Human Adipose-Derived Mesenchymal Stem Cells Ameliorate Elastase-Induced Emphysema in Mice by Mesenchymal–Epithelial Transition

Nobuhiro Fujioka¹
Masahiro Kitabatake²
Noriko Ouji-Sageshima²
Takahiro Ibaraki¹
Makiko Kumamoto¹
Shigeto Hontsu¹
Motoo Yamauchi¹
Masanori Yoshikawa¹
Shigeo Muro¹
Toshihiro Ito²

¹Department of Respiratory Medicine, Nara Medical University, Kashihara, Nara, Japan; ²Department of Immunology, Nara Medical University, Kashihara, Nara, Japan

Purpose: Chronic obstructive pulmonary disease (COPD) is a worldwide problem because of its high prevalence and mortality. However, there is no fundamental treatment to ameliorate their pathological change in COPD lung. Recently, adipose-derived mesenchymal stem cells (ADSCs) have attracted attention in the field of regenerative medicine to repair damaged organs. Moreover, their utility in treating respiratory diseases has been reported in some animal models. However, the detailed mechanism by which ADSCs improve chronic respiratory diseases, including COPD, remains to be elucidated. We examined whether human ADSCs (hADSCs) ameliorated elastase-induced emphysema and whether hADSCs differentiated into alveolar epithelial cells in a murine model of COPD.

Methods: Female SCID-beige mice (6 weeks old) were divided into the following four groups according to whether they received an intratracheal injection of phosphate-buffered saline or porcine pancreatic elastase, and whether they received an intravenous injection of saline or hADSCs 3 days after intratracheal injection; Control group, hADSC group, Elastase group, and Elastase-hADSC group. We evaluated the lung function, assessed histological changes, and compared gene expression between hADSCs isolated from the lung of Elastase-hADSC group and naïve hADSCs 28 days after saline or elastase administration.

Results: hADSCs improved the pathogenesis of COPD, including the mean linear intercept and forced expiratory volume, in an elastase-induced emphysema model in mice. Furthermore, hADSCs were observed in the lungs of elastase-treated mice at 25 days after administration. These cells expressed genes related to mesenchymal–epithelial transition and surface markers of alveolar epithelial cells, such as TTF-1, β-catenin, and E-cadherin.

Conclusion: hADSCs have the potential to improve the pathogenesis of COPD by differentiating into alveolar epithelial cells by mesenchymal–epithelial transition.

Keywords: chronic obstructive pulmonary disease, mesenchymal–epithelial transition, adipose derived mesenchymal stem cell, pulmonary function test

Introduction

Chronic obstructive pulmonary disease (COPD) induces respiratory symptoms such as dyspnea, coughing, and sputum because of abnormalities in airways and alveoli caused by smoking, exposure to biomass fuels, and air pollution. It has been reported that about 3.2 million people died of COPD in 2015 and COPD ranked third among the global age-standardized death rates for both sexes. In particular, advanced COPD causes pathological changes such as emphysema in the lungs, which is a major pathological change. Current treatments for COPD, such as bronchodilators, theophylline, and corticosteroids, are effective to reduce dyspnea...
and prevent exacerbations, which have succeeded in improving quality of life to reduce mortality. However, there is no treatment that reduces or normalizes the pathological changes in the lungs of COPD patients, because these treatments cannot prevent disease progression.4

Recently, the efficacy of mesenchymal stem cells (MSCs) has been reported in rodent models of lung disease.5–10 Moreover, therapeutic efficacy has been shown in models of emphysema established by cigarette smoke exposure and elastase administration.11–16 It has been suggested that the therapeutic effect of MSCs is mediated by their ability to differentiate into tissues,17 immunomodulatory functions,18 paracrine effects,14 and anti-apoptotic effects.19 Since bone marrow-derived MSCs (BM-MSCs) were first discovered, bone marrow has been considered to be the main source of MSCs for clinical applications. Subsequently, MSCs have been isolated from adipose tissue that is attracting attention as an alternative to bone marrow. Adipose-derived MSCs (ADSCs) can be isolated more easily and at considerably larger amounts than bone marrow.20 It has been reported that MSCs derived from mouse adipose tissue differentiate into alveolar epithelial cells and ameliorate lung parenchymal destruction caused by elastase-induced emphysema in mice.21 Moreover, it has been reported that systemic administration of non-HLA-matched allogeneic human MSCs is safe in patients with moderate-to-severe COPD,22 which suggests the potential of MSC therapy in COPD patients.

