

Title: Rat tracheal cartilage regeneration using mesenchymal stem cells derived from human iPS cells

Short running title: Tracheal cartilage regeneration using hiPSC-derived MSCs

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Abstract

Tracheal cartilage provides structural support to the airways to enable breathing. However, it can become damaged or impaired, sometimes requiring surgical resection and reconstruction. Previously, we clinically applied an artificial trachea composed of a polypropylene mesh and collagen sponge, with a favorable postoperative course. However, the artificial trachea presents a limitation, as the mesh is not biodegradable and cannot be used in pediatric patients. Compared to a polypropylene mesh, regenerated cartilage represents an ideal material for reconstruction of the damaged trachea. The use of mesenchymal stem cells (MSCs) as a source for cartilage regeneration has gained widespread acceptance, but challenges such as the invasiveness of harvesting and limited cell supply, persist. Therefore, we focused on the potential of human induced pluripotent stem cell (hiPSC)-derived mesenchymal stem cells (iMSCs) for tracheal cartilage regeneration. In this study, we aimed to regenerate tracheal cartilage on an artificial trachea as a preliminary step to replace the polypropylene mesh. iMSCs were induced from hiPSCs through neural crest cells and transplanted with a polypropylene mesh covered with a collagen sponge into the damaged tracheal cartilage in immunodeficient rats. Human nuclear antigen (HNA)-positive cells were observed in all six rats at 4 weeks and in six out of seven rats at 12 weeks after transplantation, indicating that transplanted iMSCs survived within the tracheal cartilage defects of rats. The HNA-positive cells co-expressed SOX9, and type II collagen was detected around HNA-positive cells in four of six rats at 4 weeks and in three of seven rats at 12 weeks after transplantation, reflecting cartilage-like tissue regeneration. These results indicate that the transplanted iMSCs could differentiate into chondrogenic cells and promote tracheal cartilage regeneration. iMSC transplantation thus represents a promising approach for human tracheal reconstruction.

Impact Statement

The artificial trachea previously developed by our group comprises a polypropylene mesh coated with a collagen sponge and cannot be used in pediatric patients because of growth considerations. However, it can be used for tracheal cartilage regeneration in children if the polypropylene mesh is removed. Here, we examined the efficacy of human induced pluripotent stem cell (hiPSC)-derived MSCs (iMSCs) for tracheal cartilage regeneration. iMSCs were derived from hiPSCs and transplanted into the damaged tracheal cartilage of rats. The transplanted iMSCs survived and differentiated into cartilage, indicating that these hold promise for tracheal reconstruction.

Introduction

The trachea is a tube-like organ that plays a vital role in respiration and indirect roles in swallowing and speech.¹ Tracheal cartilage rings provide radial rigidity and prevent collapse of the tracheal lumen during respiratory movements.² Tracheal defects are usually caused by infections, inflammatory disorders, malignancy, and trauma. These conditions can lead to structural issues such as narrowing and constriction of the upper airway, affecting a patient's quality of life. In most cases, primary anastomosis is performed after a tracheotomy for reconstruction. However, for long-segment tracheal defects (50% of the total tracheal length in adults and approximately 30% in children), reconstructing the defect using end-to-end anastomosis is almost impossible.³ In such situations, we must consider the structural reconstruction of the trachea as well as the preservation of its rigidity. Several approaches have been explored to reconstruct tracheal cartilage. Autologous tissue, such as costal cartilage⁴, has been used for tracheal cartilage reconstruction; however, its shape and amount represent disadvantages for airway reconstruction. Further, harvesting autologous cartilage is invasive. Our group clinically applied an artificial trachea created by Nakamura et al.,⁵ which is composed of a polypropylene mesh and collagen sponge.⁶ The collagen sponge scaffold provides favorable conditions for tissue regeneration in vivo, in combination with infiltrated host cells and regulatory factors released from the surrounding tissue,⁷ while the polypropylene mesh provides the artificial trachea with sufficient rigidity. After transplantation of the artificial trachea into tracheal defects in dogs, the harvested graft was subjected to mechanical testing. The stress-strain curve of the regenerated tissue closely resembled that of normal tracheal cartilage, indicating a robustly supported airway framework.⁸ Therefore, an artificial trachea composed of a collagen sponge and polypropylene mesh holds great value for tracheal regeneration. However, the artificial trachea presents a limitation as the mesh is not biodegradable and cannot be used in pediatric patients. Therefore, the first step towards adapting the artificial trachea for pediatric patients is the removal of the polypropylene mesh from it. However, using a collagen sponge alone would result in collapse during respiration. Use of biodegradable materials should thus be considered for tracheal cartilage regeneration. From this perspective, cell-based therapy

