ORIGINAL

Lung regeneration by fetal lung tissue implantation in a mouse pulmonary emphysema model

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Abstract : The mortality and morbidity of chronic obstructive pulmonary disease are high. However, no radical therapy has been developed to date. The purpose of this study was to evaluate whether fetal mouse lung tissue can grow and differentiate in the emphysematous lung. Fetal lung tissue from green fluorescent protein C57BL/6 mice at 16 days' gestation was used as donor material. Twelve-month-old pallid mice were used as recipients. Donor lungs were cut into small pieces and implanted into the recipient left lung by performing thoracotomy under anesthesia. The recipient mice were sacrificed at day 7, 14, and 28 after implantation and used for histological examination. Well-developed spontaneous pulmonary emphysema was seen in 12-month-old pallid mice. Smooth and continuous connection between implanted fetal lung tissue and recipient lung was recognized. Air space expansion and donor tissue differentiation were observed over time. We could clearly distinguish the border zones between injected tissue and native tissue by the green fluorescence of grafts. Fetal mouse lung fragments survived and differentiated in the emphysematous lung of pallid mice. Implantation of fetal lung tissue in pallid mice might lead to further lung regeneration research from the perspective of respiratory and exercise function. J. Med. Invest. 63 : ●-●, August, 2016

Keywords **:** *Pallid mouse, COPD, lung regeneration, fetal tissue, pulmonary emphysema*

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) characteristically manifests as chronic airflow obstruction due to terminal bronchiolar and alveolar destruction. In 2007, the World Health Organization estimated that 64 million people have COPD, and that 3 million people died off the disease. Its incidence is currently increasing, and it is estimated to become the third leading cause of death in 2020 (1, 2). Destruction of pulmonary alveoli is considered irreversible, and no curative therapy has been developed to repair the apoptotic lesion. In 1990s, interest grew in the proposal to perform lung volume reductions surgery for COPD. However, the National Emphysema Treatment Trial, which was a large-scale clinical trial conducted in the USA in 2003, found limitations in the appropriateness of this treatment (3). In 1997, Massaro *et al.* reported that administration of all-trans retinoic acid caused a reduction of emphysematous lesions in rat lung (4). While their research reached the stage of clinical trials, unfortunately it did not yield the expected therapeutic results (5). Currently, the only effective radical treatment for COPD is lung transplantation from a live donor or brain-dead healthy body. However, the serious shortage of donors limits the practicality of whole-lung transplantation and highlights the urgent need to develop new treatment methods.

Advances in regenerative sciences in recent years have been remarkable. In addition to the bone marrow stem cells and progenitor cells that were originally recognized in the field, tissue stem cells have been subsequently found to exist in many organs. Stem cells and potential applications have also been discovered in the field of pulmonology (6, 7). However, in comparison to solid organs such as liver or heart, regeneration of the lung with stem cells is considered difficult. A single type of lung cell is insufficient to restore the complicated structure of the lung. A cell source with the highest potential and a scaffold suitable for lung development are also necessary. To attempt to solve this problem, we focused on fetal lung, which has excellent potential for further growth, differentiation, and proliferation, and mesenchymal tissue as a suitable scaffold. We have previously shown that fetal rat lung tissues were able to survive, grow, and differentiate inside adult rat lung (8).

In this study, we investigated whether fetal lung tissue was also able to grow and differentiate in the adult lung of a mouse strain used as a disease model of pulmonary emphysema. While many pulmonary emphysema models exist (4, 9-11), we chose the pallid mice as recipients. In a previous study, we examined the exercise function of pallid mice and reported that they show decreased exercise capacity and diminished respiratory function antecedent to histological changes (12). If pulmonary regeneration can be achieved in pallid mice, it may lead to further functional investigation of lung regeneration with fetal tissue.

MATERIALS AND METHODS

Animals

Pallid mice (C57 pa+/pa+), which were used as recipient mice, were purchased from Jackson Laboratory (Bar Harbor, ME, USA) via Charles River Japan (Kanagawa, Japan). Green fluorescent protein (GFP)-C57BL/6 mice were used as donors and provided by the Research Institute for Microbial Diseases, Osaka University, Japan (13). These mice were bred in a pathogen-free environment

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with a 12 h light-dark cycle, and provided free access to water and food. All animal care and experiments were carried out in accordance with the institutional and national guidelines (Japanese Ministry of Education, Culture, Sports, Science and Technology) and were approved by the Animal Care Committee of the University of Tokushima.

