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ORIGINAL ARTICLE

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Orthotopic foetal lung tissue direct injection into lung showed a preventive effect against paraquat-induced acute lung injury in mice

Ryo Okabe[a], Toyofumi F. Chen-Yoshikawa[a,b], Akihiko Yoshizawa[c], Tsuyoshi Hirashima[d], Masao Saito[a], Hiroshi Date[a] and Takanori Takebe [e,f,g,h,*]

[a] Department of Thoracic Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[b] Department of Thoracic Surgery, Graduate School of Medicine, Nagoya University, Nagoya, Japan
[c] Department of Diagnostic Pathology, Graduate School of Medicine, Kyoto University, Kyoto University Hospital, Kyoto, Japan
[d] Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[e] Institute of Research, Tokyo Medical and Dental University, Tokyo, Japan
[f] Division of Gastroenterology, Hepatology & Nutrition, Developmental Biology, Center for Stem Cell and Organoid Medicine (CuSTOM), Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA
[g] Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA
[h] Advanced Medical Research Center, Yokohama City University Graduate School of Medicine, Yokohama, Japan

* Corresponding author. Division of Gastroenterology, Hepatology & Nutrition, Developmental Biology, Center for Stem Cell and Organoid Medicine (CuSTOM), Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, MLC 2010, Cincinnati, OH, USA. Tel: +1-513-8037807; fax: +1-513-6365581; e-mail: takanori.takebe@cchmc.org (T. Takebe).

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Key question

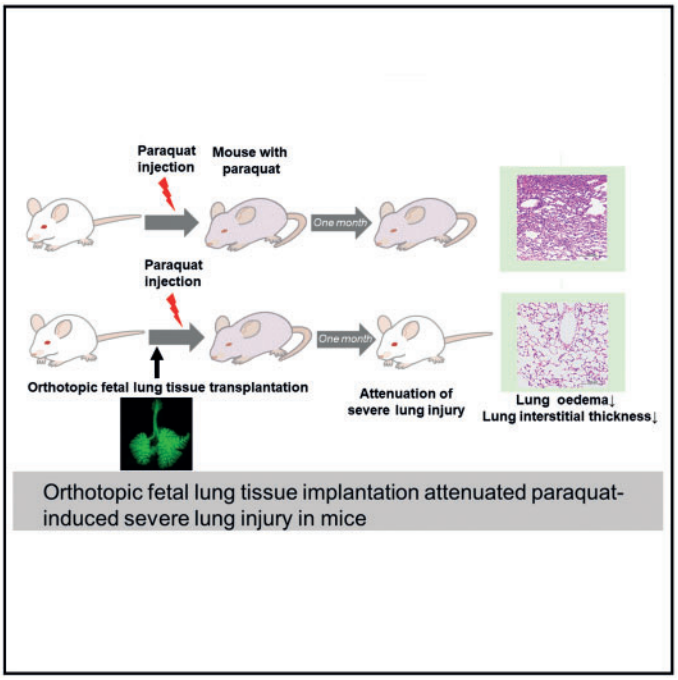
Does immature mouse foetal lung tissue protect the lungs from the effects of the injured lung?

Key finding(s)

Administration of immature foetal lungs into mice showed reduced lung inflammatory cells and lung oedema.

Take-home message

Immature foetal lungs may be useful as a new opinion for treatment of severe lung injury.



Abstract

OBJECTIVES: Lung transplantation is the only effective therapy for patients with end-stage lung disease but an organ shortage crisis necessitates the development of alternative therapies. Recent studies have highlighted the potential of foetal tissue transplantation to facilitate

the regeneration of vital organs such as liver that have been damaged by lethal diseases. Herein, with the aim of restoring pulmonary function, we hypothesized that allogenic foetal lung tissue implantation would attenuate severe respiratory failure.

METHODS: Lung tissue from the foetuses of pregnant green fluorescent protein-C57BL/6 mice at 13.5 days of gestation was injected into the left lungs of recipient mice. Severe lung injury was induced by paraquat, and we analysed the survival rate and pathohistological findings after 1 month.

RESULTS: The survival rate of the therapy group was 39%, which was significantly higher than the vehicle group at 5.9% ($P = 0.034$). Immunochemical staining showed that positive cytoplasmic stained cells with anti-interleukin-10 antibody were identified in the gland-like structure of embryonic day 13.5 foetal lung. At 4 weeks after orthotopic implantation, haematoxylin and eosin staining showed reduced lung inflammatory cells, reduced lung oedema and increased active cell proliferation of foetal lung cells. Lung injury score showed that the airway septal thickening revealed statistically significant differences between vehicle and foetal lung therapy ($P < 0.001$).

CONCLUSIONS: Immature foetal lungs improved the survival rate of mice with paraquat-induced severe lung injury, establishing the need for systematic follow-up studies. The anti-inflammatory cytokine in the tissue from embryonic day 13.5 foetal lung might suppress severe lung injury.