has been recognized as a potentially effective approach for tracheal reconstruction. In particular, mesenchymal stem cells (MSCs) have gained attention as a cell source for cartilage regeneration. MSCs form cells of the mesodermal lineage and can differentiate into osteoblasts, adipocytes, and chondrocytes.⁹ MSCs and secreted exosomes promote cartilage regeneration through balancing the metabolic activity of cartilage and chondrogenic differentiation, as reported in animal studies, with positive outcomes described for clinical applications.¹⁰ Thus, MSCs represent good candidates for regenerative therapy, and their utilization as a cell source for cartilage regeneration has gained widespread acceptance.¹¹ However, MSCs are found at a low ratio compared to other bone marrow mononuclear cells, and their proliferation capacity is limited. Thus, the problem of limited cell supply must be overcome for future clinical applications. One effective way to solve this problem is to use human-induced pluripotent stem cells (hiPSCs). Various methods for inducing MSCs from hiPSCs have been established.¹² For example, MSCs can be directly induced from hiPSCs^{13,14} or indirectly via neural crest cells (NCCs).¹⁵ Our group previously transplanted hiPSC-derived MSCs (iMSCs) into the damaged thyroid cartilage of rats, and demonstrated successful cell survival and cartilage regeneration.^{16,17} In this study, we aimed to regenerate tracheal cartilage on an artificial trachea as a preliminary step to replace the polypropylene mesh. The primary objective of this study was to achieve engraftment of iMSCs and facilitate tracheal cartilage regeneration, thereby demonstrating the efficacy of iMSCs in tracheal cartilage regeneration and advancing the development of artificial tracheas.

Materials and methods

Induction of iMSCs

These processes were performed according to the modified version of a method described previously.¹⁵⁻¹⁷ Briefly, hiPSCs (cell line 1231A3¹⁸) were cultured on iMatrix-511 (Nippi, Tokyo, Japan)-coated cell culture plates in AK02N medium (StemFit AK02N, Ajinomoto, Tokyo, Japan). NCCs were induced in AK02N medium without supplement C, with added 10 μ M SB431542 (Selleck Chemicals, Houston, TX, USA) and 1 μ M CHIR99021 (Axon Medchem, Groningen, the Netherlands) for 10 days. After NCC induction, CD271-

positive cells were sorted using a BD FACS Aria II cytometer (BD Biosciences, San Jose, CA, USA), since CD271^{high} cells were reported to express NCC markers (NGFR, SOX10, TFAP2A, and RHOB) at higher levels compared to those in CD271^{low} cells.¹⁵ After sorting, NCCs were cultured on fibronectin-coated plates in AK02N medium without supplement C, with added 10 μ M SB431542, 20 ng/mL EGF (R&D systems, a Bio-Techne brand, MN, USA), and 20 ng/mL FGF2 (Wako, Osaka, Japan) for 17 days. The cells were then differentiated into iMSCs by switching the medium to MSC medium (PRIME-XV MSC Expansion XSFM, FUJIFILM Irvine Scientific, Tokyo, Japan) and culturing for 7 days.

Flow cytometry

After iMSC induction, cells were analyzed via flow cytometry using a Human MSC Analysis kit (BD Pharmingen, Tokyo, Japan). CD73, CD90, and CD105 were used as positive markers for MSCs, whereas CD 11b, CD34, CD45, CD79A, and HLA-DR were used as negative markers. After iMSC induction, the cells were treated with these antibodies and analyzed using a BD LSRFortessa Cell Analyzer (BD Biosciences) and FlowJo software (Tree Star Inc., OR, USA).