Measurement of mean linear intercept to evaluate pulmonary emphysema

Two groups of 6 naïve pallid mice were killed at 3 and 12 months of age, respectively. The left lungs were removed and fixed with 10% phosphate-buffered formaline solution through the trachea at a pressure of 15 cm H_2O. Specimens obtained from the upper, middle and lower fields of the left lung were cut into 4-μm-thick sections and stained with hematoxylin and eosin.

We used the mean linear intercept (Lm) method to quantify the alveolar space and evaluated pulmonary emphysema (14). Five fields under a light microscope at ×200 magnification were randomly selected from each sections (i.e. 15 fields in each left lung sample), and Lm was calculated on the basis of the total number of a alveolar intercepts encountered along the length of a right-angled crosshair (500 μm). The differences among Lm values of the upper, middle, and lower fields of the left lung were analyzed by one-way analysis of variance (ANOVA) with Bonferroni correction. The difference between the Lm values of the left lung at 3 and 12 months of age was analyzed by Mann Whitney U test. All statistical analyses were performed with SPSS version 23.0. $P < 0.05$ was considered to indicate statistical significance.

Implantation

Pregnant GFP-C57BL/6 mice at 16 days' gestation were euthanized under ether anesthesia, and fetuses were removed. Donor fetal lungs were dissected under a microscope and then finely cut with scissors into small pieces in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 200 U/ml penicillin, and 200 μl/ml streptomycin (GIbco-BRL).

Fifteen 12-month-old pallid mice were used as recipients. They were intubated with 22-G Surflo external cylinders (Terumo, Tokyo, Japan) under anesthesia by intraperitoneal injection of xylazine (8 mg/kg) and ketamine hydrochloride (80 mg/kg), and then placed in a lateral position. A small animal respirator (KN-55, Natsume Factory, Tokyo, Japan) was used to establish ventilation at a respiratory rate of 150 cycles/min and a tidal volume of 0.2 ml/body. After left thoracotomy, the finely minced fetal lung pieces were injected into the lower half of the left lung using a 25-μl microsyringe (Hamilton, USA) with a 26-gauge needle. To prevent pneumothorax, the pinhole of the lung at the injection site was closed off with a silk ligature, and the peripheral lung tissue from the ligature was resected. To control for rejection of the GFP antigen, 10 mg/kg cyclosporine (CyA, Novartis) was administered intramuscularly into the quadriceps daily from the day before surgery until the day of sacrifice.

Histological study

The recipient mice were sacrificed at day 7, 14, and 28 after implantation (5 animals per time point) and used for histological examination. After cutting the left atrium, lungs were perfused with saline through the pulmonary artery and then removed en bloc with the heart and fixed in a fixing solution (4 : 1 ratio of 4% paraformaldehyde to Optimal Cutting Temperature [OCT] compound) through the trachea at a pressure of 15 cm H_2O. After fixation for 5 h at 4°C, the implanted tissue was identified and fixed with OCT compound at -80°C. Sections were prepared at 10-μm thickness for fluorescence microscopy to evaluate green fluorescence, and 4-μm thickness for hematoxylin and eosin staining. Both non-stained histological images and green fluorescent images were captured using a Biozero BZ-8000 microscope (Keyence Co, Ltd, Osaka, Japan), and light microscopic images from histologic specimens with hematoxylin-eosin stain were captured using an Olympus BX51-FL microscope (Olympus Co, Ltd, Tokyo, Japan).

RESULTS

Evaluation of emphysematous change in pallid mice using the Lm method.

To evaluate whether our pallid mice were suitable as a pulmonary emphysema models, we first compared the histological changes between 3- and 12-month-old pallid mice and evaluated Lm. Compared with 3-month-old pallid mice, 12-month-old pallid mice demonstrated well-developed morphological features of pulmonary emphysema (Figure 1A). The Lm of pallid mice at 12 months of age was significantly higher than that of pallid mice at 3 months of age ($P < 0.01$). We also observed whether any bias of Lm was present within the upper, middle, and lower fields of the lung. Little variation was observed in the distribution of emphysematous lesions within each field (Figure 1B).

Morphologic changes of GFP-fetal lung tissues in the emphysemic lung.

The morphology of lungs from fetal mice at day 16 and neonatal mice at days 7 and 14 is presented to provide landmarks for lung maturation (Figure 2A-C). Day 16 corresponds to the pseudoglandular period of mouse lung development, which covers the establishment of the undifferentiated primordial system of the developing lung and the appearance of the prospective bronchial and respiratory systems. Development of the bronchial and respiratory systems proceeds in the canalicular, terminal sac, and alveolar periods. The alveolar period lasts for 4 weeks after birth (15).