Keywords: Mouse foetal lung tissue • Orthotopic implantation • Engraftment

ABBREVIATIONS

IL	Interleukin
PBS	Phosphate-buffered saline

INTRODUCTION

Lung transplantation is an effective therapy for end-stage respiratory lung diseases. The registry of the International Society for Heart and Lung Transplantation contains data from 64 803 lung transplants in adults before June 2017 [1]. In Japan, the average waiting time is >800 days due to the organ shortage crisis, resulting in a considerable number of deaths while on the waiting list [2]. There is therefore a critical need to develop alternative therapies.

Recent reports have highlighted the promise of foetal tissue transplantation to restore organ dysfunction. For example, Pietrosi *et al.* [3] have reported that intrasplenic foetal liver cell infusion is a safe and well-tolerated procedure in patients with end-stage chronic liver disease. With respect to the lungs, although 1 study has reported that foetal lung fragment implant survived and differentiated in the emphysematous lung of a pallid mouse [4], the question of whether this treatment provides an effective cure for life-threatening pulmonary dysfunction remains largely untested.

Paraquat (1,1-dichloro-4,4-bipyridine) is one of the most widely used herbicides in the world. Intoxication with paraquat as a quaternary ammonium herbicide can lead to severe lung oedema and fibrotic lung disease. Many studies have demonstrated that paraquat-induced acute lung injury causes life-threatening respiratory failure due to an inflammatory response of the lungs [5–8]. There is no available treatment for paraquat poisoning, although a report by Tang *et al.* [9] described a successful case with extracorporeal membrane oxygenation therapy as a bridge to sequential bilateral lung transplantation for a patient after severe paraquat poisoning. In addition, there have been reports that mesenchymal cell therapy ameliorated severe lung injury induced by paraquat in small animals [10].

We herein hypothesized that immature mouse foetal lung tissue may protect the lungs from the effects of the injured lung and attenuate severe respiratory failure induced by paraquat via inflammatory modulation in the recipient lungs.

MATERIALS AND METHODS

Animals

C57BL/6J mice were purchased from SLC (Shizuoka, Japan) as implant recipients, whereas green fluorescent protein-C57BL/6-Tg (CAG-EGFP) mice were used as donors. These mice were bred in a pathogen-free environment 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 institutional and national guidelines (Japanese Ministry of Education, Culture, Sports, Science and Technology) and were approved by the Animal Care Committee of Kyoto University (authorization number: Med Kyo 17614).

Implantation

Pregnant green fluorescent protein-C57BL/6-Tg (CAG-EGFP) mice at 13.5 days gestation were euthanized under isoflurane anaesthesia, and foetuses were removed. Five donor foetal lungs per mouse were dissected under a microscope and then minced with scissors into small pieces in Dulbecco's modified Eagle's medium® (Sigma, St Louis, MO, USA) containing 10% foetal bovine serum, 200 U/ml penicillin and 200 µl/ml streptomycin.

We used 6–10-week-old C57BL/6 mice as recipients. They were anaesthetized with isoflurane inhalation. After a left thoracotomy, the finely minced foetal lung pieces with 50 µl of Matrigel® (Becton Dickinson, Erembodegem, Belgium) were injected into the left lung using a 25-G needle (Terumo, Tokyo, Japan). Five days after the mice were pretreated with tissue from foetal lungs, 40 mg/kg of paraquat dichloride (Wako Pure Chemical Industries, Osaka, Japan) was injected into the left quadriceps muscle under anaesthesia by isoflurane inhalation.

Experimental groups

The animals were randomly assigned into 3 groups: non-paraquat group ($n = 3$), vehicle group ($n = 17$) and therapy group

($n = 18$). Only skin incision under anaesthesia with isoflurane inhalation was performed in the non-paraquat group. We performed intramuscular paraquat injection 5 days after skin incision and Matrigel® (50 µl) injection into the left lung in the vehicle group. In the therapy group, we performed intramuscular paraquat injection 5 days after skin incision, Matrigel® and mouse foetal lung (50 µl including foetal lungs and 5 foetal lungs/animal) injection into the left lung. We gave 5 foetal lungs per mouse to assess the therapeutic effect at maximum dosing when we injected the parenchyma into the lung.

Histopathological study and immunochemical staining

We analysed 13.5 days mouse foetal lungs with E-cadherin staining and anti-interleukin (IL)-10 antibody staining. The recipient mice were sacrificed at 4 weeks after paraquat injection for histopathological examination. After tracheal intubation with a 20-G needle (Terumo, Tokyo, Japan), samples were fixed with a 1:1 ratio of phosphate-buffered saline (PBS) to optimal cutting temperature compound through the trachea at a pressure of 21 cmH_2O, infiltrated with isopentane (Wako, Tokyo, Japan) in liquid nitrogen and preserved at -80°C. The sections were prepared at 12-µm thickness for fluorescence microscopy to evaluate green fluorescence. Both non-stained histological images and green fluorescent images were captured using a Biorevo BZ-9000® (Keyence Co. Ltd., Osaka, Japan).