In this study, we investigated whether human ADSCs (hADSCs) improved lung functional and histological changes in an elastase-induced mouse model of emphysema. We also examined whether hADSCs migrated into emphysematous lungs and their differentiation characteristics.

## Methods

### Ethics Statement

All animal experiments were approved by The Animal Care and Use Committee at Nara Medical University (Nara, Japan). All methods were performed in accordance with the Policy on the Care and Use of Laboratory Animals, Nara Medical University. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University (approval number 12582).

### Elastase-Induced Emphysema Mice Model

Female SCID-beige mice (6 weeks old) were purchased from Oriental Bio Service (Kyoto, Japan) and kept under specific pathogen-free conditions in the animal care facility of Nara Medical University. To establish the COPD model, mice were injected intratracheally (i.t.) with 1.25 U porcine pancreatic elastase (EC134, Elastase-High Purity, porcine, Elastin Products Company, Inc., Owensville, MO) in 50 μL saline (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) following anesthesia induced by pentobarbital (Nacalai Tesque Inc., Kyoto, Japan). Control mice were i.t. injected with 50 μL saline. Three days after injection, 1×10⁶ hADSCs (Lonza, Basel, Switzerland) in 200 μL phosphate-buffered saline (PBS; Wako, Osaka, Japan) were administered intravenously (i.v.). Control mice were i.v. administered 200 μL PBS.

In some experiments, 1×10⁶ hADSCs were labeled with PKH26 (Sigma-Aldrich Inc., St. Louis, MO) in accordance with manufacturer’s protocol. Mice were divided into four groups as follows: (i) Control group (Saline i.t. + PBS i.v.), (ii) hADSC group (Saline i.t. + hADSCs i.v.), (iii) Elastase group (Elastase i.t. + PBS i.v.), and (iv) Elastase-hADSC group (Elastase i.t. + hADSCs i.v.).

### Evaluation of Lung Functions

After the mice were anesthetized with 0.1 mg/g pentobarbital by intraperitoneal injection, tracheal intubation was performed with a 19-G catheter, followed by measurement of lung functions, such as forced vital capacity (FVC), forced expiratory volume and flow at 0.1 s (FEV₀.₁, FEF₀.₁), static compliance (Cst), peak expiratory flow (PEF), Newtonian resistance (Rn), tissue damping (G), tissue elastance (H), inspiratory capacity (IC), and respiratory system resistance (Rrs) using a flexiVent® ventilation system (SCI REQ Inc., Montreal, Canada) as described previously.23

### Histology

After evaluating lung functions, mice were euthanized by blood collection from the left ventricle of the heart and then their lungs were collected for analysis. The left lobe of the lung was inflated by injecting 4% paraformaldehyde into the trachea at a constant pressure of 25 cm H₂O for 20 min and fixed overnight at room temperature. Fixed lungs were embedded in paraffin, cut into 2-μm-thick sections, and then stained with Mayer haematoxylin (Sakura

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Finetek Japan Co. Ltd., Tokyo, Japan) and eosin (Nacalai Tesque, Inc.).