At 7 days after implantation, the grafted tissue still contained some structures with pseudoglandular-like shape. However, some air-filled structures were also observed, with thick septal walls similar to those seen in day-7 postnatal lung. The border between donor and host tissue were clearly existed, however, continuous connections between them were detected. The host alveoli adjacent to transplanted fetal tissues were compressed (Figure 3A-C).

At 14 days after implantation, alveoli were further developed. The alveolar wall became thinner, and the alveolar space became larger. A relatively thick septal wall remained between alveoli composed of cuboidal epithelial cells and matrix. The host alveoli adjacent to transplanted fetal tissues were also compressed at this time (Figure 3D-F).

At 28 days after implantation, the alveolar wall in the injected tissue became thinner than that at day 14. Moreover, the alveolar spaces were expanded, mimicked to that in a normal adult lung. In the border zone between the implanted fetal lung tissue and the adult lung tissue, the connection of alveolar wall between them became smoother. The compression of host alveoli by transplanted fetal tissues does not observed (Figure 3G-I). This sequence of events indicated that fetal lung tissue also grows and differentiates in the adult lung of a mouse genetic model of pulmonary emphysema.

With the use of a fluorescence microscope, the border and distribution of the recipient lung could be clearly distinguished by the luminescence of grafts. The differentiated fluorescent tissue was seen inside the implanted tissue area. However, no fluorescent tissue spreading into the recipient lung was detected. The extremely light fluorescence in the recipient lung was the autofluorescence.

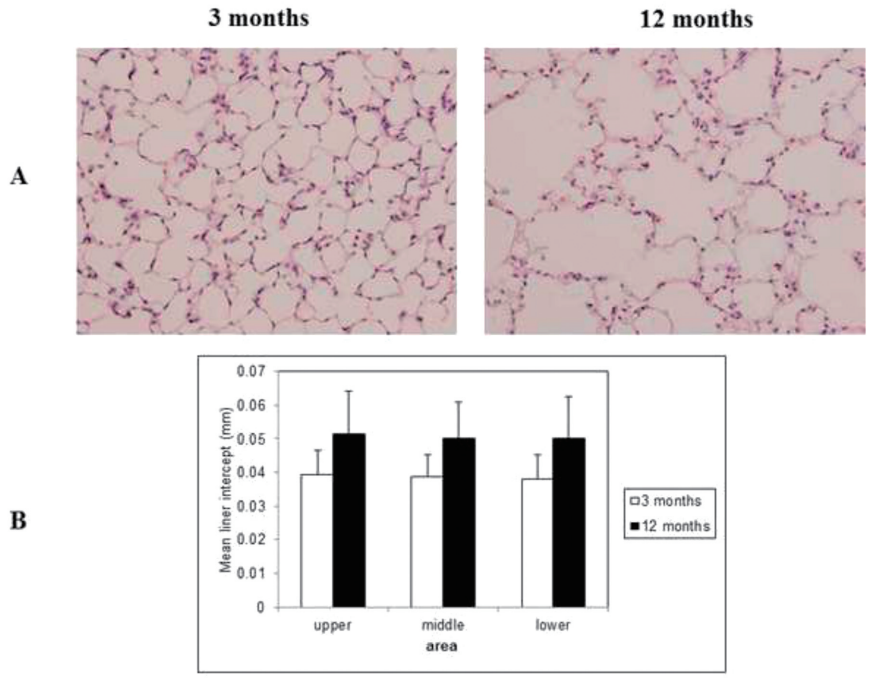

Figure 1
(A) Representative hematoxylin and eosin-stained specimens of pallid mice at 3 and 12 months of age (×200). 12-month-old pallid mice demonstrated well-developed morphological features of pulmonary emphysema.
(B) Evaluation of emphysematous change in pallid mice using the Lm method. The Lm of 12-month-old pallid mice was significantly higher than that of 3-month-old pallid mice ($P < 0.01$). Data are presented as mean±standard deviation in each group of 6 animals. There was little bias of Lm within the upper, middle, and lower fields of the lung.

