mice after MSCs transplantation, to verify whether MSCs transplantation can promote the proliferation of endogenous lung stem cells in a mouse COPD model.

1 MATERIALS AND METHODS

1.1 Isolation and Expansion of Mouse Bone Marrow MSCs

C57/B6 mice bone marrow MSCs were isolated by the method previously described[20]. MSCs of passage 2 in a density of 4×10^6 cells/mL were used in the transplantation experiments.

1.2 Establishment of COPD Model and Bone Marrow MSCs Transplantation

All animal procedures and protocols were reviewed and approved by the Institution Animal Care and Use Committee. The COPD model was established by cigarette smoke exposure method[21]. Eighteen 6-week-old female C57/B6 mice (Beijing Vital River Laboratory Animal Co., Ltd., China) were randomly divided into three groups: control group, COPD group, COPD+MSCs group (n=6 each). The animals in COPD group and COPD+MSCs group were exposed to cigarette smoke for 12 weeks (cigarette smoke exposure: 5 min/time, 3 times/day, and 6 days a week). The mice in COPD+MSCs group was injected with MSCs 50 μL (total cell number: 4×10^6) via the tail vein from 5 to 12 weeks of cigarette smoke exposure at the 7th day every week. The mice in COPD group were injected with PBS 50 μL. The mice were sacrificed after 14 weeks.

1.3 BALF Collection and Lung Tissue Treatment

The mouse thoracic cavity was opened and the right lobe was ligated. 0.3 mL 0.9% saline was injected into the left lung via the tube, and BALF was gently retracted, three times. The cells in BALF were classified and counted. The right upper lobe was soaked in 4% paraformaldehyde solution, fixed for 2 h, embedded in paraffin, and sectioned. The rest right lobe was stored at −70°C for later use.

1.4 Morphologic Analysis of Lungs

Sections were processed for staining with hematoxylin and eosin (HE). The emphysematous extent was assessed by the mean linear intercept (MLI) and alveolar destructive index (DI). Two slices were selected in each specimen, 5 fields were selected in each slice, and bronchia and large vessels were avoided. MLI was obtained by dividing the total length of all lines in the frames counted by the total number of intercepts encountered in the counted lines, to indicate the average inner diameter of the alveoli[22]. The DI was calculated according to the method previously described[23]. Each counting was performed by two independent observers who were blinded to the subjects' profiles.

1.5 Pulmonary Function Tests

The lung function of mice was measured by PLY3211 small animal spirometer (Buxco Electronics, Inc., USA). Airway resistance (Raw), dynamic lung compliance (Cdyn) and peak expiratory flow (PEF) were recorded.

1.6 Measurement of Alveolar Wall Cell Apoptosis

Paraffin sections were stained with TUNEL method for detection of alveolar wall cell apoptosis. TUNEL was performed with commercially available kit (Roche, USA) following the manufacturer's instructions. The percentage of TUNEL-positive cells was calculated by dividing the number of TUNEL-positive cells by the total cell number in 10 fields at 400× magnification randomly sampled in 5 sections for each mouse. Apoptosis index (AI)=The number of apoptosis cells/The number of total cells×100%.

1.7 Measurement of Alveolar Wall Cell Proliferation

Paraffin sections were deparaffinized and added with PCNA antibody (1:100, Beijing Zhongshan Biotechnology Co., Ltd., China) at 4°C overnight, then biotin-labeled anti-mouse IgG (1:100 dilution), incubated at 37°C for 15 min, streptavidin affinity biotin peroxidase complex (1:100 dilution) incubated at 37°C for 15 min. The slides were immersed in diaminobenzidine (DAB, Vector Laboratories, USA) solution and counterstained with hematoxylin. PBS instead of primary antibody served as control. The cell nuclei were colored by PCNA. The proliferation index (PI)=The number of proliferation cells/The number of total cells×100%.

