



Transplantation of multi-ciliated airway cells derived from human iPS cells using an artificial tracheal patch into rat trachea

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Title

Transplantation of multi-ciliated airway cells derived from human iPS cells using an artificial tracheal patch into rat trachea

Running title

Transplantation of hiPSC-airway cells using artificial tracheal patch

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For Peer Review

Abstract

Tracheal resection is often performed for malignant tumors, congenital anomalies, inflammatory lesions, and traumatic injuries. There is no consensus on the best approach for the restoration of tracheal functionality in patients with tracheal defects. Artificial grafts made of polypropylene and collagen sponge have been clinically used by our group. However, two months are required to achieve adequate epithelialization of the grafts in humans. This study aimed to investigate the feasibility of transplantation therapy using an artificial trachea with human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs). Collagen vitrigel membrane, a biocompatible and absorbable material, was used as a scaffold to cover the artificial trachea with hiPSC-MCACs. Analyses of hiPSC-MCACs on collagen vitrigel membrane were performed by immunocytochemistry and electron microscopy and by assessing ciliary beat frequency. Along with the artificial trachea, hiPSC-MCACs were transplanted into surgically created tracheal defects of immunodeficient rats. The survival of transplanted cells was histologically evaluated at 1 and 2 weeks after the transplantation. The hiPSC-MCACs exhibited motile cilia on collagen vitrigel membrane. The surviving hiPSC-MCACs were observed in the endotracheal epithelium of the tracheal defect at 1 and 2 weeks after transplantation. These results suggest that hiPSC-MCAC is a useful candidate for tracheal reconstruction.

Keywords: human induced pluripotent stem cell; tracheal reconstruction; multi-ciliated airway cell; artificial trachea; collagen vitrigel membrane; tracheal epithelialization

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1. Introduction

Ciliated epithelium of the trachea plays an important role in the removal of foreign substances and protects the airways against external invasion by mucociliary clearance (Lopez-Vidriero, 1989). Tracheal defects due to surgical resection for malignant tumors, congenital anomalies, inflammatory lesions, and trauma impair the basic biological functions such as speech, respiration, and deglutition. Therefore, these patients have a markedly diminished quality of life. Post-surgical reconstruction of trachea with complete functionality is challenging. Autologous tissue implantation using cartilage, muscle, and skin grafts has been used for the treatment of post-surgical tracheal defects (Caputo et al., 1961; Grillo, 2002). However, these techniques are invasive and inevitably involve multiple-staged surgeries. Moreover, complete functional restoration is difficult because reconstructed trachea is not covered with mucociliary surface. An airway framework with an optimal stiffness and endotracheal mucociliary surface is necessary for an ideal tracheal regeneration (Belsey, 1950; Neville et al., 1990).

Nakamura et al. have produced an artificial trachea using *in situ* tissue engineering technique (Teramachi et al., 1995, 1997; Nakamura et al., 2000), which was made of a polypropylene mesh and spiral stent as a framework with collagen sponge. The polypropylene mesh is a conventional material developed for clinical application and has good biocompatibility and morphological adjustability. The collagen sponge scaffold used for artificial trachea comprised type I and III collagen of the porcine origin. It provides favorable conditions for the infiltration of host cells and regulatory factors released from the surrounding tissues. Therefore, it is an appropriate material for use as a framework for tracheal reconstruction.

In a clinical study by Omori et al., the polypropylene scaffold with collagen sponge has been shown to possess adequate mechanical strength to support human tracheal regeneration

(Omori et al., 2005, 2008). Because 2 months are required for sufficient epithelialization on the luminal side of the scaffold, further promotion of epithelialization is desirable. To reduce the risk of infection and respiratory complications during the early postoperative stage, epithelial regeneration on the artificial graft should be completed as early as possible.

Autologous transplantation of fibroblasts and adipose-derived stem cells has been performed in combination with artificial grafts for tracheal epithelial regeneration in animal experiments (Nomoto et al., 2008; Okano et al., 2009; Tani et al., 2012; Kobayashi et al., 2010). However, these cells only promote the extension of epithelial layer in the area surrounding the tracheal defect. Therefore, this technique is effective only in a limited range of tracheal defects. Owing to these limitations, here we examined the efficacy of covering the luminal surface of the artificial graft with airway epithelial cells. Autologous airway epithelial cells and human induced pluripotent stem cell (iPSC)-derived airway epithelial cells are readily available cell sources. The use of autologous tissues involves invasive procedures at the donor site, and the supply of cells is limited. In contrast, iPSC-derived cells may be obtained from the patient or a human leukocyte antigen (HLA)-matched donor. iPSCs have the capacity for unlimited proliferation and multi-lineage differentiation. The use of iPSC-derived airway epithelial cells to cover the transplanted artificial graft may minimize the complications of infection and transplant rejection. Therefore, iPSC-derived cells are an ideal cell source in tracheal regenerative medicine. In our previous studies, embryoid bodies, including ciliated epithelium derived from mouse iPSCs, were transplanted to tracheal defects of nude rats with artificial trachea (Ikeda et al., 2017). Although ciliated epithelial structures were confirmed, their attributes were not similar to those of endotracheal epithelium, but appeared as cystic tissue under the epithelium, indicating that transplanted epithelial cells need to engraft at the appropriate site and that there is a need to improve the induction efficiency by minimizing the inclusion of other cell types during transplant.

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Gotoh et al. have established a method to generate hiPSC-derived alveolar epithelial progenitor cells via purified progenitor cells using an antibody to carboxypeptidase M (CPM) as a surface marker of NKX2-1⁺ ventralized anterior foregut endoderm cells (VAFECs) (Gotoh et al., 2014). They reported a method for an efficient induction of functional multi-ciliated airway cells (MCACs) from hiPSC-derived CPM⁺ cells (Konishi et al., 2016). They employed immunofluorescent staining for marker proteins, electron microscopy, and ciliary function analyses to demonstrate that hiPSC-derived MCACs (hiPSC-MCACs) are similar to normal MCACs. They used a polyethylene terephthalate (PET) membrane as a scaffold and prepared the epithelial cell sheet. For the successful clinical application of hiPSC-derived cells in tracheal regenerative medicine, a biocompatible and absorbable material is required as a scaffold. Collagen vitrigel membrane is a transparent biomaterial characterized by a highly organized fibrillar nanostructure (Takezawa et al., 2004, 2007). Tracheal reconstruction studies have indicated the feasibility of using collagen vitrigel membrane for accelerating epithelial regeneration (Tada et al., 2008, 2012). Therefore, collagen vitrigel membrane is considered as a candidate scaffold for organizing iPSC-derived airway epithelial sheet graft.

There were two primary aims of this study: study 1 (*in vitro*) aims to demonstrate the induction of hiPSC-MCACs on collagen vitrigel membrane, and study 2 (*in vivo*) aims to transplant the cell sheet with an artificial trachea into the tracheal defects of immunodeficient rats and to examine the survival of cells. Herein, we investigated the potential of transplantation therapy using an artificial trachea loaded with hiPSC-MCACs.