Preparation of the artificial trachea

Collagen type I from porcine skin-derived collagen (Nippon Meat Packers, Inc., Osaka, Japan) was dissolved in pure water (6 mg/mL), and the pH was adjusted to 7. After centrifugation, the collected pellet was frozen at -80 °C and subjected to lyophilization for 10 days. The lyophilized collagen was then fragmented into small pieces, dissolved in ultrapure water (106.4 mg/mL) with HCl added to adjust the pH to 3. The collagen solution was poured into a mold with a polypropylene mesh coated with the collagen solution placed on top, and an equal volume of the collagen solution was poured again. The entire assembly was then frozen at -80°C overnight. After one week of lyophilization, collagen, including the polypropylene mesh, was cross-linked by heating and sterilized with ethylene oxide gas (EOG).

Preparation of grafts for transplantation

The iMSCs were seeded in 12-well culture dishes at a density of 1.6×10^6 cells/well and cultured in MSC medium without passage for two weeks. The cell sheet was gently detached from the culture dish using forceps. The peeled cell sheet was folded in half and placed on one side of the artificial trachea. Then, a collagen Vitrigel membrane (ad-MED Vitrigel 2, Kanto Chemical Co., Tokyo, Japan) was placed on the cell sheet (defined as the “cell graft”). We also created a graft using only vitrigel on the artificial trachea (defined as the “no-cell graft”) as a control.

Animals

Transplantation experiments were performed in male nude rats (F344/NJcl-rnu/rnu) purchased from CLEA Japan (Osaka, Japan). The rats were 13 weeks old. The animal experimental protocols were approved by the Animal Experimentation Committee of Kyoto University (MedKyo 23113). All animal experiments were performed in accordance with the “Guidelines for Animal Experiments of Kyoto University.”

Transplantation

In this study, 17 nude rats were used as transplant recipients. The experimental animals were divided into the following four groups: a group in which cell grafts were transplanted and the animals were sacrificed after 4 weeks ($n = 6$), a group in which cell grafts were transplanted and the animals were sacrificed after 12 weeks ($n = 7$), a group in which the no-cell grafts were transplanted and the animals were sacrificed after 4 weeks ($n = 2$), and a group in which no-cell grafts were transplanted and the animals were sacrificed after 12 weeks ($n = 2$). General anesthesia was induced via an intraperitoneal injection with a mixture of midazolam (0.2 mg/100 g), butorphanol tartrate (0.25 mg/100 g), and medetomidine hydrochloride (0.015 mg/100 g). Under general anesthesia, the trachea was exposed by making a midline skin incision and separating the bilateral sternohyoid and sternothyroid muscles. Subsequently, a square-shaped tracheal defect was created by removing one cartilage ring and excising the lower half of the cartilage above it and the upper half of the cartilage below it using a scalpel. The defect was approximately 2.5 mm in length and 2 mm in width. An artificial trachea covered with a

cell sheet and a collagen vitrigel membrane was positioned over the tracheal defect, with the collagen vitrigel membrane facing the tracheal lumen. The top and bottom of the graft and the tracheal cartilage of the rat were sutured together using a single stitch with 9-0 nylon (Bear Medic Co., Ibaraki, Japan). Subsequently, the sternohyoid and sternothyroid muscles and the incised skin were also sutured. After 4 or 12 weeks of transplantation, the nude rats were humanely euthanized using CO₂, and histological examination was performed. A schematic protocol of the procedure is illustrated in Fig. 1, and the timeline after transplantation is shown in Fig. 2.

Histological analyses

The cell sheets before transplantation and tracheae after transplantation were immersed in 4% PFA overnight. Subsequently, they were immersed in 10%, 20%, and 30% sucrose solutions overnight. They were then embedded in an optimal cutting temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan). Cryosections of 10 µm thickness were prepared and stained using hematoxylin and eosin (HE), Alcian blue, and Safranin-O. For Alcian blue staining, sections were treated with 3% acetic acid and then stained with Alcian Blue 8GX solution (Waldeck GmbH & Co., KG, Munster, Germany) for 30 min. The sections were immersed in 3% acetic acid for 3 min. Nuclear fast red dye (Sigma-Aldrich Japan G.K.) was used as a counterstain. The sections were then dehydrated and cleared with ethyl alcohol before mounting. For Safranin-O staining, the sections were immersed in Weigert's iron hematoxylin solution for 10 min and stained with 0.05% Fast Green FCF (Sigma-Aldrich, St. Louis, MO, USA) solution for 5 min. The sections were treated with 1% acetic acid. The sections were then stained again with a 0.1% Safranin-O solution (Sigma-Aldrich) for 5 min, dehydrated, and cleared with ethyl alcohol before mounting. Images were captured using a BZ-X810 All-in-One fluorescence microscope (Keyence, Osaka, Japan).