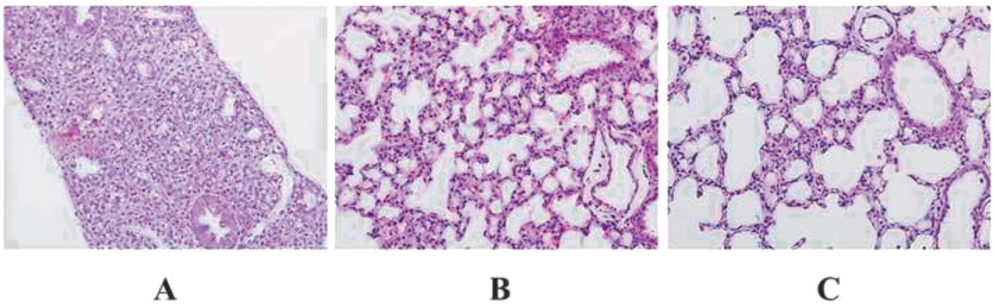

Figure 2
Landmarks of lung maturation . The morphology of fetal mouse lung at day 16 (A) and neonatal mouse lung at days 7 (B) and 14 (C) is presented to indicate landmarks of lung maturation. (hematoxylin and eosin staining, ×200)

DISCUSSION

To date, several studies regarding pulmonary regeneration have been reported, with some describing transplantation of individual cells, such as alveolar type 2 cells (16-18). However, individual pulmonary cells transplanted into lung are presumed insufficient to achieve regeneration at the organ level. Concomitant regeneration of the vascular system, scaffold, and interstitial components are additionally required.

To surmount this difficulty, we previously used fetal rat lung fragments for implantation (8), which contains various cells with the potential to differentiate into pulmonary tissue. In that study, we demonstrated that fetal rat lung tissues were capable of differentiation inside host adult lungs. In the present study, we used mice that spontaneously develop pulmonary emphysema as recipients and showed that implanted lung tissues from fetal mice survived and differentiated in this disease model of pulmonary emphysema.

While many pulmonary emphysema models exist (4, 9-11), those induced by intra-tracheal administration of chemical agents show some bias in emphysematous grade and distribution, which are inadequate models for tissue implantation because of the possibility of little emphysematous grade at the site of implantation. Pallid mice have a C57BL/6 origin, and they carry a mutation in the pallid gene, which is located on chromosome 2. They characteristically develop spontaneous pulmonary emphysema at 12-15 months of age due to a deficiency in α-antitrypsin (19, 20). Kei *et al.* measured Lm at 1, 12, and 24 months of age in pallid and C57BL/6 mice and reported a significant difference at 12 months (21). Consistent with their data, in our study, we observed significantly higher Lm at 12 months of age in pallid mice and little variation in the distribution of