E-cadherin staining of foetal mouse lung was performed as follows. We blocked the left foetal lungs with 10% normal goat serum for 3–4 h at 37°C, washed them in PBST (PBS + 0.1% Triton X-100) and infiltrated them with E-cadherin (24E10, rabbit mAb, # 3195S, 100 µl; Cell Signaling Technology) as the first antibody overnight at 4°C on day 1. On day 2, we washed them in PBST 3 times on a rotator and infiltrated them with Alexa 488 (Thermo Fisher Scientific) as the second antibody. On day 3, we washed in PBST 3 times on a rotator and observed them under a fluorescence stereomicroscope and light fluorescence microscopy.

Anti-IL-10 antibody staining of foetal lung was performed as follows. We used the standard avidin–biotin–peroxidase method, rat monoclonal antibody (Abcam, Clone: JES5-2A5, Cat #: AB189392) as the first antibody and rat IgG (VECTOR LABORATORIES, Cat #: PK-6104) as the second antibody.

Tissue-clearing-based 3-dimensional imaging

We conducted clear, unobstructed brain/body imaging cocktails and computational (CUBIC®)-based foetal lung analysis with a Carl Zeiss Light Sheet Z.1® microscope [11–16]. Details are presented as follows. For the preparation of whole-organ clearing samples, adult mice (C57BL/6J) were sacrificed by an overdose of pentobarbital (100–150 mg/kg, Kyoritsu Seiyaku, Tokyo, Japan) and then perfused with 10 ml of 4% (w/v) paraformaldehyde (PFA) in PBS, 10 ml of PBS to wash out PFA and 10 ml of 50% (v/v) CUBIC-L reagent, which is a 1:1 mixture of CUBIC-L and milli-Q water through right ventricle perfusion. Two millilitres of 50% (v/v) CUBIC-L was also injected into the trachea, and the cardiopulmonary block was harvested. As a pretreatment, we infiltrated the trachea and the cardiopulmonary block with 50% CUBIC-L, which is a 1:1 mixture of CUBIC-L and milli-Q water, for 6–24 h at 37°C with gentle shaking (60/min).

Table 1: Lung injury scoring system [17]

Parameter	Score per field		
A. Neutrophils in the alveolar space	None	1–5	>5
B. Neutrophils in the interstitial space	None	1–5	>5
C. Hyaline membrane	None	1	>1
D. Proteinaceous debris filling the airspaces	None	1	>1
E. Alveolar septal thickening	<2×	2×-4×	>4×

'Hyaline membrane' is synonymous with 'pulmonary oedema'.
Score = $[(20 \times A) + (14 \times B) + (7 \times C) + (7 \times D) + (2 \times E)]/(\text{number of fields} \times 100)$.

For delipidation, we infiltrated them with CUBIC-L for 3 days at 37°C with gentle shaking (60/min). For washing, we infiltrated them with PBS for >2 h on a rotator. After washing, samples were stored for 1 day at room temperature. For immunochemical staining, we blocked them with 10% normal goat serum for 3–4 h at 37°C, washed them in PBST (PBS + 0.1% Triton X-100) and infiltrated them with E-cadherin (24E10, rabbit mAb, 3195S, 100 µl) as the first antibody overnight on day 1. On day 2, we washed them in PBST 3 times on a rotator at room temperature and infiltrated with goat anti-rabbit IgG H&L (Alexa Fluor 568, ab 175471) as the second antibody overnight at 4°C. For washing, we infiltrated them with PBST for >2 h 3 times on a rotator. After washing, samples were stored for 1 day at room temperature. For pretreatment, we infiltrated them with 50% CUBIC-R, which is a 1:1 mixture of CUBIC-R and milli-Q water, for 6–24 h at 37°C with gentle shaking (60/min). For refractive index matching, we infiltrated them with CUBIC-R for 3 days at room temperature with gentle shaking (60/min).

Measuring histological evidence of tissue injury

Slides for evaluation were prepared from suitably inflated, formalin-fixed, paraffin-embedded tissue that had been stained with haematoxylin and eosin. There were 20 high-power fields (400×) randomly selected in the slide, and each parameter was scored in a blinded fashion. Each of 5 histological findings was graded using a 3-tiered schema summarized in Table 1 according to a previous paper [17]. The resulting injury score is a continuous value between 0 and 1. There were 3 interpreters to read the slides including an experienced pathologist and 2 surgeons. The 2 surgeons read the slides after training under the guidance of the pathologist. The pathologist confirmed the result of the interpretation of the slides read by the surgeons.

Pulmonary function test

Airway resistance and compliance were measured with a flexiVent® (SCIREQ, Montreal, QC, Canada), and arterial partial oxygen pressure of the left ventricle was also measured under fraction of inspired oxygen 1.0 to compare the therapy group with the vehicle group 4 weeks after foetal lung tissue implantation. The surviving numbers in the non-paraquat-treated, vehicle and therapy groups 4 weeks after lung implantation were 3, 1 and 7 mice, respectively.

Statistical analysis of results

Statistical analyses were performed using JMP-Pro 14® software (Cary, NC, USA). Analyses were exploratory in nature. Survival rates were estimated using the Kaplan–Meier method, and differences between groups were tested using the log-rank test. The differences of lung injury score among the non-paraquat-treated, vehicle and therapy groups were each analysed by the Wilcoxon signed-rank test. A *P*-value of <0.05 was considered statistically significant. All data are shown as mean ± standard deviation.