Immunofluorescence staining

To detect anti-type I collagen and anti-type II collagen antibody reactions, cryosections were treated with 0.5% pepsin (Sigma-Aldrich) diluted with 0.5 mM hydrochloric acid for 10 min at 37 °C for epitope retrieval. For anti-SOX9 antibody staining,

sections were incubated for 30 min at 65 °C, and heated at 98 °C for 10 min in citrate buffer solution (0.01 mol/L, pH = 6.0, LSI Medience Co., Tokyo, Japan). After blocking with Blocking One (Nacalai) for 1 h, the sections were incubated overnight at 4 °C with anti-human nuclear antigen (HNA) (Mouse, MAB1281, 1:1000; Millipore), anti-type I collagen (COL1) (Rabbit, ab34710, 1:200; Abcam), anti-type II collagen (COL2) (Mouse, MA5-12789, 1:100; Invitrogen), and anti-SOX9 (Rabbit, AB5535, 1:5000; Millipore) antibodies. Sections were then incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor-conjugated secondary antibodies; Invitrogen, Tokyo, Japan) and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) stain for 1 h at room temperature. Secondary antibodies (1:1000) were diluted with Can Get Signal Solution B (Toyobo, Osaka, Japan) for anti-COL1 and anti-COL2 staining, and with 10% Blocking One in PBS containing 0.05% Tween 20 (PBST) for anti-SOX9 staining. Images were captured using an OLYMPUS BX 50 fluorescence microscope equipped with an Olympus DP70 camera (Olympus, Tokyo, Japan), ORCA-spark digital COMS camera C11440-36U (HAMAMATSU, Hamamatsu, Japan), and CellSens Standard software (Olympus).

To confirm the iNCC induction, immunostaining was performed on the 10th day of NCC induction. Following fixation with 4% PFA in PBS for 15 min and washing with PBS, iNCCs were treated with 0.2% Triton X-100 in PBS for 15 min at 4 °C. The cells were then immersed in Blocking One for 1 h at 4 °C to block non-specific antibody binding. The cells were then incubated with anti-TFAP2A (Mouse, AB528084: 1:100; DSHB) diluted with 10% Blocking One in PBST overnight at 4 °C. The cells were incubated with a fluorophore-labeled secondary antibody and DAPI for nuclear staining for 1 h at room temperature. Images of undifferentiated iPSCs and iNCCs were obtained using a fluorescence microscope (Nikon Eclipse Ti-S), an Olympus DP73 camera (Olympus), and CellSens Standard software (Olympus).

Results

Induction of iMSCs from hiPSCs through NCCs

Figure 3a shows the pre-culture of hiPSCs, and Fig. 3b shows iNCCs after NCC induction. Immunohistochemistry revealed that the induced iNCCs expressed NCC marker

TFAP2A (Fig. 3b). The induced iMSCs showed MSC-like morphology (Fig. 3c). Further, they exhibited expression of human MSC surface markers (Fig. 3d) (positive for CD73, CD90, and CD105, while negative for CD11b, CD19, CD34, CD45, and HLA-DR).

Evaluation of iMSC sheets and grafts before transplantation

Figure 4a shows the gross appearance of the iMSC sheets prior to being placed on the artificial trachea. Figure 4b, c presents cryosections of the iMSC sheet. The iMSC sheet appeared stratified rather than monolayered and contained an extracellular matrix (ECM) stained with Alcian Blue.