1.8 Flow Cytometry

The right lung tissue of mice was mixed with 3 mL Dispasel, filtrated by 40 μm filter, then cell suspension was obtained by washing with DMEM, HEPES (GibcoCo., USA) and DNasel (Invitrogen, USA). The cells were resuspended and counted after 1600 r/min centrifugation at 4°C, then adjusted to a density of 1×10^6 cells/100 μL with buffer. The cell suspension was mixed with $CD45^-$PE (1:1000), $CD31^-$PE (1:200) and

Sca-1⁻FITC (1:200, BD, USA) in dark at 4°C for 30 min, centrifuged and resuspended, then LSRH flow cytometer (BD, USA) was done for cell analysis.

1.9 Immunofluorescence Staining for BASCs

The primary antibodies were used with rabbit anti-mouse CCSP antibody (1:1000, Abcam, USA), SP-C antibody (1:250, sc-7705, Santa Cruz, USA). All fluorescent staining was performed with appropriate secondary antibodies, anti-rabbit 555 (1:200, Abcam, USA), anti-goat FITC (for SP-C, JacksonImmunoResearch, USA), nuclear staining with DAPI (Invitrogen, USA). BASCs are double positive cells which were stained with CCSP and SPC. The positive cells of BASCs were counted at least in 5 BADJs.

1.10 Statistical Analysis

All values were expressed as $x\pm s$. Statistical analyses were performed by using analysis of variance (ANOVA) with GraphPad Prism 5.0 (GraphPad, USA). $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Effects of MSCs Transplantation on Emphysematous Changes in COPD Mice

HE staining of lung tissue in control group showed integrated airway and alveolar epithelium, and alveolar morphology was uniform. Some bronchial epithelial cells were shed in COPD group, accompanied with inflammatory cell infiltration in the bronchial and lung tissues, alveolar wall thinning, alveolar septum fracture and emphysematous changes. As compared with COPD group, inflammatory cell infiltration and emphysematous changes were significantly ameliorated in COPD+MSCs group (fig. 1A–1F). The MLI and DI were significantly higher in COPD group and COPD+MSCs group than in control group with the difference being statistically significant ($P<0.01$). However, the MLI and DI were significantly reduced in COPD+MSCs group when compared with COPD group with the difference being statistically significant ($P<0.01$, fig. 1A–1E). As compared with control group, the total cell number of BALF in COPD group and COPD+MSCs group was significantly increased ($P<0.01$). The total cell number of BALF in COPD+MSCs group was less than in COPD group ($P<0.01$, fig. 1F).