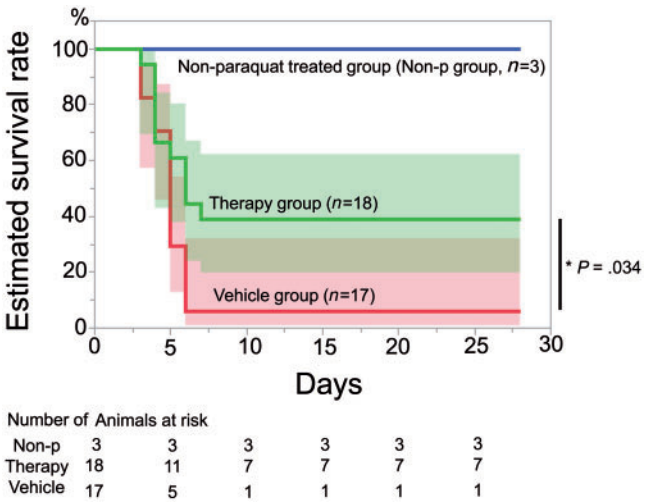


Figure 1: The survival rate in the non-paraquat-treated, vehicle and therapy groups. The 4-week survival rate in the non-paraquat, therapy and vehicle groups were 100%, 39% (95% confidence interval 19.8–62.1%) and 5.9% (95% confidence interval 0.8–32.0%), respectively.

RESULTS

Survival rate

To investigate the survival benefit of foetal lung tissue implantation before the induction of severe lung injury by paraquat treatment, 3 animals in the non-paraquat-treated group, 17 animals in the vehicle group and 18 animals in the therapy group were assigned in the following manner (Fig. 1). The surviving numbers in the non-paraquat-treated, vehicle and therapy groups 4 weeks after lung tissue implantation were 3, 1 and 7 mice, respectively. The 4-week survival rate in the non-paraquat group was 100% (3/3). The 4-week survival rate in the therapy group was significantly higher at 39%, compared with the vehicle group at 5.9% (vehicle group: 1/17, therapy group: 7/18, $P = 0.034$ by Kaplan–Meier method and log-rank test).

Histopathological changes in green fluorescent protein-foetal lung tissues in the paraquat-induced damaged lung and tissue-clearing-based 3-dimensional imaging

We initially analysed the mouse foetal lung after 13.5 days gestation, known as the pseudoglandular period. Haematoxylin and eosin staining showed the appearance of a gland-like structure. E-cadherin staining showed that planar bifurcation formed thin edges and orthogonal bifurcation created lobes and filled the interior (Fig. 2A and B). Anti-IL-10 antibody staining showed that positive cytoplasmic stained cells were identified in the gland-like structure (Fig. 2C).

At 5 days after foetal lung tissue injection into normal lung, gland-like structures and alveolar-like structures of the foetal