**Immunofluorescence Staining of the Lung**

Left lungs were removed, embedded in optimum cutting temperature compound (Sakura Finetek Japan Co. Ltd.), and frozen in liquid nitrogen. Sections were cut at 5 μm thicknesses and fixed in PBS with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the sections were placed in blocking solution that contained 10% normal goat serum in PBS at room temperature, incubated in PBS for 1 hr, and then incubated overnight at 4°C in a primary antibody solution. After washing three times with PBS, the sections were incubated with the secondary antibody for 1 hr at room temperature. Nuclei were counterstained with DAPI (2 μg/mL, Dojindo Laboratories, Kumamoto, Japan). We used the following primary antibodies: rabbit anti-TTF-1 antibody (1:200; Abcam, Cambridge, UK; ab76013), rabbit anti-β-Catenin antibody (1:100; Cell Signaling Technology Inc., MA; #8480), rabbit anti-pro-SP-C antibody (1:100; Abcam; ab170699), and rabbit anti-CDH1 (E-cadherin) antibody (1:100; Thermo Fisher Scientific, Waltham, MA; #143249). Staining was visualized by Alexa Fluor 488 goat anti-rabbit IgG (1:400; Thermo Fisher Scientific; A32731) as the secondary antibody. Finally, the sections were analyzed under an FV1000 confocal microscope (Olympus, Tokyo, Japan).

**Lung PKH26-Labeled Cell Isolation**

To prepare a single cell suspension from the right lung, the lungs were dissociated using a Lung Dissociation Kit (Miltenyi Biotec, Cologne, Germany) with a gentle MACS Dissociator. The suspension was filtered through a 70 μm cell strainer and the cells were collected by centrifugation following the manufacturer’s instructions. After red blood cells were lysed with BD Pharm Lyse™ (BD Biosciences, Franklin Lakes, NJ), the cells were centrifuged (400 ×g) and collected. PBS containing 0.5% bovine serum albumin (Wako) and 2 mM EDTA (Wako) was added to the cells and PKH26-labeled cells were sorted using a FACSAria™ Ilu Cell Sorter (BD Biosciences). Sorted PKH26-positive cells were collected in RLT buffer (Qiagen, Hilden, Germany) for RNA extraction.

**RNA Extraction and PCR Array**

Total RNA was extracted using a RNeasy Micro Kit (Qiagen) following the manufacturer’s instructions. The amount of extracted RNA was measured using a NanoDrop™ (Thermo Fisher Scientific). The gene expression of hADSCs and sorted PKH26-positive cells from mouse lungs were analyzed using a RT² Profiler™ PCR Array Human Epithelial to Mesenchymal Transition (Qiagen). RNA was reverse transcribed into cDNA by incubation with reverse transcriptase at 42°C for 15 min, followed by incubation at 95°C for 5 min using an RT² First Strand Kit (Qiagen). cDNA was amplified with RT² SYBR Green ROX qPCR Mastermix (Qiagen) in accordance with the manufacturer’s instructions. Gene expression was normalized to multiple housekeeping genes. The results were analyzed using RT² Profiler PCR Array Data Analysis v3.5 (Qiagen) by comparing gene expression between hADSCs and sorted hADSCs from the lungs of the Elastase-hADSC group.

**Statistical Analysis**

Statistical differences were analyzed by one-way analysis of variance with Tukey’s multiple comparison test and the Student’s t-test. A P-value of <0.05 was considered significant. Graphs and statistical tests were made using GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA).

**Results**

**hADSC Administration Improves the Pathogenesis of Lung Emphysema in Mice**

We administered elastase or saline intratracheally to SCID-beige mice, followed 3 days later by intravenous administration of PBS or hADSCs. At 28 days after elastase administration, mice were euthanized after pulmonary function tests and their lungs were collected (Figure 1A). Lung histology in the Control group (Saline i.t. + PBS i.v.) did not show histological changes and damages, and that in the Elastase group (Elastase i.t. + PBS i.v.) showed air space enlargement and destruction of the alveolar walls compared with the Control group. However, in the Elastase-hADSC group (Elastase i.t. + hADSCs i.v.), the destruction of alveolar walls and the enlargement of alveolar spaces were milder than in the Elastase group (Figure 1B). The mean linear intercept (Lm) method, which was used to evaluate the emphysematous change, showed a significant change in the Elastase group (Lm: 20.5–34.5 μm) compared with the Control group (Lm: 14.5–19.0 μm) and significant reduction in the Elastase-hADSC group (Lm: 17.1–
32.1 μm) compared with the Elastase group. However, Lm showed no significant change between the hADSC group (Saline i.t. + hADSCs i.v.) (Lm: 12.4–14.2 μm) and the Control group (Figure 1C).

hADSC Administration Improves the Lung Functions of Emphysema in Mice

To evaluate the effect of hADSC administration, we performed a pulmonary function test. FEV$_{0.1}$ was
significantly lower in the Elastase group than in the Control group and FEV$_{0.1}$ was markedly improved in the Elastase-hADSC group (0.8534 ± 0.0148 mL) compared with the Elastase group (0.7682 ± 0.0226 mL) (*Figure 2*).