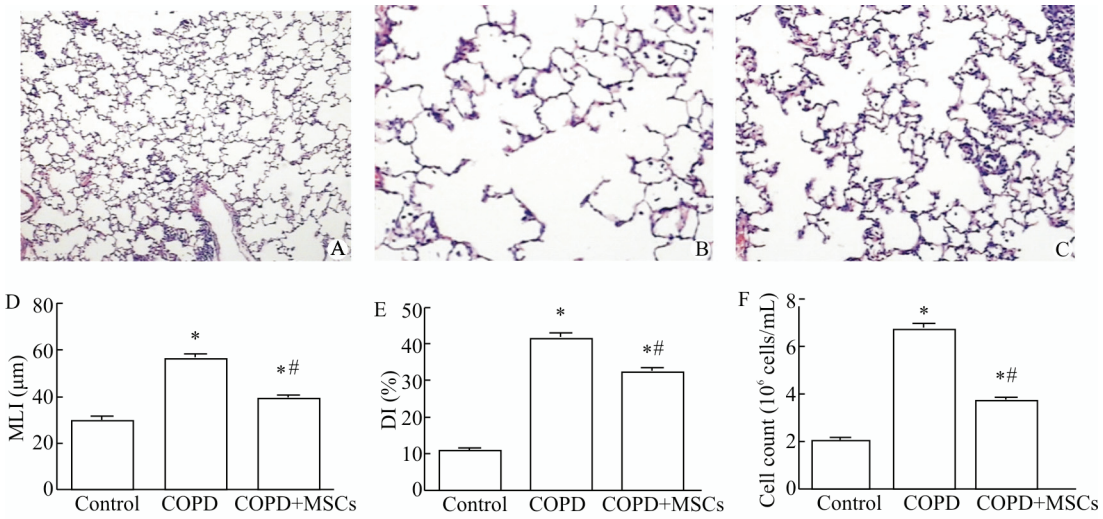

Fig. 1 MSCs transplantation ameliorated emphysematous changes in COPD mice

Lung sections were stained with hematoxylin and eosin (×100). A: control group; B: In COPD group induced by cigarette smoke, lung section exhibited emphysematous changes; C: In COPD+MSCs group treated with cigarette smoke plus MSCs, emphysematous changes were ameliorated; D: The MLI in COPD group and COPD+MSCs group was significantly higher than that in control group. The MLI in COPD+MSCs group was lower than in COPD group; E: The DI in COPD group and COPD+MSCs group was significantly higher than that in control group. The DI in COPD+MSCs group was lower than that in COPD group; F: As compared with control group, the total cell number of the BALF in COPD group and COPD+MSCs group was significantly increased. The total cell number of BALF in COPD+MSCs group was less than that in COPD group.

Data are expressed as $\bar{x}\pm s$ for six mice in each group. $^{*}P<0.01$ *vs.* control group, $^{\#}P<0.01$ *vs.* COPD group

2.2 Measurement of Lung Function

Raw (cmH_2O/mL/min) in COPD group and COPD+MSCs group was increased as compared with that in control group ($P<0.01$). There was no significant difference in Raw between COPD+MSCs group and COPD group ($P>0.05$). Cdyn (mL/cmH_2O) and PEF (mL/s) in COPD group and COPD+MSCs group were reduced as compared with those in control group, but there were no significant differences in Cdyn and PEF between COPD+MSCs group COPD group ($P>0.05$). The changes of Raw, Cdyn and PEF in COPD group and COPD+MSCs group were consistent with the pathophysiologic changes of COPD. The change of lung function in COPD+MSCs group was ameliorated but there was no significant difference from COPD group ($P>0.05$, fig. 2A–2C).

2.3 Effects of MSCs Transplantation on Alveolar Epithelial Cell Apoptosis and Proliferation in COPD Mice

To observe the changes of alveolar epithelial cell injury and repair, we measured apoptosis of alveolar epithelium by TUNEL and the proliferation by PCNA. The AI in alveolar walls of COPD group and COPD+MSCs group was significantly higher than that of

control group (P<0.01), but the AI in COPD+MSCs group was significantly lower than that of COPD group (P<0.01, fig. 3A–3C, 3G). As compared with control group, the PI of alveolar wall cells in COPD group was slightly elevated but the difference was not statistically significant (P>0.05). The PI of COPD+MSCs group was significantly increased as compared with COPD group and control group (P<0.01, fig. 3D–3F, 3H). The result suggests the apoptosis of alveolar epithelium is more than proliferation and there was repair deficiency in COPD mice, but alveolar epithelial repair can be promoted by MSCs.

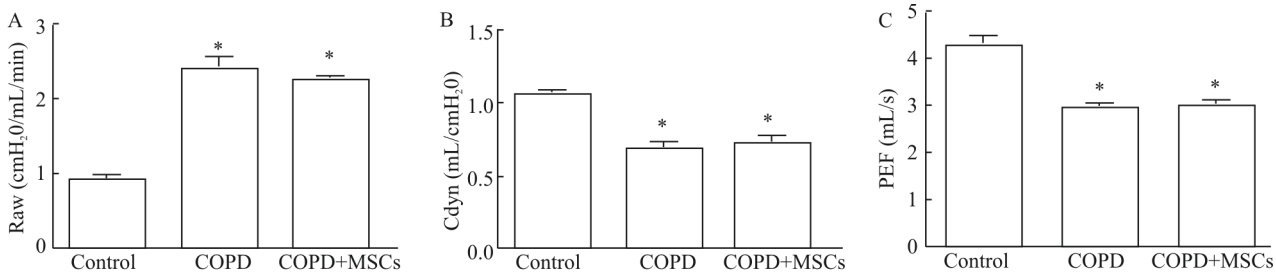

Fig. 2 The measure of Raw (A), Cdyn (B) and PEF (C)

The changes of Raw, Cdyn, and PEF in COPD group and COPD+MSCs group were consistent with the pathophysiologic changes of COPD. There were no significant differences in the changes of these lung functions between COPD+MSCs group and COPD group.