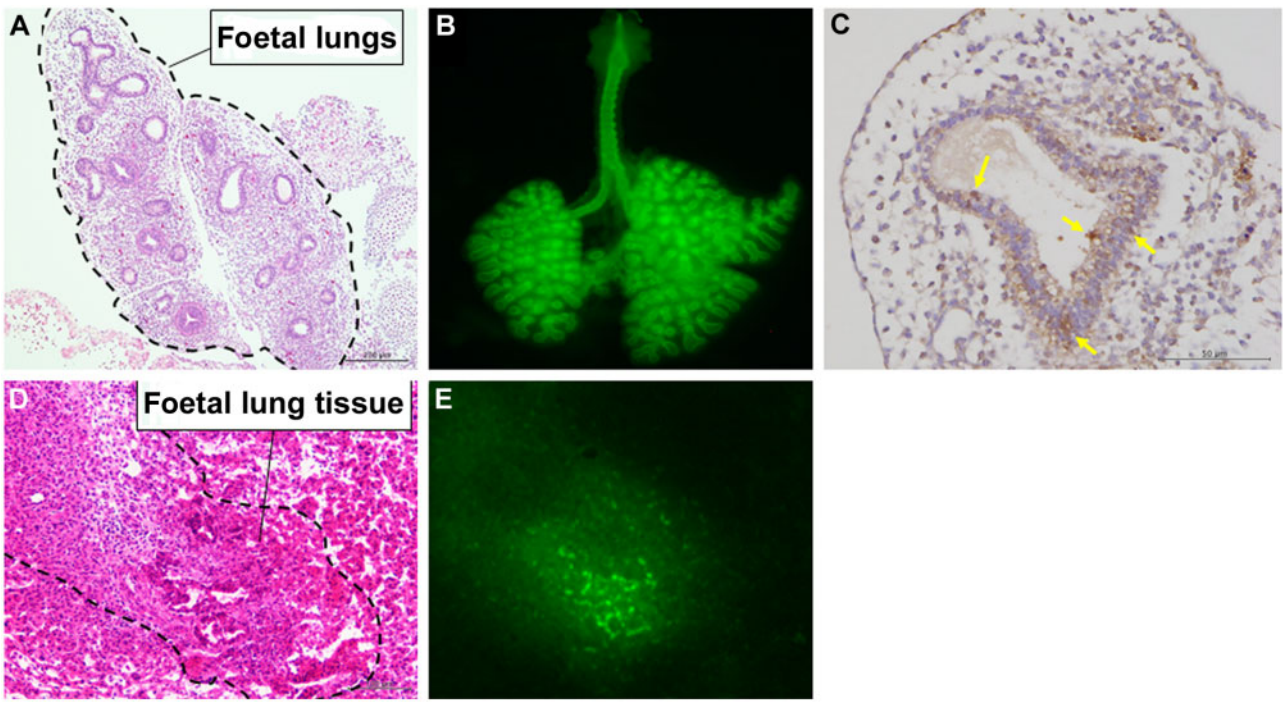


Figure 2: (**A**) 13.5 days mouse foetal lungs with haematoxylin and eosin staining. (**B**) 13.5 days mouse foetal lungs with E-cadherin staining. (**C**) 13.5 days mouse foetal lung with anti-interleukin-10 antibody. Yellow arrows indicate positive cytoplasmic stained cells. (**D**) Foetal lung in recipient lung 5 days after orthotopic transplantation. (**E**) Green fluorescence images of foetal lung in recipient foetal lungs at 5 days after orthotopic transplantation captured using the Biorevo BZ-9000®.

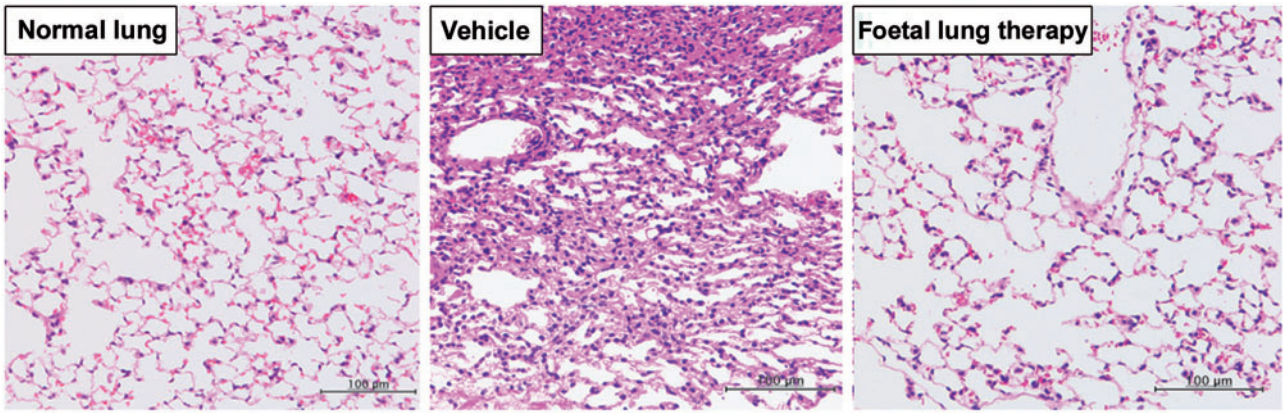


Figure 3: The histopathological therapy effect of the foetal lungs (normal lung). The normal lung structure in the non-paraquat-treated group (vehicle). Interstitial thickness 4 weeks after paraquat injection (foetal lung tissue therapy). The normal lung structure in the therapy group.

lung tissue were mixed with the recipient lung (Fig. 2D and E). Pathohistological findings showed pulmonary oedema and inflammatory cells infiltration 3–4 days after paraquat injection. At 4 weeks after orthotopic implantation, haematoxylin and eosin staining showed reduced lung inflammatory cells, reduced lung oedema and increased active cell proliferation of foetal lung cells in the recipient lung (Fig. 3). Right lung tissue also showed a normal lung structure in the therapy group. A tissue-clearing-based fluorescence stereomicroscopy image showed implanted foetal lung tissue successfully engrafted in the left lung (Fig. 4A). Light sheet microscopy showed a cluster of foetal-derived lung cells and no clear airway connection to recipient lung cells (Videos 1 and 2).

Haematoxylin and eosin staining showed that green fluorescent protein-foetal lung tissue appeared in the extrapleural engraftment (Fig. 4B–E). There were some cells of foetal lungs differentiated into surfactant protein-C-positive cells, constructing alveolar-like structures (Fig. 4F).

Measuring histological evidence of tissue injury

The lung injury score was 0.015 in the normal lung, 0.18 in the vehicle and 0.16 in the foetal lung therapy (all Fig. 5). There was no statistical difference in total injury score between vehicle and therapy groups, but the airway septal thickening revealed statistically significant differences between vehicle and foetal lung therapy (total injury score: $P = 0.25$, airway septal thickening: $P < 0.001$, Fig. 5). We analysed each parameter and the injury score per field by Student's *t*-test with the violin plot.