Histological evaluation of cartilage like regeneration

Figure 5a-c shows the representative images of cartilage-like regeneration following iMSC transplantation 4 weeks postoperatively. Regenerated tissue (indicated by an asterisk) was observed in the transplanted region (between the yellow arrowheads) after HE staining (Fig. 5a). The regenerated tissue was then stained with Alcian blue and Safranin O (Fig. 5b, c). Alcian blue and Safranin O are cationic dyes used to stain proteoglycans, which are the main components of the cartilage ECM.^{19,20} Our results indicated that the ECM of the regenerated tissue was comparable to that of normal cartilage. Figure 5j-l shows images of the control group transplanted with the no-cell graft upon HE, Alcian blue, and Safranin-O staining. Cartilage-like regenerated tissue positive for Alcian blue or Safranin O was not observed in the tracheal defect. Figure 5d-i shows higher-magnification images of the regenerated tissue following iMSC transplantation, with small lacunae formed in the regenerated tissues indicating cartilage formation.

Evaluation of chondrocyte marker expression and ECM production via immunofluorescence staining

Figure 6 shows a representative case of chondrocyte marker expression and ECM production following iMSC transplantation. The regenerated tissue (asterisk in Fig. 6b) was HNA-positive, whereas the rat trachea, outlined with white dashed lines, was HNA-negative, indicating that the regenerated tissue was derived from the transplanted iMSCs. SOX9 is a marker of chondroprogenitor cells and chondrocytes.²¹ Within the regenerated

tissue, most cells co-expressed HNA and SOX9 (Fig. 6c-f), indicating that the transplanted cells survived and differentiated into the chondrogenic lineage. COL2 constitutes a significant component of the ECM in hyaline cartilage, whereas COL1 is not expressed in hyaline cartilage but is expressed in fibrocartilage and fibrous tissue.^{22,23} The ECM surrounding the surviving cells expressed COL2 (Fig. 6g), but not COL1 (Fig. 6h). Considering the findings from COL2 and COL1 staining, along with those from Safranin O and Alcian blue staining, the ECM within the regenerated tissue appeared equivalent to normal hyaline cartilage.

Evaluation of transplanted cell engraftment and cartilage-like regeneration

HNA-positive surviving transplanted cells were detected in all six rats at 4 weeks after transplantation and in six out of seven rats at 12 weeks after transplantation. Regeneration of cartilage-like tissue, characterized by the presence of surviving transplanted cells expressing SOX9, ECM stained with Safranin-O and Alcian blue, and ECM expressing COL2 but not COL1, was observed in four of six rats at 4 weeks and in three of seven rats at 12 weeks post-transplantation. Figure 7 shows the histology and immunofluorescence staining of all the rats with regenerated cartilage-like tissue. The histology and immunofluorescence staining sections of the rats without cartilage regeneration are shown in Supplemental Fig. 1.

Discussion

In this study, we induced iMSCs through NCCs, transplanting them into the tracheal cartilage defects of nude rats using an artificial trachea composed of a polypropylene mesh and collagen sponge, which resulted in the regeneration of cartilage-like tissues. Engraftment of iMSCs was observed in all six rats at 4 weeks and in six of seven rats (86%) at 12 weeks after transplantation. Cartilage-like tissue regeneration was observed in four out of six rats (67%) at 4 weeks and in three of seven rats (43%) at 12 weeks (Table 1). To the best of our knowledge, only one study has investigated tracheal cartilage regeneration using hiPSCs.²⁴ In the study, iMSCs were seeded onto three-dimensional (3D) printed tube grafts and transplanted into tracheal defects in rabbits. Although engraftment of iMSCs and cartilage regeneration were observed, the engrafted

iMSCs did not survive in the regenerated cartilage. The authors thus considered that iMSCs contributed to cartilage regeneration through paracrine effects rather than through direct differentiation. Therefore, our study is the first to demonstrate the engraftment of iMSCs in tracheal cartilage defects, their differentiation into chondrocytes, and tracheal cartilage regeneration *in vivo*.