However, there were no significant differences in other pulmonary function parameters including FVC, FEV$_{0.1}$/FVC, Cst, FEF$_{0.1}$, PEF, Rn, G, H, IC, and Rrs (*Figure S1*).

**hADSCs That Migrate to the Emphysematous Lung Express Genes Related to Mesenchymal-to-Epithelial Transition**

Next, we investigated how hADSCs had changed their phenotype after migration into emphysematous lungs. PKH26-labeled hADSCs were identified at around 1% of whole lung cells in the Elastase-hADSC group at day 28 (*Figure 3A*). We sorted PKH26-labeled hADSCs and compared gene expression between naïve hADSCs directly thawed from frozen stock and hADSCs sorted from the lungs of the Elastase-hADSC group using a RT$^2$ profiler$^\text{TM}$ PCR array Human Epithelial to Mesenchymal Transition.

Gene expression of epithelial markers, such as Cdh2, Colla2, Col3a1, Fn1, Snai2, Twist1, Vim, Wnt5a, Wnt5b, and Zeb1, was lower in hADSCs from emphysematous lungs compared with naïve hADSCs (*Figure 3B*). These results suggest that hADSCs migrated into the emphysematous lungs and changed their phenotype by mesenchymal-to-epithelial transition.

**hADSCs That Migrate to the Emphysematous Lung Express Pulmonary Epithelial Surface Markers**

To define where hADSCs had migrated into the lungs, we next performed immunofluorescence staining of the lungs. We found very few PKH26-positive cells in the hADSC group, whereas PKH26-positive cells were sufficiently found in the Elastase-hADSC group (*Figure 4A*). Next, lung sections were stained with several alveolar epithelial markers, including TTF-1, Pro SP-C, β-Catenin, and CDH1 (E-cadherin) to investigate the characteristics of PKH-positive hADSCs in the lungs of the Elastase-hADSC group. PKH-positive hADSCs were merged with TTF-1, β-Catenin, and CDH1, but negative for Pro SP-C, a type II epithelial marker (*Figures 4B*, S2 and S3). These data indicate that hADSCs might differentiate into type I alveolar epithelial cells in emphysematous lungs.

**Discussion**

In this study, we demonstrated that administration of hADSCs, which were migrated into emphysematous lungs, improved the pathogenesis and pulmonary function of emphysematous lungs in mice. Moreover, hADSCs attracted to the emphysematous lungs showed increased epithelial gene expression and decreased mesenchymal gene expression compared with naïve hADSCs, which indicated that hADSCs may have acquired the characteristics of type I alveolar epithelial cells by mesenchymal–epithelial transition.

hADSCs are less invasive to collect than BM-MSCs, collected at larger numbers, and more effective suppressors of immune responses. Furthermore, hADSCs have been reported to be genetically and morphologically more stable in long-term culture, exhibit lower senescence rates and higher proliferative capacity, and retain differentiation potential for longer periods in culture than human BM-MSCs, suggesting that hADSCs are more suitable for clinical application among the MSC types. In fact, many preclinical studies using rodent models of...
Figure 3 Gene expression of epithelial and mesenchymal genes in hADSCs isolated from lungs.

Notes: SCID-beige mice were intratracheally injected with 1.25 U elastase and then intravenously injected with $1 \times 10^6$ PKH26-labeled hADSCs on day 3. On day 28, lungs were collected for analyses. (A) PKH26-labeled hADSCs were identified and sorted from the lungs of the Elastase-hADSC group (Elastase i.t. + hADSCs i.v.). (B) Gene expression changes in hADSCs isolated from the lungs of the Elastase-hADSC group (n=4) compared with naïve hADSCs (n=4) using RT²® profiler™ PCR array human epithelial-to-mesenchymal transition. Relative values to the average of naïve hADSCs are presented as means ± SEM with individual plots.