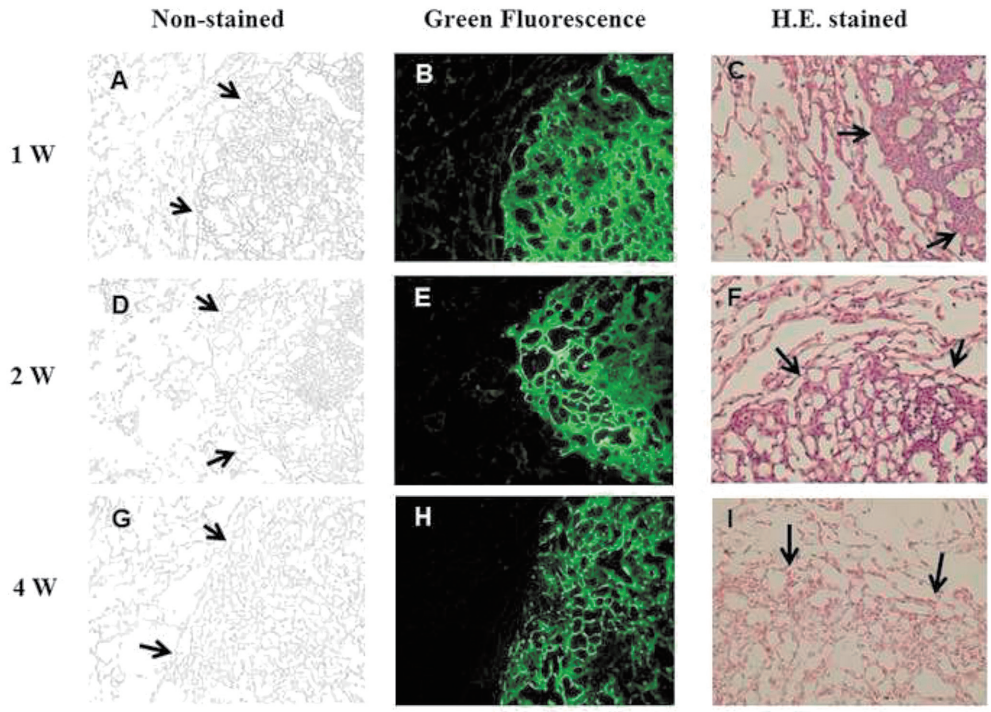

Figure 3
Morphologic change of fetal lung tissue implanted in emphysematous lungs of pallid mice. Fetal lung tissues and surrounding areas were examined at 7 (A-C), 14 (D-F), and 28 days (G-I) after implantation. Unstained specimens were observed under white light (A, D, G) and fluorescence (B, E, H) microscopy (100×). Green fluorescence clearly demonstrates the border zones and smooth connection between the donor and recipient tissue. Hematoxylin and eosin-stained specimens (C, F, I) are shown at 200× magnification. Arrows indicate the border zone between injected and native tissue. Air space expansion and morphologic changes of the donor tissue were observed over time. Each photo is representative of 5 animals that were treated as described in the Materials and Methods.

emphysematous lesions within each field. Therefore, we regarded pallid mice as feasible models of pulmonary emphysema for experiments with fetal lung implantation.

The development course of fetal mouse and rat lung are normally almost identical (15, 22), however, compared with the implanted grafts in normal rats we previously reported, which demonstrated the beginning of alveolar expansion at 4 weeks (8, 23), grafts implanted in pallid mice demonstrated expanded air spaces earlier. We postulate two possible reasons for this difference. (1) The turnover of alveolar wall cells is enhanced in emphysematous lungs (24). This environment may produce some positive effect on fetal tissue differentiation. (2) Although the fetal mouse lung tissues were cut into small pieces under the same procedure used for the rat models, the size of the implanted grafts in proportion to body size was greater in the mouse than in the rat models because the former are smaller. Therefore, the courses in mice might have been similar to those of organ transplantation models.

In addition, in the rats, the implanted alveolar units of the fetal lung became extremely larger than the surrounding alveolar units in the recipients. In contrast, in the pallid mice, the implanted alveolar units were slightly smaller than the surrounding alveolar units (those without emphysematous change). The reasons for this may be that the alveoli in the pallid mice were compressed by the surrounding emphysematous tissue, and that the mechanical stimulation produced by partial lung resection was not effective in the implanted fetal lung tissue, with the surrounding emphysematous tissue instead showing expansion of the high compliance area.

We previously reported that the pallid mouse showed decreased exercise capacity and diminished respiratory function from approximately 6 months of age (12). We attempted to investigate the exercise capacity of fetal-lung-implanted pallid mice in comparison to pallid mice that had not undergone surgery. However, due to the invasive nature of the large thoracotomy, the exercise function of the recipient mice decreased postoperatively. (deta not shown) We are left with the issue of how to establish more diffuse and less invasive transplantation methods to carry out postoperative evaluations of respiratory and exercise function. Transplanting fetal lung tissues through the airway or lung blood vessels may be a possible solution ; however, suffocation or pulmonary embolism was unavoidable with massive fetal lung tissue injection. We must now identify which cells in the fetal mouse lung play key roles for tissue growth and aim to implant the minimum number of those cells necessary to differentiate in recipient lung. Takebe *et al.* reported organ bud formation including lung by combining tissue specific progenitor cells, endothelial cells, and mesenchymal stem cells (25).

The ultimate goal of tissue engineering of the lung may be to induce differentiation of stem cells such as induced pluripotent stem cells into various respiratory cells, reconstructing 3-dimensional architectural features of alveoli with these cells and a scaffold, and transplanting them into lung. A recent report described differentiation of human pluripotent stem cells to basal, goblet, Clara, ciliated, type I and type II alveolar epithelial cells (26), however, the issue of reconstructing complex alveolar structure still remains unresolved. Petersen *et al.* reported a method of developing a scaffold from extracted rat lungs ; the cellular components were removed with detergent solutions and remaining airway and vasculature structures served as a scaffold. However, generating a scaffold from the cellular level remains difficult (27). Our method, to regenerate lung using fetal lung tissue, resolves both problems.

We believe that our success regenerating emphysematous lungs of pallid mice using fetal lung tissue, along with further investigation

of respiratory and exercise function in this model, will lead to further research and advances in the possibility of regenerating lung with stem cells.

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