Currently, studies on tracheal cartilage regeneration are limited to animal models. Further, primary cultured chondrocytes are commonly used.²⁵⁻²⁷ Many studies on knee cartilage regeneration have focused on MSCs owing to their multipotent differentiation and immunomodulatory effects.²⁸⁻³⁰ Therefore, MSCs are also considered attractive candidates for treating tracheal cartilage defects. We used iMSCs for transplantation without differentiating them into chondrocytes before transplantation. Transplantation of autologous and allogeneic MSCs is performed in the clinic.^{31,32} However, certain challenges remain, including the invasiveness of the MSC harvesting process, loss of proliferative capacity over repeated passages,³³ and the requirement of matching HLA types in allogeneic transplantation. In this regard, hiPSCs theoretically have an unlimited proliferative capacity, making it possible to easily obtain the required quantity of iMSCs. HLA mismatch issues can be overcome by using HLA-homozygous hiPSCs or CRISPR-Cas9 to create HLA-edited hiPSCs.³⁴ Furthermore, iMSCs also have immunomodulatory properties, similar to those of primary cultured MSCs harvested from tissues such as the bone marrow,³⁵ and are an ideal cell source for cartilage regeneration.

The method of transplanting iMSCs in a spherical form, as performed in previous studies, poses challenges in their fixation to the tracheal defect, leading to them dropping into the tracheal lumen. Therefore, we modified the graft preparation method. We focused on the transplantation of MSC sheets, which have been clinically applied to knee cartilage injuries in a previous study.³¹ Briefly, autologous synovial MSCs were cultured for 7–21 days, detached from the culture dish in a sheet form, and transplanted into knee cartilage defects. Based on these methods, we established a model for the transplantation of iMSCs in a contracted sheet form with the artificial trachea. Multiple studies have shown that MSCs exhibit greater potential for chondrogenesis in 3D structures compared to that in two dimensional cultures.^{36,37} Contracted cell sheets, although not 3D structures,

retain ECM, receptors, and adhesive proteins³⁸ and are widely used in cartilage regeneration research through techniques such as layering to create stacked structures.^{39,40} Although stacking of cell sheets was not performed in our study, a folded cell sheet was transplanted, which may have contributed to cartilage regeneration.

This study had several limitations. First, the relatively short observation period following transplantation limited the histological analysis. Histological examination revealed the formation of small lacunae in the regenerated cartilage-like tissue. Prolonged observation periods, such as six months or one year, are desirable to allow maturation during cartilage-like tissue regeneration. However, in this study, fewer rats tended to exhibit cartilage-like regeneration in the 12 weeks post-transplantation group than in the 4 weeks group. In our previous study, wherein iMSCs were transplanted into the thyroid cartilage defects of nude rats, cartilage-like regeneration was observed in five out of six rats (83%) at four weeks post-transplantation and in three out of six rats (50%) at eight weeks post-transplantation.¹⁷ This study also showed a trend of fewer rats showing cartilage-like regeneration with longer post-transplantation observation periods. In our previous study, involving iMSC transplantation into thyroid cartilage defects of X-linked severe combined immunodeficient (X-SCID) rats, cartilage-like regeneration was observed in five of seven rats (71.4%) at both 4 and 8 weeks post-transplantation.¹⁶ X-SCID rats were established by interleukin (IL)-2 receptor gamma (Il2rg) gene knockout, and their immunodeficient phenotypes were characterized by a nearly complete lack of T cells, B cells, and natural killer (NK) cells.⁴¹ Meanwhile, nude rats had no mature T cells but preserved B and NK cell function.⁴² Therefore, the immune response in nude rats may have resulted in rejection of the regenerated cartilage. Thus, for long-term observations, using rats with a more severe immune deficiency, such as SCID rats with mutations in Il2rg and recombination activating gene (Rag2), would be ideal.⁴³

Second, the biomechanical strength of regenerated cartilage-like tissue has not been determined. Owing to the small size of the rat trachea and variability in the degree of cartilage regeneration, sufficient cartilage for evaluating biomechanical strength was not obtained in this study. Therefore, future studies should aim for transplantation into larger animals, such as rabbits or dogs, to ensure adequate cartilage and to evaluate

biomechanical strength. This study is a preliminary study for replacing the polypropylene mesh in the future. Therefore, a collagen sponge containing polypropylene mesh was used. In future studies, we aim to explore the use of collagen sponges without a polypropylene mesh. However, the use of a collagen sponge alone may pose a risk of collapse owing to its insufficient physical strength against inhalation. Although iMSC sheets show promise for cartilage regeneration, their physical properties remain uncertain. A potential approach involves creating an artificial trachea using biodegradable polycaprolactone (PCL) rings covered with a collagen sponge. The iMSC sheets, which are rod-shaped and rounded, can be placed between the PCL rings, which are expected to biodegrade within several months to years,⁴⁴ during which time iMSC sheets are anticipated to differentiate into mature cartilage and maintain tracheal patency.