Pulmonary function test

Effects on pulmonary function and partial pressure on arterial oxygen in the left ventricle. The partial pressure of oxygen/fraction of inspired oxygen ratio in the left ventricle in the vehicle and therapy groups was almost the same. The maximum airway pressure was 8.91 ± 1.47 cmH_2O in the non-paraquat-treated group ($n = 3$), 9.75 cmH_2O in the vehicle group ($n = 1$) and 9.21 ± 1.22 cmH_2O in the therapy group ($n = 6$). The compliance was 0.041 ± 0.017 ml/cmH_2O in the non-paraquat-treated group, 0.036 ml/cmH_2O in the vehicle group and 0.037 ± 0.008 ml/cmH_2O in the therapy group. The arterial partial oxygen pressure under fraction of inspired oxygen 1.0 was 449 ± 13.5 mmHg in the non-paraquat-treated group and 517 mmHg in the vehicle group, whereas it was 427 ± 28.4 mmHg in the therapy group.

DISCUSSION

Here, we demonstrate, for the first time, the therapeutic effects of mouse foetal lung tissue on acute lung injury induced by paraquat. In this experiment, the main cause of death was paraquat-related respiratory failure in the vehicle and therapy groups due to severe lung injury and pulmonary oedema. A previous report showed that foetal mouse lung fragments engrafted and differentiated in the emphysematous lung of pallid mice, yet it was unclear if the foetal tissue transplant offers the efficacy through improved respiratory function in a diseased condition [4]. Although the in-depth therapeutic mechanism remained unclear in the current study, the survival rate of the therapy group was significantly higher than the vehicle group. In agreement with this, histopathological findings showed a massive reduction in infiltration inflammatory cells and the improvement in lung oedema. Lung injury score showed an improvement in airway septal thickening. In addition, immunochemical staining showed that there were positive cytoplasmic cells in the 13.5 days mouse foetal lung with anti-IL-10 antibody.

To the best of our knowledge, therefore, this is the first report to show the efficacy of foetal lung tissue implantation in the treatment of acute lung injury.

Several known toxic mechanisms of paraquat include: (i) the generation of the superoxide anion, which can lead to the formation of more toxic reactive oxygen species, such as hydrogen peroxide and hydroxyl radical; (ii) the oxidation of cellular reduced nicotinamide adenine dinucleotide phosphate (NADPH), the major source of reducing equivalents for the intracellular reduction in paraquat, which results in the disruption of important NADPH-dependent requiring biochemical processes; and (iii) lipid peroxidation, which results in the oxidative degeneration of cellular polyunsaturated fatty acids [18]. Recent studies have attempted to target these mechanisms to treat a paraquat-induced injury condition [5–8]. For example, artificial surfactants, chymostatin curcumin, prednisolone and pirfenidone have been used in preclinical studies on acute injury induced by paraquat.