2. Materials and Methods

2.1. Human iPS cells

The hiPSC line 201B7 used in this study was donated by RIKEN BioResource Center. hiPSCs were maintained according to the methods published by the Center for iPS Cell

Research and Application, Kyoto University. In brief, the cells were initially maintained on mitomycin C (Kyowa Hakko Kirin Co. Ltd, Tokyo, Japan)-treated SNL feeder cell layers in human iPS cell medium. Cells were cultured in the feeder-free system for 3–10 passages prior to the induction. The cells were maintained on dishes coated with Geltrex (Life Technologies, Tokyo, Japan) in Essential 8 medium (Life Technologies). These were passaged with a split ratio of 1:3–1:12 after being washed with PBS, incubated in 0.5 mM EDTA/PBS for 10 minutes at 37°C, suspended in Essential 8 medium with 10 μ M of Y-27632 (Wako, Osaka, Japan), and seeded on Geltrex-coated dishes.

2.2. Airway cell induction

The induction of MCACs from hiPSCs was performed according to the method described by Konishi et al. (Konishi et al., 2016). In brief, hiPSCs cultured in the feeder-free system were seeded on Geltrex-coated plates in the medium containing RPMI1640 medium (Nacalai Tesque, Kyoto, Japan), 1 $\times$ B27 supplement (Thermo Fisher, Kanagawa, Japan), 50 U/mL of penicillin/streptomycin, 100 ng/mL of human activin A (R/D system, Osaka, Japan), 1 μ M of CHIR99021 (Axon Medchem, Groningen, The Netherlands), 10 μ M of Y-27632 (day 0–1), and 0.25 mM (day 1) and 0.125 mM (day 2–6) of sodium butyrate (Wako) to allow the differentiation of cells into endoderm. After 5 days of differentiation, FOXA2⁺ endoderm cells were obtained. From days 6 to 28, the basal medium comprised DMEM/F-12 with GlutaMAX (Life technologies), 1 $\times$ B27 supplement, 50 U/mL of penicillin/streptomycin, 0.05 mg/mL of L-ascorbic acid (Wako), and 0.4 mM of monothioglycerol (Wako). From day 6, the endoderm cells were cultured for 4 days in the basal medium containing 100 ng/mL of human recombinant noggin (Human Zyme, Chicago, IL, USA) and 10 μ M of SB-431542 (Stem RD, Burlingame, CA, USA) to differentiate into anterior foregut endoderm cells. From day 10, the cells were cultured for 4 days in basal medium containing 20 ng/mL of human recombinant

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BMP4 (Human Zyme), 2.5 μ M of CHIR99201, and 0.5 μ M of all-trans retinoic acid (Sigma-Aldrich, Tokyo, Japan). On day 14, NKX2-1⁺ VAFECs expressing CPM on their surface were induced. CPM⁺ cells were purified from the cells cultured for 14 d by magnetic activated cell sorting (MACS) using mouse anti-human CPM antibodies (Leica Microsystems, Tokyo, Japan). Subsequently, CPM⁺ cells were mixed in 1:1 growth factor-reduced Matrigel (Corning, NY, USA)/basal medium containing 3.0 μ M of CHIR99021, 100 ng/mL of FGF10 (Wako), and 10 μ M of Y-27632 and embedded on a 12-well cell culture insert made from PET (Corning, #353292). The cells embedded with Matrigel were maintained by changing the basal medium containing 3.0 μ M of CHIR99021, 100 ng/mL of FGF10, and 10 μ M of Y-27632 in the lower chamber for 14 days. Proximal airway epithelial progenitor cell (PAEPC) spheroids were induced from CPM⁺ VAFECs on day 28.

PAEPC spheroids were differentiated into mature airway epithelial cells after culture in PneumaCult-ALI Maintenance medium (STEMCELL Technologies, Vancouver, Canada), 10 μ M of Y-27632, 4 μ g/mL of heparin (Nacalai Tesque), 1 μ M of hydrocortisone (Sigma-Aldrich), and 10 μ M of DAPT(Wako) for 14 days. On day 42, single cell suspension obtained by enzymatic treatment of the proximal airway epithelial cell spheroid was seeded on cell culture insert. By reducing the medium in the upper chamber, air-liquid interface (ALI) condition was started. Airway epithelial cells were induced on PET or collagen vitrigel membrane in the form of a monolayer cell sheet by culturing in the ALI condition for 14 days.

In the original method (Konishi et al., 2016), a cell culture insert with a membrane made from PET was used. We used a cell culture chamber in which the membrane was made from a collagen vitrigel (ad-MED Vitrigel; Kanto Chemical Co. Inc., Tokyo, Japan) by vitrifying a disk of conventional collagen gel and subsequently rehydrating the material (Takezawa et al., 2004, 2007).

2.3. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes. After permeabilization with 0.2% Triton X-100 for 5 minutes, the cells were treated with 1% bovine serum albumin (BSA; Wako) for 10 minutes at room temperature. After overnight treatment with primary antibodies (Table 1) at 4°C, the specimens were rinsed thrice with PBS and treated with fluorescent-labeled secondary antibodies for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Tokyo, Japan). Signals were visualized and captured using an Olympus BX50 microscope equipped with fluorescent filters (Olympus, Tokyo, Japan) or a BioRevo fluorescent microscope (BZ-X710, Keyence, Osaka, Japan). To quantify the induction efficiency of MCACs, the rest of each graft was utilized. The total epithelial cell number in each field was counted based on the expression of E-cadherin using Hybrid Cell Count software (BZ-X710, Keyence).

2.4. Analysis of ciliary beat frequency (CBF)

The hiPSC-derived airway epithelial sheet prepared on PET membrane and collagen vitrigel membrane were analyzed for CBF. The airway epithelial sheets were attached on 35-mm dishes (Corning). The dishes were filled with PneumaCult-ALI Maintenance medium and laid on the stage of an upright microscope (BX-51; Olympus, Tokyo, Japan) equipped with a stage heater (ST-71; Blast, Kanagawa, Japan) and a high-speed camera (FASTCAM misi UX50; Photron, Tokyo, Japan). Apical surfaces of the airway epithelial sheets were observed using the stage heater set at 37°C. The movement of motile cilia in airway epithelial sheets was recorded at 500 frames per second, and mean CBFs in the region captured by the high-speed camera were analyzed by Cilia FA, which is a plugin software of ImageJ.

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2.5.Electromicroscopy

After the induction of MCAC on PET membrane or collagen vitrigel membrane, the apical surface structure of hiPSC-MCACs was confirmed by scanning electron microscopy and transmission electron microscopy.