Data are expressed as $\bar{x}\pm s$ for six mice in each group. $^{*}P$<0.01 *vs.* control group

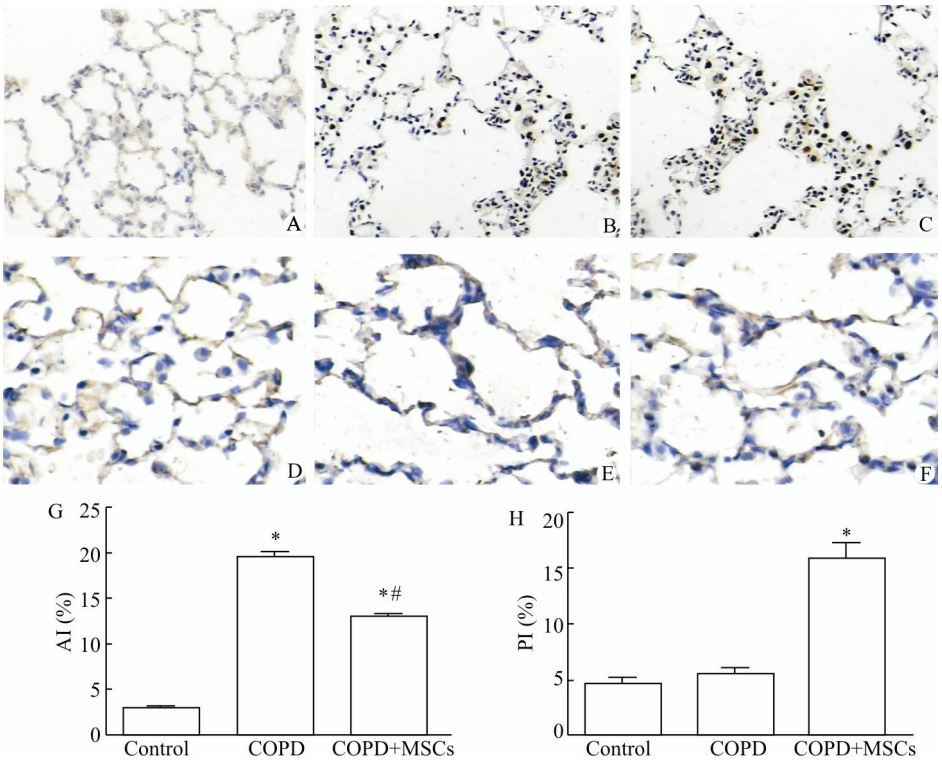

Fig. 3 Effects of MSCs transplantation on alveolar epithelial cell apoptosis and cell proliferation in COPD mice

A–C: detection of cell apoptosis by TUNEL in alveolar wall cells (×400). A: control group; B: COPD group; C: COPD+MSCs group. D–F: detection of cell proliferation by PCNA in alveolar wall cells (×400). D: control group; E: COPD group; F: COPD+MSCs group; G: the AI in different groups; H: the PI in different groups; H: the PI in different groups

Data are expressed as $\bar{x}\pm s$ for six mice in each group. $^{*}P$<0.01 *vs.* control group, $^{\#}P$<0.01 *vs.* COPD group

2.4 Effects of MSCs Transplantation on Proliferation of Endogenous Lung Stem Cells and the Number of BASCs in COPD Mice

The number of $CD45^{-}/CD31^{-}/Sca\text{-}1^{+}$ population was measured by flow cytometry to assess the role of MSCs transplantation in endogenous lung stem cells in COPD mice. As compared with control group, the number and the ratio of $CD45^{-}/CD31^{-}/Sca\text{-}1^{+}$ population in lung tissue of COPD group were slightly increased, but the difference was not statistically significant (P>0.05). The number and the ratio of $CD45^{-}/CD31^{-}/Sca\text{-}1^{+}$ population of COPD+MSCs group were significantly increased as compared with those of COPD group and control group, and the difference was statistically significant (P<0.01, fig. 4A and 4B).