Abbreviations: hADSCs, human adipose-derived mesenchymal stem cells; SEM, standard error of the mean.
pulmonary diseases including COPD have reported the effects of MSCs including BM-MSCs and ADSCs.\textsuperscript{11–16,21,30–36} Several clinical trials in COPD patients have shown that administration of BM-MSCs or ADSCs is safe, but there is no report showing therapeutic effects such as improved the pathogenesis and lung functions using human ADSCs. Therefore, we here focused on the effects of hADSCs for COPD. And it has been reported that human non-autologous stem cells may be immunogenic in animal models, so we used SCID-beige mice as the recipient experimental animals in this study.\textsuperscript{37} Emphysema is a characteristic histological change in

**Figure 4** Fluorescence staining of epithelial markers in the lungs.

**Notes:** Lung sections from SCID-beige mice administered intravenously with PKH26-labeled hADSCs were observed under a confocal laser microscope. (A) Fluorescence of PKH-26 (red) was observed with nuclear counterstaining by DAPI (blue) (×200). (B) Expression of epithelial cells markers TTF-1, Pro SP-C, β-Catenin, and CDH1 visualized by Alexa Fluor 488 (green) in PKH26-labeled hADSCs (red) (×400). Arrow shows Alexa Fluor 488 and PKH26 merged cells. Scale bar shows 50 μm. Data are representative of more than three independent experiments.

**Abbreviations:** hADSCs, human adipose-derived mesenchymal stem cells; DAPI, 4ʹ,6-diamidino-2-phenylindole; TTF-1, thyroid transcription factor-1; SP-C, surfactant protein C; CDH1, cadherin-1.
COPD, and one widely used animal model of emphysema is the elastase treatment. In this study, systemic administration of hADSCs resulted in histological changes in an elastase-induced lung emphysema model mouse. Also, COPD causes not only emphysematous changes in the lungs but also airflow obstruction. In humans, in respiratory function tests, the ratio of forced expiratory volume in one second (FEV\textsubscript{1}) to forced vital capacity (FVC) of less than 0.7 after administration of bronchodilators in respiratory function tests is essential for diagnosis of COPD.\textsuperscript{38} Recently, it has become possible to evaluate respiratory function in mice using the Forced Oscillation Technique,\textsuperscript{39} and its usefulness has been reported in mouse models of respiratory diseases.\textsuperscript{23,40–43} So we evaluated the effects of hADSCs on respiratory function using flexiVent\textsuperscript{40} in a mouse model of elastase-induced emphysema. It has been reported that forced expiratory volume at 0.1s (FEV\textsubscript{0.1}), forced vital capacity (FVC), forced expiratory flow at 0.1s (FEF\textsubscript{0.1}), inspiratory capacity (IC), Newtonian resistance (Rn), and tissue hysteresivity (G/H) increase and FEV\textsubscript{0.1}/FVC, peak expiratory flow (PEF), tissue damping (G), and tissue elastance (H) decrease in lungs with a mouse model of emphysema established by intranasal administration of porcine pancreatic elastase three times a week to male BALB/c mice.\textsuperscript{23} In another model of emphysema induced by intratracheal administration of porcine pancreatic elastase to BALB/c mice, it has been reported that IC and Rrs (respiratory system resistance) increased and H decreased.\textsuperscript{44} We found that FEV\textsubscript{0.1} was significantly decreased in the mice of elastase-induced emphysema as reported previously,\textsuperscript{23} and that systemic administration of hADSCs to the mice of elastase-induced emphysema significantly improved the decrease in FEV\textsubscript{0.1}. This indicates that hADSCs not only improve COPD histologically, but also improve pulmonary function. On the other hand, systemic administration of hADSCs to elastase-induced COPD model mice did not improve FVC, FEV\textsubscript{0.1}/FVC, Cst, FEF\textsubscript{0.1}, PEF, Rn, G, H, IC, and Rrs in this study. Compared with a previous report,\textsuperscript{23,44} the difference of elastase administered to induce emphysema and that of respiratory function measurement method, flexiVent\textsuperscript{40} and Buxco lung function analysis, might result in no significant difference in lung compliance or resistance.