For scanning electron microscopy, cells on the membrane were fixed overnight with 4% paraformaldehyde/2% glutaraldehyde in phosphate buffer at 4°C. The fixed cells were subsequently incubated in 1% osmium tetroxide (Nacalai Tesque), dehydrated, and dried using a critical point drying method and then coated with a thin layer of platinum palladium. Specimens were observed using the scanning electron microscope S-4700 (Hitachi Co., Tokyo, Japan).

For transmission electron microscopy, cells on the membrane were fixed overnight with 4% paraformaldehyde/2% glutaraldehyde in phosphate buffer at 4°C. The fixed cells were incubated in 1% osmium tetroxide for 2 hours and dehydrated by washing in ascending concentrations of ethanol. Subsequently, samples were embedded in epoxy-resin and DMP-30 (Nacalai Tesque). Thin sections were stained with uranyl acetate and lead citrate. The specimens were examined under a transmission electron microscope S-4700 (Hitachi).

2.6. RNA isolation and RT-PCR

Total RNA isolation was performed using RNeasy Mini kit (Quiagen, Hilden, Germany), and the genomic DNA was eliminated from the solution using a TURBO DNA-free™ kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instruction. For cDNA synthesis from 1 µg of total RNA, ReverTraAce-α™ kit (Toyobo, Osaka, Japan) and oligo(dT) 20 primers were used. PCR was performed using ExTaq HS (Takara Bio, Shiga, Japan). Primer sequences are shown in Table 2.

2.7. Animals

Male F344/NJcl-rnu/rnu nude rats were used for transplantation experiments, which were purchased from CLEA Japan (Osaka, Japan). The age of transplant recipients was 8–9 weeks and weight was 180–230 g. The animal experimental protocol for this study was approved by the Animal Experimentation Committee of Kyoto University. All animals received humane care in compliance with the “Guidelines for Animal Experiments of Kyoto University”.

2.8. Transplantation

A total of 20 male nude rats were used as recipients of the transplant (Fig. 1A). Fourteen nude rats received transplantation of hiPSC-MCACs cultured for 63–65 days. Cells cultured for 21–23 days on collagen vitrigel membrane in ALI condition were placed on an artificial trachea and transplanted into tracheal defects (Fig. 1B). The animals were divided into two groups with different postoperative periods and sacrificed afterwards: 1 week ($n = 7$) or 2 weeks ($n = 7$). Tracheostomy was performed under general anesthesia with intraperitoneal injection of a mixture of midazolam (2 mg/kg), butorphanol (2.5 mg/kg), and medetomidine (0.15 mg/kg). The trachea was exposed by a midline skin incision and separation of the sternohyoid and sternothyroid muscles. A square tracheal defect was created in each rat by incision with a scalpel. The size of the defect was 2.5 mm in length (equivalent to two tracheal rings) and 2 mm in width. The scaffold was laid over the tracheal defects with the cells facing into the lumen, after which the sternohyoid and sternothyroid muscles and incised skin were sutured.

As a control for the transplantation of hiPSC-MCACs, the remaining animals were transplanted with an artificial trachea covered with collagen vitrigel membrane without cells. Histological examination was performed at 1 ($n = 3$) or 2 weeks ($n = 3$) after transplantation. After euthanasia, the trachea along with the sternohyoid and sternothyroid muscles was extirpated en bloc.

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2.9. Immunohistochemistry

One or 2 weeks after transplantation, the animals were euthanized with carbon dioxide. The trachea along with the sternohyoid and sternothyroid muscles was immersed in 4% PFA for 1 day. Subsequently, these were immersed in 10%, 20%, and 30% sucrose for 8 hours each and embedded in optimal cutting temperature (OCT) Compound (Sakura Finetek Japan, Tokyo, Japan). As a cross-sectional control, hiPSC-MCACs cultured for 64 days were fixed in 4% PFA and embedded in OCT compound. The embedded samples were sliced into 10-μm sections at 200-μm intervals. For immunohistochemistry, the sections were permeabilized with 0.2% Triton X-100 for 5 minutes. These were treated with 1% BSA for 10 minutes at room temperature and incubated overnight with primary antibodies (Table 1) at 4°C, followed by washing thrice with PBS and incubation of secondary antibodies for 1 hour at room temperature. After a final rinse with PBS, nuclei were stained with DAPI.

The surviving transplanted cells were identified by the expression of human nuclear antigen (HNA) and normal nuclear morphology as demonstrated by DAPI staining. HNA-, DAPI-, and E-cadherin-positive cells were considered as the surviving transplanted epithelial cells. The number of surviving cells in the tracheal epithelium was counted in two areas with 350-μm width (at the tracheostomy side and center) to examine the influence of host epithelial regeneration.

The average of the number of surviving transplanted cells at the tracheostomy side and that at the center was used to calculate the number of surviving transplanted cells in the tracheal epithelium. All immunostained sections were photographed and calculations were performed using the BioRevo fluorescent microscope (BZ-X710, Keyence).

2.10. Statistical analysis

The proportion of acetylated α -tubulin (Ac-Tub) positive cells in the epithelial cells was compared between the two groups using a Mann–Whitney U-test. Data are expressed as mean $\pm$ standard deviation (SD); p-values < 0.05 were considered statistically significant.

3. Results

3.1. Study 1 (*in vitro*): Preparation of hiPSC-MCACs on collagen vitrigel membrane for transplantation

In this study, we used a stepwise method for the induction of MCACs from hiPSCs according to the method described by Konishi et al. (2016). The characteristics of cells were determined at each differentiation step.

On day 6, the immunocytochemical examination of cells revealed a positive expression of FOXA2, which is a transcription factor for a master regulator of endoderm development (Fig. 2A). On day 14, the cells exhibited a positive expression of NKX2-1, which is a factor that is expressed in VAFECs (Fig. 2B). On day 28, spheroids were identified in 3D culture (Fig. 2C). On days 50–56 of the 3D-ALI protocol, ciliary beats were readily observed on the apical surface of cells by phase contrast microscopy. Immunocytochemistry, electron microscopy, and the analysis of CBF were performed to elucidate whether the cells induced on PET membrane and collagen vitrigel membrane were comparable to hiPSC-MCACs reported by Konishi.

Immunocytochemistry revealed positive expressions of Ac-Tub (marker of cilia) and E-cadherin (an adherence junction marker). A number of cilia-like structures marked by Ac-Tub antibody were observed in the epithelial cell sheet composed of E-cadherin-positive cells, both on the PET membrane and collagen vitrigel membrane (Fig. 3A). We quantified the proportion of Ac-Tub positive cells among the E-cadherin-positive epithelial cells in three randomly chosen visual fields for each specimen. The proportion of Ac-Tub-positive cells on PET

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membrane and on collagen vitrigel membrane was $31.7 \pm 16.4\%$ ($n = 15$) and $16.0\% \pm 13.8\%$ ($n = 22$), respectively. The proportion of Ac-Tub-positive cells on PET membrane was significantly higher than that on collagen vitrigel membrane ($p = 0.0013$; Fig. 3B).