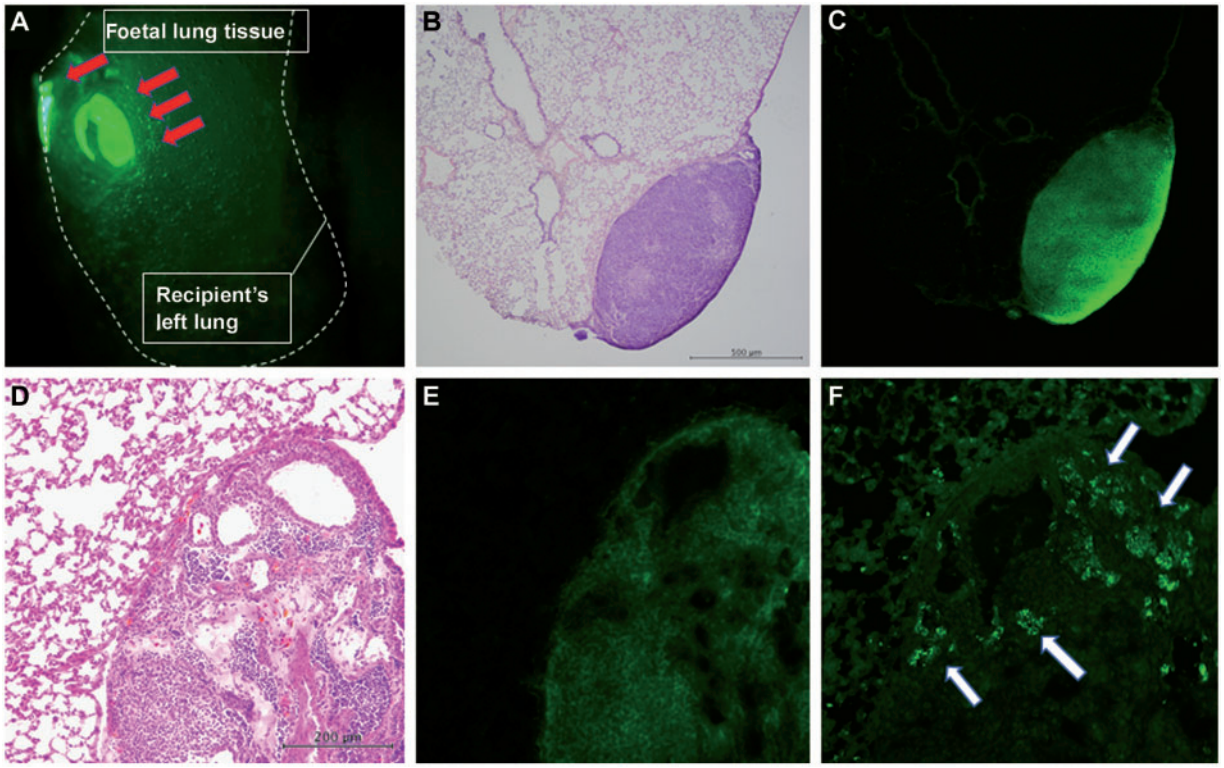


Figure 4: Images of the foetal lungs 4 weeks after orthotopic transplantation. (**A**) Light fluorescence microscopy image in the therapy group. A tissue-clearing-based fluorescence image obtained by stereomicroscopy in the therapy group (red arrows). (**B** and **C**) Dense cell population areas were seen in foetal lungs and captured using a Biorevo BZ-9000®. (**D–F**) The alveoli-like structures were seen in other areas and captured using a Biorevo BZ-9000®. Slides (**D**) and (**E**) are the same specimen, but the slides are different. There were some cells stained with SP-C (white arrows).

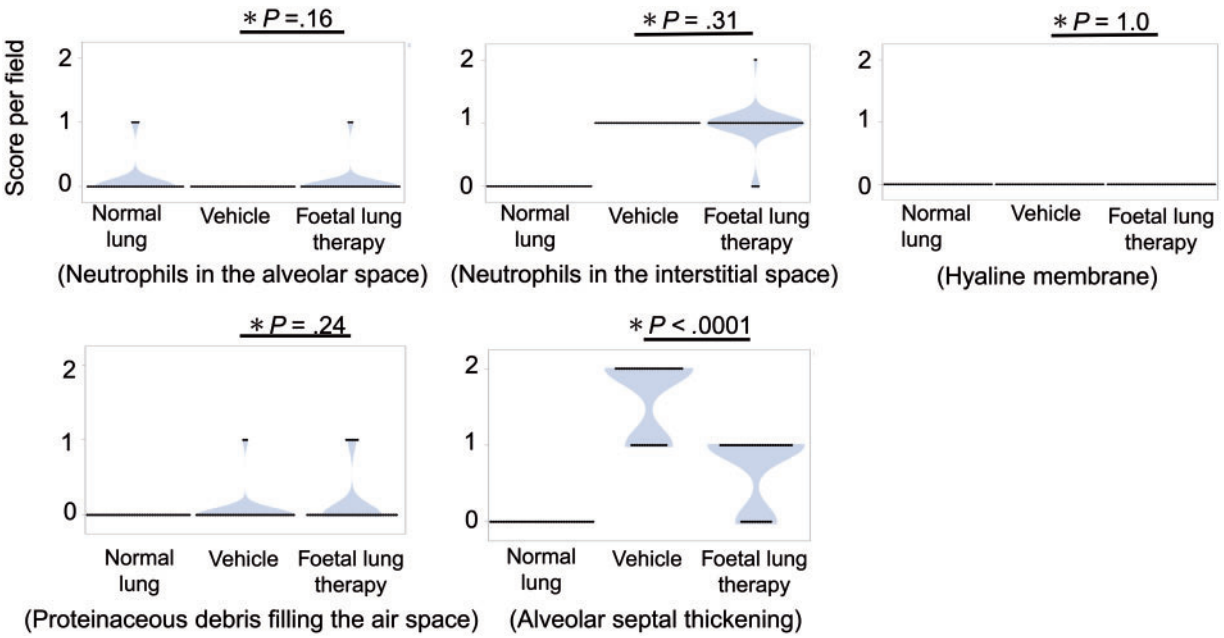
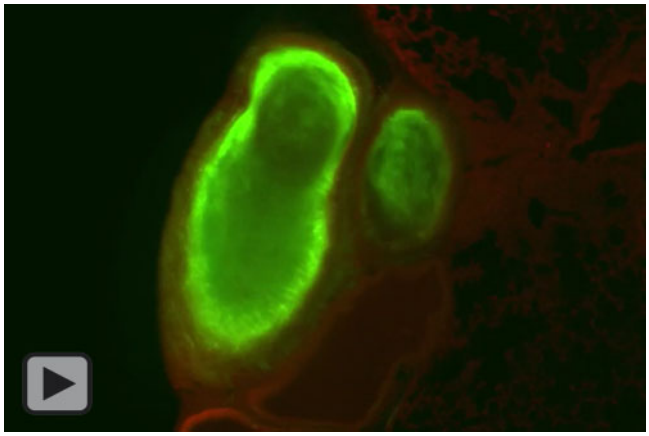
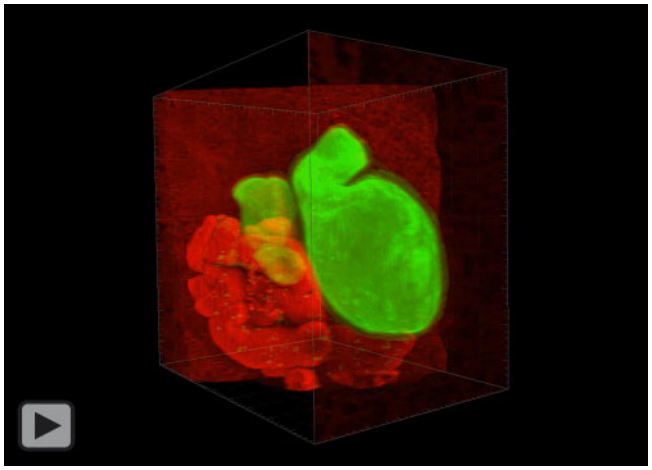