In addition, hADSCs were detected in the lung parenchyma 25 days after systemic administration of hADSCs in elastase-induced emphysema mice. Previous reports have shown that systemically administered ADSCs were detected in the lung parenchyma and airways from day 1 to 21 after administration.\textsuperscript{15} Intravenously administered hADSCs migrated into the lungs of the Elastase-hADSC group, while very few hADSCs could be detected in the lungs of the hADSC group (Saline i.t. + hADSCs i.v.), which suggests that intravenously administered hADSCs are recruited to the site of lung injury. It has been proposed that various chemokines and cytokines are involved in the mechanism of stem cell recruitment to the site of injury.\textsuperscript{45} However, there are no reports on stem cell homing to emphysematous lungs, which requires further investigation.

We found that hADSCs attracted to emphysematous lungs led to downregulate mesenchymal-related gene expression, while epithelial-related gene expression was upregulated, which suggests induction of mesenchymal–epithelial transition. Setiawan et al reported that inhibition of GSK3 and TGF-β signaling causes human ADSCs to differentiate into epithelial cells through mesenchymal–epithelial transition while smoking-induced emphysema models, whereas inhibition of Wnt5A reduces destruction of lung tissue and improves lung functions. This leads to re-epithelialization of alveoli and reactivation of Wnt/β-catenin,\textsuperscript{46} which suggests that it might be a potential target for COPD treatment.\textsuperscript{49} Similarly, in this study, gene expression of β-catenin was up-regulated and that of Wnt5A was downregulated in hADSCs migrated to the emphysematous lungs, suggesting that hADSCs may have ameliorated emphysema by reactivating Wnt/β-catenin signaling and inhibiting Wnt5A signaling.

Moreover, hADSCs attracted to emphysematous lungs expressed cell surface markers of type I alveolar epithelial cells, suggesting that hADSCs improved COPD by differentiating into type I alveolar epithelial cells. Previous studies reported that mouse BM-MSCs also differentiated into type I alveolar epithelial cells in elastase-induced emphysema,\textsuperscript{14} while mouse ADSCs differentiated into type II alveolar epithelial cells.\textsuperscript{21} As type II alveolar...
epithelial cells are progenitors of type I alveolar epithelial cells; 50–52 it is possible that human ADSCs differentiated into type II alveolar epithelial cells and then into type I alveolar epithelial cells. The long time between administration of ADSCs and the analysis of ADSCs might have influenced the results of this study, and further investigation for the time points of analysis following ADSC administration is required.

Finally, we demonstrated the therapeutic effect of human-derived ADSCs on COPD using a COPD model established by administering elastase to SCID-beige mice, which suggests that hADSCs may be a valuable candidate for COPD medical therapy. But, the present study has still some limitations. For example, SCID-beige mice were used in this study to exclude the effect of graft-versus-host disease, while we should consider the role of immunity in allograft hADSC administration for clinical trial. Second, because COPD in humans is mainly caused by smoking, our approach might also be valuable in other models of COPD, that have additional features of disease including airflow/tissue inflammation such as cigarette smoke-induced models. Further studies are required to investigate whether the similar effects can be obtained from other models of COPD.

Conclusion
We found that hADSCs ameliorated emphysema both histologically and functionally, and migrated into the emphysematous lung in an elastase-induced COPD model in SCID-beige mice. The administered hADSCs into lungs underwent mesenchymal–epithelial transition in the emphysematous lungs and differentiated into type I alveolar epithelial cells. The results of this study may provide a mechanism for the treatment of COPD by ADSCs. Human ADSCs not only improved pulmonary function, but also had the potential to differentiate into alveolar epithelial cells in elastase-induced emphysema, which might be a source of fundamental therapy for COPD.

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Author Contributions
All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure
The authors report no conflicts of interest in this work.

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