RT-PCR showed a positive expression of the pluripotent marker *OCT4* on hiPSCs (Fig. 3C). After the induction of MCACs from hiPSCs, the cells on PET membrane or collagen vitrigel membrane did not express *OCT4*, which indicated the absence of any undifferentiated iPS cell. Instead, hiPSC-MCACs differentiated on PET membrane or collagen vitrigel membrane expressed *SNTN*, which is a specific marker of MCACs. In addition, both hiPSC-MCACs expressed *p63*, which is a marker of basal cells (Fig. 3C).

The morphology of hiPSC-MCACs was examined by electron microscopy. Long cilia-like structures on the apical surface were observed by scanning electron microscopy and transmission electron microscopy (Fig. 4A). Transmission electron microscopy also showed a “9 + 2” structure [i.e., nine doublet microtubules arranged in a circle around two central singlet microtubules with dynein arms (Fig. 4A-c,d)], which is a specific and fundamental pattern of motile cilia (Gibbons et al., 1965). These findings virtually confirmed that these structures were cilia comprising complete microtubules.

Ciliary beating was recorded using a high-speed camera. Time lapse images of cilia of airway epithelial sheets, which were prepared from hiPSC-MCACs on PET membrane and collagen vitrigel membrane (Fig. 4B), are shown. Ciliary beating was observed on the apical surface of the cells (Movie 1, 2). Metachronal wave of cilia (Machemer, 1972) was observed on both membranes.

CBFs of the motile cilia observed in the airway epithelial sheets were analyzed by CiliaFA. CBF of hiPSC-MCACs differentiated on PET membrane and collagen vitrigel membrane were 10.35 ± 0.81 and 10.81 ± 0.50 Hz, respectively (Fig. 4C). A similar CBF was assessed in normal rat tracheal epithelial cells (10.93 ± 1.04 Hz, $n = 5$).

3.2. Study 2 (*in vivo*): Transplantation of hiPSC-MCACs to immunodeficient rat

hiPSC-MCACs on collagen vitrigel membrane (Supplemental Fig. 1A) were placed on an artificial trachea and transplanted into the tracheal defects of nude rats (Fig. 1). The surviving transplanted epithelial cells were identified by their expression of HNA, E-cadherin, and DAPI. Surviving transplanted epithelial cells were found in six out of seven animals at 1 week after transplantation and in all seven animals at 2 weeks after transplantation. hiPSC-MCACs with positive expressions of Ac-Tub, HNA, E-cadherin, and DAPI were found in two and six out of the seven animals at 1 and 2 weeks after transplantation, respectively (Fig. 5A, 5B). The transplanted epithelial cells were thick-layered. Conversely, in both the control models, the epithelial cells were relatively thin-layered (Fig. 5A, 5B). The proportions of surviving HNA-, E-cadherin-, and DAPI-positive cells among the E-cadherin-positive epithelial cells in the 1- and 2- week survival groups were $8.5\% \pm 13.3\%$ and $14.3\% \pm 8.61\%$, respectively (1 week, $n = 6$; 2 weeks, $n = 7$). The proportion of surviving Ac-Tub-, HNA-, E-cadherin-, and DAPI-positive cells among the E-cadherin-positive epithelial cells in the 1- and 2- week survival groups were $0.5\% \pm 0.3\%$ and $1.7\% \pm 2.8\%$ (1 week, $n = 2$; 2 weeks, $n = 6$). The proportion of Ac-Tub-, HNA-, E-cadherin-, and DAPI-positive cells among the transplanted HNA- and E-cadherin-positive epithelial cells in the 1- and 2- week survival groups were $2.8\% \pm 1.0\%$ and $7.8\% \pm 8.8\%$ (1 week, $n = 2$; 2 weeks, $n = 6$, Fig. 5C). These results indicated that hiPSC-derived cells survived as epithelial cells in tracheal defects of nude rats for 2 weeks following transplantation. Furthermore, hiPSC-MCACs were confirmed in the transplanted tissues. There was no significant difference with respect to the proportion of surviving transplanted cells between the 1- and 2-week survival groups. Since there no significant differences were noted between the tracheostomy side and center, the average number of surviving transplanted cells is shown in the graph. The proportions of Ac-Tub-, E-cadherin-, and DAPI-positive cells in E-

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cadherin-positive epithelial cells in the 1- and 2-week transplanted groups were comparable to those in the respective control models [1 week, transplanted group: $23.9\% \pm 13.2\%$ ($n = 6$) vs. control: $19.9\% \pm 16.6\%$ ($n = 3$); 2 weeks, transplanted group: $20.7\% \pm 11.5\%$ ($n = 7$) vs. control: $24.3\% \pm 2.4\%$ ($n = 3$)]. These results imply that the transplantation of hiPSC-MCACs had a limited effect on accelerating the epithelialization of tracheal defects in the present animal model, although the engraftment of transplanted hiPSC-MCACs was confirmed.

4. Discussion

In the present study, we assessed the potential of transplantation therapy using an artificial trachea loaded with hiPSC-MCACs. As mentioned above, our group has developed an artificial trachea with optimal stiffness as an airway framework. However, the results of clinical application showed that sufficient epithelialization on the luminal side took considerable time after reconstruction. To complement the endotracheal lumen with airway epithelial cells in the early postoperative stage, the transplantation of cell sheets would be desirable. Using cell sheet technology, cultured cells can be harvested as intact sheets without using proteolytic enzymes (which can result in cell damage and loss of differentiated cells). This is a promising technology for use in the field of tissue engineering and regenerative medicine. Currently, the application of cell sheets for tissue reconstruction includes direct implantation at the recipient site, superposition of cell sheets to construct three-dimensional structure, or implantation of cell sheets combined with scaffolds (Chen et al., 2015). Cell culture on a thermoresponsive polymer (poly-N-isopropyl acrylamide) surface was developed with an eye on its potential biomedical application (Takezawa et al., 1990; Yamada et al., 1990). Scaffold-free cell sheet can be harvested intact using thermoresponsive polymer grafted cell culture substrates by lowering the culture temperature (Okano et al., 1995). In the context of tracheal regeneration, it can be a useful approach in settings where epithelial regeneration is required.