Figure 5: Lung injury score per field in the left lung of the non-paraquat-treated, vehicle and therapy groups.

In contrast, mesenchymal stem cell transplant has also shown antifibrotic therapeutic effects in animal models of lung injury caused by paraquat poisoning, presumably through the suppression of inflammatory mechanisms further downstream including cytokine modulation [10]. Immunochemical staining showed that positive cytoplasmic stained cells with anti-IL-10 antibody were identified in the gland-like structure and might indicate that embryonic day 13.5 foetal lung was an appropriate method as



Video 1: Cross-section image: Light sheet microscopy showing a cluster of foetal-derived lung cells and no clear airway connection to recipient lung.



Video 2: Overall image: Light sheet microscopy showing a cluster of foetal-derived lung cells and no clear airway connection to recipient lung.

treatment option [19–21]. Therefore, this anti-inflammatory effect might be responsible for the improvement in the outcome and the airway septal thickening in the lung injury score.

Interestingly, unlike conventional mesenchymal stem cell therapy, all the survived cases harboured extrapleural tissue engraftment including stroma and alveoli in the therapy group, implying that the engrafted mesenchymal tissues provide persistent support for severe lung injury induced by paraquat. Persistent tissue engraftment could potentially prolong the therapeutic impact of foetal lung cells. One possible reason for this is the presence of abundant endothelial and other supportive lineages together with stromal cells. Despite the absence of a uniform conducting airway, our approach highlighted a potential avenue of cell therapy with the use of an immature (foetal) tissue implant approach to ameliorate pulmonary function.

Limitations

Our study has several limitations. For example, the optimal dosage for this implant approach is still unclear. Titrating the doses of foetal lung tissue in future systematic investigations will be critical to progress this therapy. The administration route also still requires careful investigation. We preferred to use direct percutaneous injection into the lung parenchyma because there were abundant recipient's vessels around the foetal lung tissue with minimal airway embolism. However, the reconstruction of the recipient airway might require other operative approaches. In addition, post-treatment of paraquat poisoning with foetal lung tissue may be needed if we intend to use this tissue as a treatment for lung injury in the future. In our study, the recipient mice were pretreated with foetal lung tissue before paraquat was injected into the left quadriceps muscle because we needed to confirm foetal lung cells engraftment before paraquat-induced severe lung injury. The method of foetal lung tissue implantation before the paraquat injection was a limitation because this sequencing would be better in reverse if we think this therapy has clinical utility. In a previous report, it was found that functional vascularization of transplant or implant cells or organoids was observed in 2 days and functional engraftment of them was observed in 3 or 4 days [22, 23]. However, mice with severe lung injury induced by paraquat were dead in 3 days in the earlier case. This is why we had to inject foetal lung tissue prior to paraquat injection for the estimation of the pathophysiological exacerbation in the acute phase. We hope to overcome this problem, but foetal lung tissue therapy is expected to be adaptable for the subacute disorders in 7–14 days. Preventive lung foetal tissue therapy might be a novel therapy to attenuate severe lung injury and improve the survival rate, even though the foetal lung tissue was injured by paraquat. In addition, it is possible that foetal tissues would undergo a cancerous change in the long term. However, the prognosis for the patients is particularly poor and this new therapy will be expected to be an option as a bridge to lung transplantation or a beneficial therapy for the patients with respiratory failure who are not suitable for lung transplantation.

CONCLUSION

In conclusion, immature foetal lung tissue improved the survival rate of mice with paraquat-induced severe lung injury, establishing the need for systematic follow-up studies. The anti-inflammatory cytokine in the tissue of foetal lungs on embryonic day 13.5 might suppress severe lung injury.

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Conflict of interest: none declared.

Author contributions

Ryo Okabe: Conceptualization; Data curation; Formal analysis; Writing—original draft. **Toyofumi F. Chen-Yoshikawa:** Supervision; Writing—review & editing. **Akihiko Yoshizawa:** Writing—review & editing. **Tsuyoshi Hirashima:** Writing—review & editing. **Masao Saito:** Writing—review & editing. **Hiroshi Date:** Writing—review & editing. **Takanori Takebe:** Funding acquisition; Supervision; Writing—review & editing.

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