BASCs located in BADJ are endogenous stem cells of bronchial and alveolar epithelial cells and can differentiate into Clara cells and alveolar epithelial cells,

express Clara cell marker CCSP$^+$ and type 2 alveolar epithelial cell marker SPC$^+$. There was no significant difference in the number of BASCs located in BADJ between COPD group and control group (P>0.05), suggesting there was no proliferation of endogenous lung stem cells in COPD mice induced by cigarette smoke. The number of BASCs in COPD+MSCs group was greater than that of COPD group (P<0.01). The result indicates MSCs transplantation can contribute to the proliferation of BASCs in COPD mice induced by cigarette smoke (P<0.01, fig. 4C and 4D).

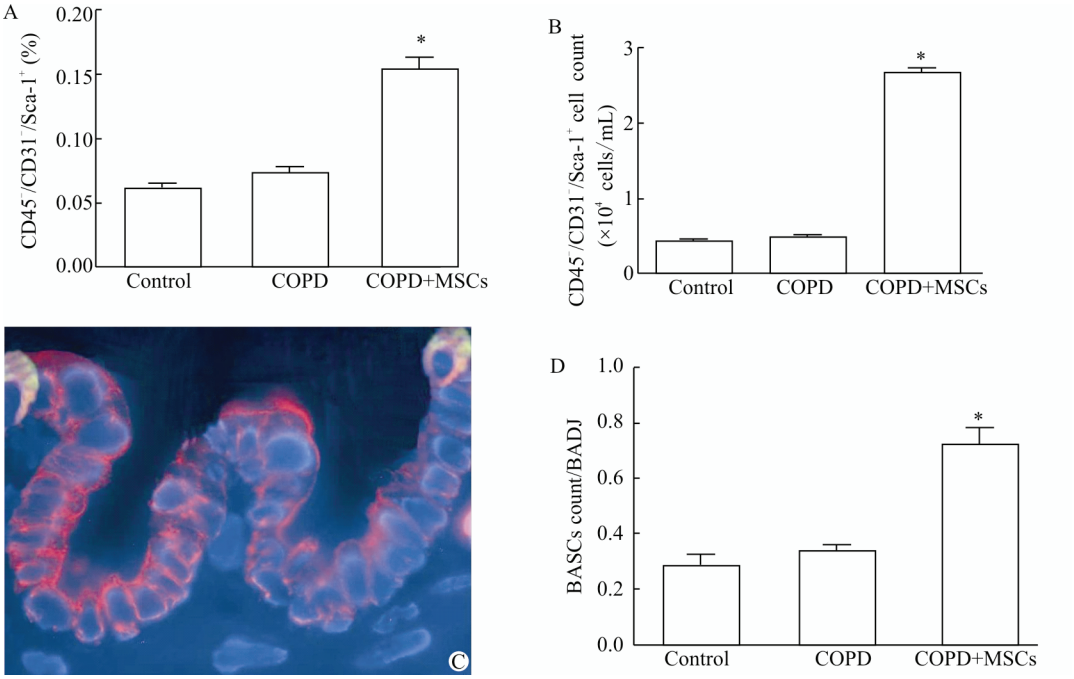

Fig. 4 Effects of MSCs transplantation on the proliferation of endogenous lung stem cells and the number of BASCs in COPD mice
A: the ratio of CD45$^-$/CD31$^-$/Sca-1$^+$population in lung tissue in different groups; B: the number of CD45$^-$/CD31$^-$/Sca-1$^+$ population in lung tissue in different groups; C: immunofluorescence for BASCs in lung tissue of COPD+MSCs group (×1000). CCSP$^+$ (red color), SPC$^+$ (green color), nuclei stained with DAPI (blue color) for BASCs. D: the number of BASCs located in BADJ in different groups
Data are expressed as $\bar{x}\pm s$ for six mice in each group. $^*P$<0.01 *vs.* control group