To utilize hiPSCs for the regeneration of tracheal defects, we used the artificial trachea as a framework and collagen vitrigel membrane as a scaffold (Takezawa et al., 2004, 2007). Collagen vitrigel membrane is commercially available as a type of cell culture insert and is compatible with the ALI culture method. It exhibits excellent transparency and permeability for high-molecular weight proteins and has, therefore, been successfully used as a cell culture scaffold in various studies. Human corneal models (Ko et al., 2008; Ambrose et al., 2009; Takezawa et al., 2011; Chae et al., 2015; Yamaguchi et al., 2016; Yoshida et al., 2017; Yamaguchi and Takezawa, 2018) and human hepatocytes models (Oshikata-Miyazaki and Takezawa, 2016; Watari et al., 2018) have been successfully reconstructed using the collagen vitrigel membrane. An artificial skin based on collagen vitrigel was shown to promote the epithelialization of regenerative skin and to inhibit scar formation and inflammation, while the existing products such as DuoACTIVEV ET of a hydrocolloid dressing and Pelnac of a collagen sponge was not able to sufficiently inhibit scar formation (Aoki et al., 2015). In a porcine model, high-density collagen vitrigel patch was shown to reduce the incidence of esophageal stricture associated with extensive endoscopic submucosal dissection (Aoki et al., 2017). Furthermore, the insertion of a novel thread type of atelocollagen xerogel into the peritoneal cavity successfully prevented peritoneal fibrosis and intestinal adhesion in mice with chlorhexidine gluconate-induced peritonitis although the infusion of atelocollagen gel did not (Aoki et al., 2018). Collagen vitrigel membrane is approximately 20 times stronger than the conventional collagen gel and withstands breathing, coughing, and secretory fluid in the endotracheal lumen (Tada et al., 2008). In addition, certain properties of the collagen vitrigel membrane, such as water retentivity and permeability of macromolecular proteins, facilitate tracheal epithelialization. Therefore, it is appropriate for use in the reconstruction of tracheal defects.

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We successfully demonstrated the induction of hiPSC-MCACs on collagen vitrigel membrane as well as on PET membrane. The ciliary function analysis of hiPSC-MCACs is also an important aspect of the present study. The ciliary movement of murine embryonic stem cell-derived MCACs has been reported (Nishimura et al., 2006; Shojaie et al., 2015). Konishi et al. have reported that CBF of hiPSC-MCACs in the 3D-ALI protocol approximates the normal CBF of human MCACs (range, 10–14 Hz) (Konishi et al., 2016; Rutland et al., 1982). In the present study, CBF on both membranes was comparable (Fig. 4C). However, the induction efficiency of MCACs on collagen vitrigel membrane was less than that on PET membrane (Fig. 3B). The difference between PET membrane and collagen vitrigel membrane is partly attributable to the difference in strength, physiological activity, and cell attachment of the membrane. Therefore, further investigations are required to improve the induction efficiency; potential approaches may include increasing the collagen cross-linkage of the membrane and reinforcement of extracellular matrix (e.g., with the use of laminin and fibronectin). Previous studies have indicated that eliminating pluripotent stem cells from the transplanted cells is essential to prevent tumor formation (Fukuda et al., 2006). In this induction method of MCACs, target cells were sorted by MACS. The results of RT-PCR demonstrated the absence of any undifferentiated hiPSC after MCAC induction from hiPSCs (Fig. 3C). To exclude the possibility of tumor formation *in vivo*, the development of a long-term model is a key future research imperative.

in vivo studies in a nude rat model of tracheal defects showed the presence of hiPSC-derived cells in endotracheal epithelia for up to 2 weeks after transplantation. Furthermore, hiPSC-derived ciliated cells were observed for the same period. In the present study, epithelialization by native rat cells was observed on collagen vitrigel membrane in the control group; similar findings were reported from a previous study (Tada et al., 2008). However, the epithelial cells were relatively thin-layered. The normal tracheal epithelia of nude rats show pseudostratified

columnar epithelia, which is a special type of upper respiratory tract epithelium comprising a falsely-stratified single layer of epithelial cells resting on a basement membrane (Crystal et al., 2008). The tracheal defects in the hiPSC-MCACs transplantation group were covered with thick epithelia, which was similar to that observed in normal trachea (Supplemental Fig. 1B). It should be taken into consideration that the size of the tracheal defects in this study was smaller than that typically encountered in clinical medicine. It is necessary to apply this technique to large tracheal defects in larger animal models for a longer period. Another study limitation is that the functionality of the transplanted hiPSC-MCACs was not elucidated. In some cases, the regenerative epithelial cells of rats were observed among transplanted hiPSC-MCACs. Transplanted hiPSC-MCACs need to be distinguished from rat-derived MCACs. For a more precise functional evaluation, further studies using fluorescent reporter hiPSCs are required (Matsui et al., 2014). The results of this study envisage good potential prospects for transplantation therapy using hiPSCs. Our transplantation experiment using hiPSCs may provide a good platform for future clinical application of tracheal reconstruction.

5. Conclusions

In this study, hiPSC-MCACs were induced on collagen vitrigel membrane, which enabled the hiPSC-MCACs to survive on the endotracheal epithelium at the tracheal defect site in nude rats. However, the assessment of long-term survival and functional evaluation of transplant-derived cells are subjects for future research. Further evaluation using a larger animal model of tracheal defect is required to clarify the extent of its contribution to epithelialization.

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Conflict of interest

The authors declare no conflicts of interest.

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Tables

Table 1. Antibodies used in this study

Antibodies	Source	Dilution
FOXA2	R&D systems, AF2400	1:500
Nkx2_1	Thermo scientific, MS-699-P	1:500
E-Cadherin (24E10)	Cell Signaling Technology, 3195S	1/500
Acetylated α -tubulin	Sigma, T7451	1/1000
Human nuclei	Millipore, MAB1281	1/1000

Table 2. Antibodies used in this study

Gene	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')
<i>SNTN</i>	GCTGCAAACCCAATTTAGGA	TGCTCATCAAGTTCAGAAAGGA
<i>P63</i>	ACTGCCAAATTGCAAAGACA	TGACTAGGAGGGGCAATCTG
<i>OCT4</i>	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCAAAC
<i>GAPDH</i>	GACCCCTTCATTGACCTCAAC	CTTCTCCATGGTGGTGAAGA

Figure legends

Figure 1. Transplantation of human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) into nude rats. hiPSC-MCACs were cultured on collagen vitrigel membrane. The luminal surface of an artificial trachea was covered with hiPSC-MCACs sheet together with collagen vitrigel membrane and transplanted into tracheal defects of nude rats.

Figure 2. Differentiation of human induced pluripotent stem cell (hiPSC) into airway cells. Results of immunocytochemical analyses showing the expression of FOXA2 in endoderm cells (day 6) (A) and NKX2-1 in VAFECs (day 14) (B). (C) Phase contrast image of spheroids in 3D culture (day 28). Scale bar = 100 μm .

Figure 3. Comparison of human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) on PET membrane and on collagen vitrigel membrane. (A) Immunocytochemistry analyses exhibit the expression of E-cadherin and Ac-Tub on PET membrane (a, b, c) and collagen vitrigel membrane (d, e, f). Scale bar = 20 μm . (B) Quantitative analysis of the proportion of acetylated α -tubulin (Ac-Tub)-positive cells among E-cadherin-positive epithelial cells on PET membrane and collagen vitrigel membrane (** $p = 0.0013$, Mann–Whitney U-test). Data are presented as mean $\pm$ SD (PET membrane, $n = 15$; collagen vitrigel membrane, $n = 22$). (C) Results of RT-PCR analysis of airway epithelial cell markers. hiPSC-MCACs exhibit positive expression of MCAC marker gene, *SNTN*, and basal cell marker, *p63*, but not of the pluripotent marker gene, *OCT4*. Undifferentiated iPSCs were used as controls.

Figure 4. Characterization of the cilia of human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) on PET membrane and collagen vitrigel membrane. (A) Cilia-like structures are observed on differentiated cells on PET membrane (a) or collagen vitrigel membrane (b) by scanning electron microscope. Scale bar = 5 μ m. Transmission electron microscopy images show a “9 + 2” structure, which includes nine doublet microtubules arranged in a circle around two central singlet microtubules with dynein arms, on PET membrane (c) and collagen vitrigel membrane (d). Scale bar = 1 μ m. (B) Cilia motility in iPSC-MCACs. Time lapse images of cilia in iPSC-MCACs sheets on PET membrane or collagen vitrigel membrane are shown. (C) Ciliary beat frequencies (CBFs) of the motile cilia observed in iPSC-MCACs sheets were analyzed by CiliaFA. Data are shown as mean $\pm$ SD (PET membrane, 10.35 ± 0.81 Hz; collagen vitrigel membrane, 10.81 ± 0.50 Hz).

Figure 5. Survival of human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) in tracheal defects of nude rats 1 and 2 weeks after transplantation. (A) Representative immunostained cross-sectional images of whole trachea from the control and transplantation groups. Scale bar = 500 μ m. (B) In high-magnification images of the transplanted center part, human nuclear antigen (HNA)-, E-cadherin-, and 4',6-diamidino-2-phenylindole (DAPI)-positive cells exhibit a positive expression of acetylated α -tubulin (Ac-Tub) in the transplantation group. Scale bar = 30 μ m. (C) On quantitative analysis, the proportion of surviving HNA-, DAPI-, and E-cadherin-positive cells among the E-cadherin-positive epithelial cells in the 1- and 2-week survival groups were $8.5\% \pm 13.3\%$ and $14.3\% \pm 8.61\%$, respectively (1 week, n = 6; 2 weeks, n = 7). The proportion of surviving Ac-Tub-, HNA-, E-cadherin-, and DAPI-positive cells among the E-cadherin-positive epithelial cells in the 1- and 2-week survival groups were $0.5\% \pm 0.3\%$ and $1.7\% \pm 2.8\%$ (1 week, n = 2; 2 weeks,

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n = 6). The proportion of surviving Ac-Tub-, HNA-, E-cadherin-, and DAPI-positive cells among the transplanted HNA- and E-cadherin-positive epithelial cells in the 1- and 2-week survival groups were $2.8\% \pm 1.0\%$ and $7.8\% \pm 8.8\%$ (1 week, n = 6; 2 weeks, n = 7).

See also Movie 1 and 2.

(Movie 1) Ciliary movement in the human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) sheet prepared on PET membrane. Ciliary movement in the sheets was captured at 500 fps and is shown at 30 fps. Scale bar = 20 μm .

(Movie 2) Ciliary movement in the human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) sheet prepared on collagen vitrigel membrane. Ciliary movement in the sheets was captured at 500 fps and is shown at 30 fps. Scale bar = 20 μm .

Supplementary Figure 1. (A) Immunostained images of human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) cultured on collagen vitrigel membrane before transplantation. Scale bar = 30 μm . (B) Immunostained images of normal trachea of a nude rat. Normal tracheal epithelia appear to comprise multiple layers. Scale bar = 500 μm . Scale bar in high-magnification images = 30 μm .

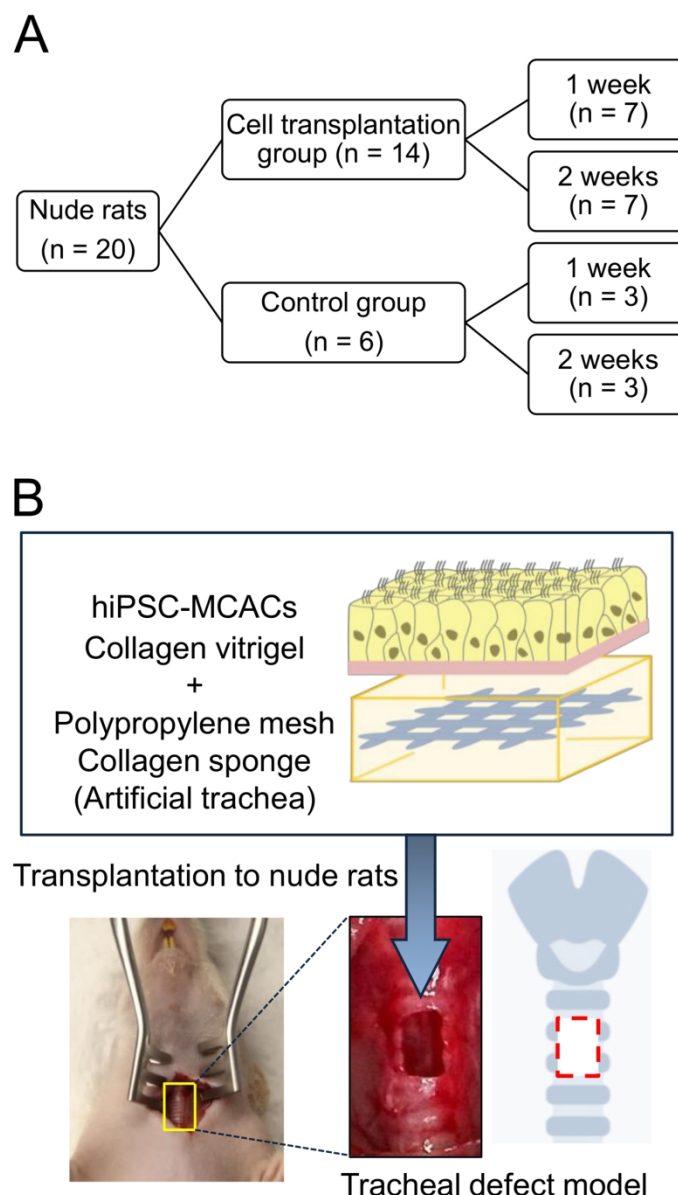


Figure 1. Transplantation of human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) into nude rats. (A) A total of 20 male nude rats were used as recipients of the transplant. Fourteen nude rats received transplantation of hiPSC-MCACs. As a control for the transplantation of hiPSC-MCACs, the remaining animals were transplanted with an artificial trachea covered with collagen vitrigel membrane without cells. (B) hiPSC-MCACs were cultured on collagen vitrigel membrane. The luminal surface of an artificial trachea was covered with hiPSC-MCACs sheet together with collagen vitrigel membrane and transplanted into tracheal defects of nude rats.