3 DISCUSSION

There are various types of endogenous lung stem/progenitor cells in airways and lung and can self-renew and differentiate into mature cells in lung tissue. After lung injury, endogenous lung stem cells can supplement damaged cells locally, but their proliferation potential is low. Then the exogenous stem cells such as MSCs repair the lung tissue further[11−13, 24]. COPD is a chronic inflammation disease of airways and lung parenchyma, accelerates aging of mesenchymal precursor cells, accompanied with reduction of extracellular matrix and increased apoptosis of alveolar components, and damages regenerative capacity, leading to alveolar structure damage and emphysematous formation[25−27]. Studies have shown there was no proliferation of endogenous lung stem/progenitor cells in the lung in cigarette smoke-induced COPD models. Bone marrow MSCs have the ability of repairing lung injury and have the ability of anti-inflammation, immunomodulation and anti-apoptosis mediated by paracrine mechanism. The clinical application of MSCs in ALI, asthma and other diseases also achieves certain effect. Recent study has suggested that MSCs in myocardial injury model could stimulate the proliferation of endogenous cardiomyocyte stem cells and differentiation into cardiomyocytes, thereby further improving cardiac function[14]. The research of bronchopulmonary dysplasia model also indicated that MSC transplantation promoted BASC proliferation in lung injury[15]. MSC transplantation has the roles of anti-inflammation, anti-oxidation and anti-apoptosis in COPD[16], but no studies reported about whether MSCs can promote the proliferation of endogenous lung stem cells in COPD.

The purpose of this study is to find out the effect of MSCs transplantation on endogenous lung stem cells in the COPD mice induced by cigarette smoke. The data suggested that in the lung tissue of COPD mice, the inflammation cells increased, alveolar septa damaged, emphysema-like changes formed and lung function declined. Meanwhile, apoptosis of alveolar epithelial cells was increased and proliferation was insufficient, indicating that lung tissue was damaged by cigarette smoke. The proliferation of alveolar epithelial cells was induced by endogenous lung stem cells mainly by BASCs in BADJ and AT Ⅱ in the alveoli. The CD45$^-$/CD31$^-$/Sca-1$^+$ cells measured by flow cytometry are lung epithelial stem cells/progenitor cells that can promote lung tissue repair. 85% of this group is BASCs, and also contains a small number of AT Ⅱ and Clara cells. The population of CD45$^-$/CD31$^-$/Sca-1$^+$ cells was slightly increased in the COPD mice, showing no significant difference from control group. BASCs are pluripotent stem cells and can differentiate into Clara

cells, ATⅡ and ATⅠ, and promote bronchial and alveolar epithelial regeneration. The data indicated that BASCs in COPD mice were increased slightly, showing insignificant difference from control group. These results suggested that the proliferation of endogenous lung stem cells was damaged by cigarette smoke, then the increased apoptosis of alveolar wall cells and inadequate proliferation of alveolar wall cells resulted in the alveolar wall spoil. After bone marrow MSCs transplantation, there were less inflammation cells and apoptosis of alveolar wall cells, more $CD45^-/CD31^-/Sca\text{-}1^+$cells, BASCs and cell proliferation in the lung than COPD group, which relieved emphysematous changes and improved lung function.

In summary, MSCs transplantation can repair lung damage by promoting the proliferation of endogenous lung stem cells in COPD mice induced by cigarette smoke. This study also suggests that in chronic diseases, MSCs transplantation can play a role in injured tissue by promoting proliferation and differentiation of endogenous stem cells, but further researches are needed to find out the mechanism and signalling pathways of endogenous lung stem cell proliferation after MSCs transplantation.

Conflict of Interest Statement

The authors declare that there are no competing interests and financial relationship with the commercial identities mentioned in this paper.

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