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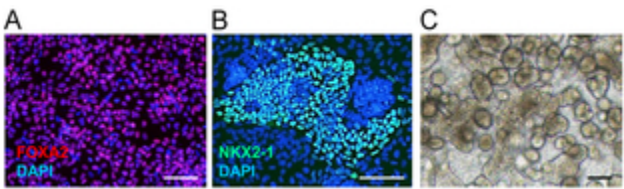


Figure 2. Differentiation of human induced pluripotent stem cell (hiPSC) into airway cells. Results of immunocytochemical analyses showing the expression of FOXA2 in endoderm cells (day 6) (A) and NKX2-1 in VAFECs (day 14) (B). (C) Phase contrast image of spheroids in 3D culture (day 28). Scale bar = 100 μ m.

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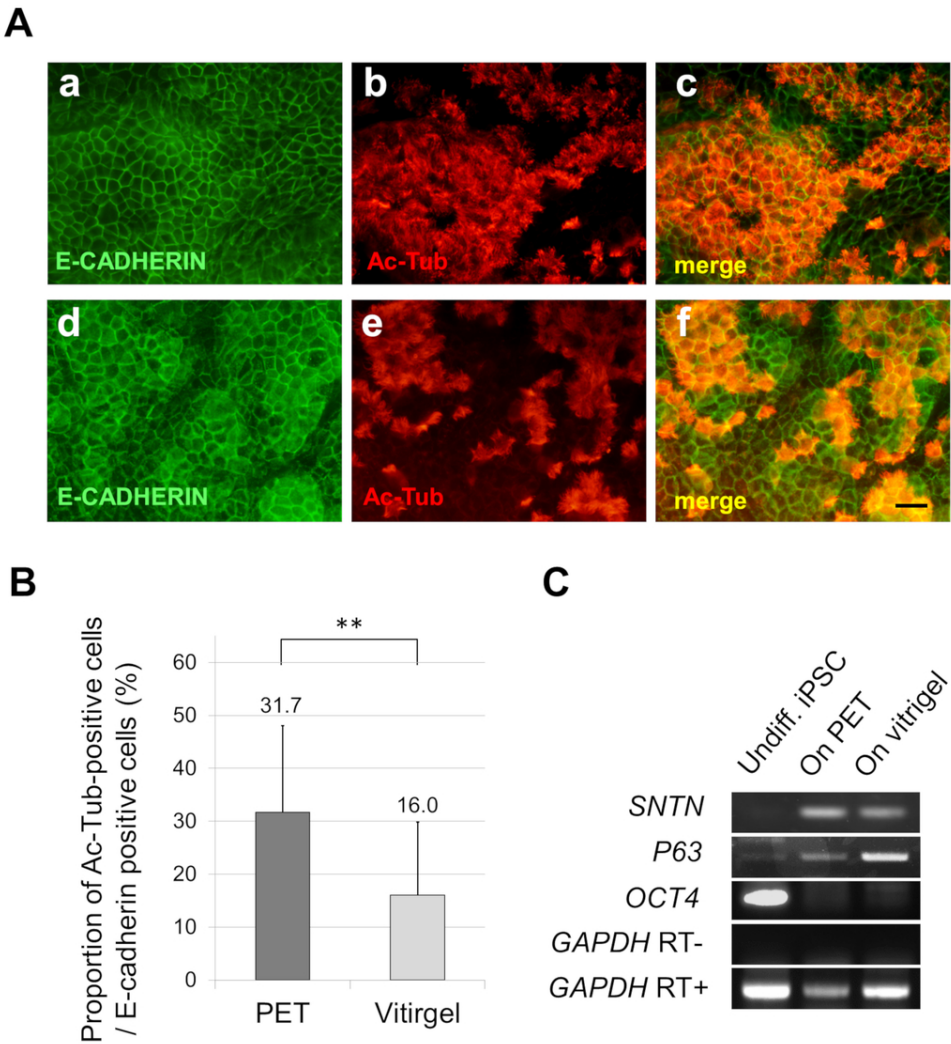


Figure 3. Comparison of human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) on PET membrane and on collagen vitrigel membrane. (A) Immunocytochemistry analyses exhibit the expression of E-cadherin and Ac-Tub on PET membrane (a, b, c) and collagen vitrigel membrane (d, e, f). Scale bar = 20 μ m. (B) Quantitative analysis of the proportion of acetylated α -tubulin (Ac-Tub)-positive cells among E-cadherin-positive epithelial cells on PET membrane and collagen vitrigel membrane (** p = 0.0013, Mann-Whitney U-test). Data are presented as mean $\pm$ SD (PET membrane, n = 15; collagen vitrigel membrane, n = 22). (C) Results of RT-PCR analysis of airway epithelial cell markers. hiPSC-MCACs exhibit positive expression of MCAC marker gene, SNTN, and basal cell marker, p63, but not of the pluripotent marker gene, OCT4. Undifferentiated iPSCs were used as controls.

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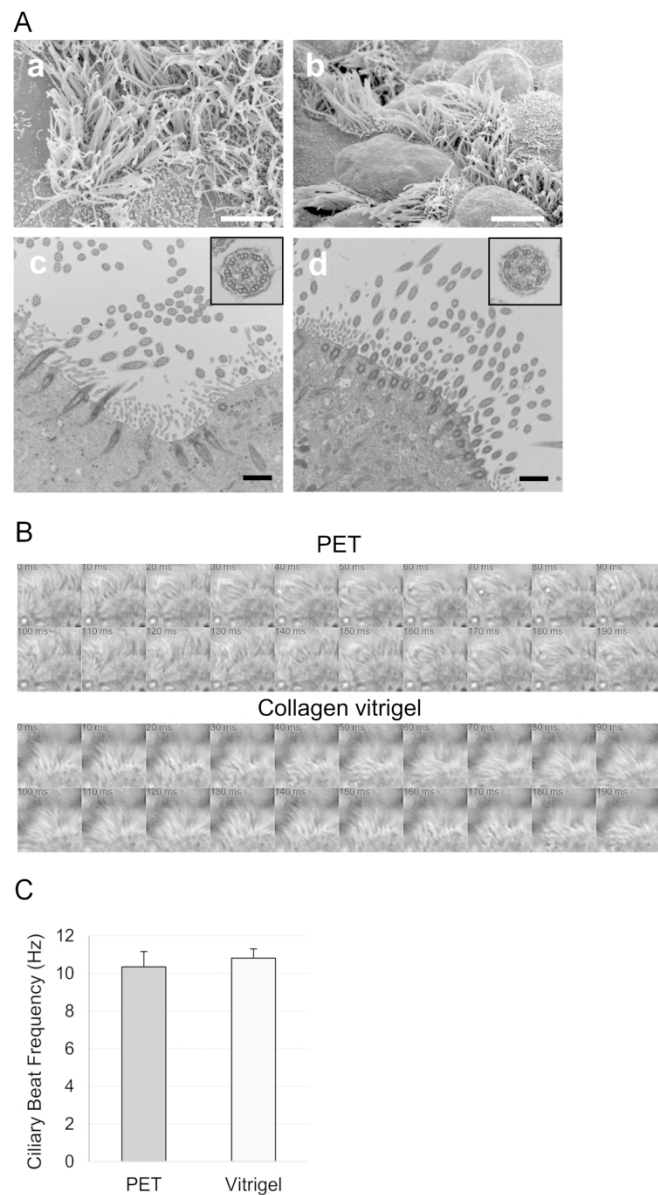


Figure 4. Characterization of the cilia of human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACS) on PET membrane and collagen vitrigel membrane. (A) Cilia-like structures are observed on differentiated cells on PET membrane (a) or collagen vitrigel membrane (b) by scanning electron microscope. Scale bar = 5 μm. Transmission electron microscopy images show a “9 + 2” structure, which includes nine doublet microtubules arranged in a circle around two central singlet microtubules with dynein arms, on PET membrane (c) and collagen vitrigel membrane (d). Scale bar = 1 μm. (B) Cilia motility in iPSC-MCACS. Time lapse images of cilia in iPSC-MCACS sheets on PET membrane or collagen vitrigel membrane are shown. (C) Ciliary beat frequencies (CBFs) of the motile cilia observed in iPSC-MCACS sheets were analyzed by CiliaFA. Data are shown as mean ± SD (PET membrane, 10.35 ± 0.81Hz; collagen vitrigel membrane, 10.81 ± 0.50 Hz).

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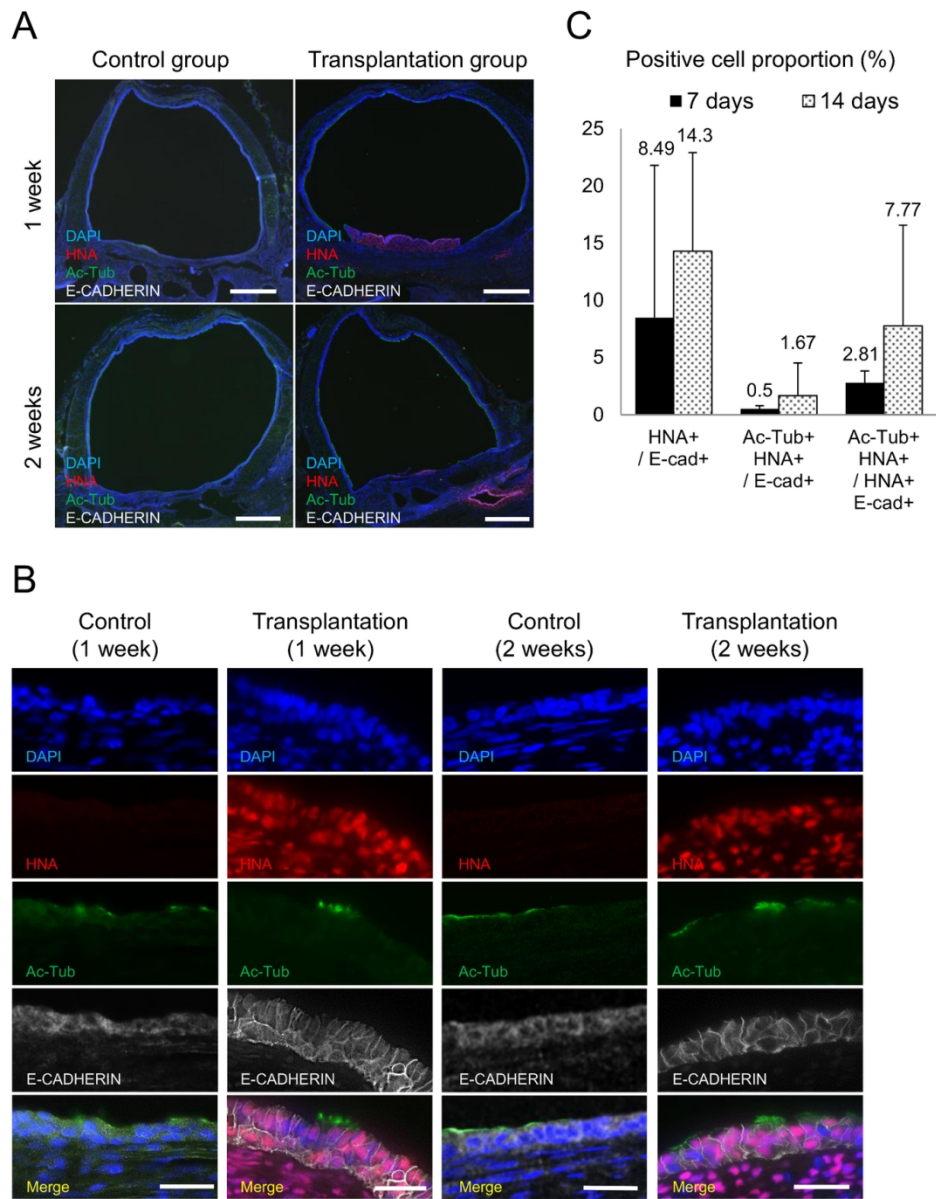


Figure 5. Survival of human induced pluripotent stem cell (hiPSC)-derived multi-ciliated airway cells (hiPSC-MCACs) in tracheal defects of nude rats 1 and 2 weeks after transplantation. (A) Representative immunostained cross-sectional images of whole trachea from the control and transplantation groups. Scale bar = 500 μ m. (B) In high-magnification images of the transplanted center part, human nuclear antigen (HNA)-, E-cadherin-, and 4',6-diamidino-2-phenylindole (DAPI)-positive cells exhibit a positive expression of acetylated α -tubulin (Ac-Tub) in the transplantation group. Scale bar = 30 μ m. (C) On quantitative analysis, the proportion of surviving HNA-, DAPI-, and E-cadherin-positive cells among the E-cadherin-positive epithelial cells in the 1- and 2-week survival groups were 8.5% $\pm$ 13.3% and 14.3% $\pm$ 8.61%, respectively (1 week, n = 6; 2 weeks, n = 7). The proportion of surviving Ac-Tub-, HNA-, E-cadherin-, and DAPI-positive cells among the E-cadherin-positive epithelial cells in the 1- and 2-week survival groups were 0.5% $\pm$ 0.3% and 1.7% $\pm$ 2.8% (1 week, n = 2; 2 weeks, n = 6). The proportion of surviving Ac-Tub-, HNA-, E-cadherin-, and DAPI-positive cells among the transplanted HNA- and E-cadherin-positive epithelial cells in the 1- and 2-week survival groups were 2.8% $\pm$ 1.0% and 7.8% $\pm$ 8.8% (1 week, n = 6; 2 weeks, n = 7).

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