Third, iMSCs may represent a heterogeneous cell population. In this study, flow cytometry analysis of iMSCs showed high expression of CD73 and CD105, but a low expression of CD90 at 64.3%. One of the minimal criteria for defining MSCs proposed by the International Society for Cellular Therapy is that CD73, CD90, and CD105 must be positive in more than 95% of the cells.⁴⁵ For clinical application, it will be necessary to meet these criteria, and efforts to increase purification efficiency, such as by selectively collecting iNCCs with even further higher expression levels of CD271, are considered.

We also focused on the possible paracrine effects of iMSCs on tracheal epithelial regeneration. Okuyama et al. transplanted multiciliated airway cells derived from hiPSCs (iMCACs) along with an artificial trachea into a rat trachea and confirmed their engraftment.⁴⁶ However, the engraftment efficiency was insufficient. Several studies have also been conducted to promote epithelialization of the inner cavity surface of the artificial trachea.^{47,48} Although transplantation of iMCACs was not performed in these studies, transplanting artificial tracheas with fibroblast cells and adipose-derived stem cells was reported to enhance epithelialization. Considering these points, we anticipate that transplanting a combination of iMCACs and iMSCs may improve the engraftment rate of iMCACs. Our future goals include advancing tracheal regeneration using iMSCs, including both tracheal cartilage and airway epithelium, and translating our research to patients with tracheal defects, including pediatric patients.

Conclusions

In this study, we investigated the efficacy of iMSCs in tracheal cartilage regeneration. Engraftment of iMSCs and cartilage-like tissue regeneration were observed at 4 and 12 weeks after transplantation. These results indicate that iMSCs could be a promising option for tracheal reconstruction and the current research provides a basis for the future development of artificial tracheas suitable for pediatric use.

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Authorship contribution

K. Omori, H. Ohnishi and K. Mizuno designed the study. K. Omori and M. Ikeya, Y. Kishimoto, T. Kojima, S. Fujimura, Y. Kawai provided critical advice. H. Ohnishi and K. Mizuno prepared hiPSC-derived mesenchymal stem cells and collagen sponge. K. Mizuno performed the transplantation and the histological analyses. M. Kitano provided advice on surgical technique. K. Mizuno, H. Ohnishi, and K. Omori wrote the main manuscript text and all figures. All authors participated in revising the manuscript.

Disclosure Statement

No competing financial interests exist.

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References

1. Udelsman B, Mathisen DJ, Ott HC. A reassessment of tracheal substitutes-a systematic review. *Ann Cardiothorac Surg* 2018;7(2):175–182; doi: 10.21037/ACS.2018.01.17.
2. Park JH, Hong JM, Ju YM, et al. A novel tissue-engineered trachea with a mechanical behavior similar to native trachea. *Biomaterials* 2015;62:106–115; doi: 10.1016/J.BIOMATERIALS.2015.05.008.
3. Li D, Yin Z, Liu Y, et al. Regeneration of trachea graft with cartilage support, vascularization, and epithelization. *Acta Biomater* 2019;89:206–216; doi: 10.1016/J.ACTBIO.2019.03.003.
4. Fabre D, Kolb F, Fadel E, et al. Successful tracheal replacement in humans using autologous tissues: an 8-year experience. *Ann Thorac Surg* 2013;96(4):1146–1155; doi: 10.1016/J.ATHORACSUR.2013.05.073.
5. Nakamura T, Teramachi M, Sekine T, et al. Artificial trachea and long term follow-up in carinal reconstruction in dogs. *Int J Artif Organs* 2000;23(10):718–24.
6. Omori K, Nakamura T, Kanemaru S, et al. In situ tissue engineering of the cricoid and trachea in a canine model. *Ann Otol Rhinol Laryngol* 2008;117(8):609–613; doi: 10.1177/000348940811700811.
7. Omori K, Nakamura T, Kanemaru S, et al. Regenerative medicine of the trachea: the first human case. *Ann Otol Rhinol Laryngol* 2005;114(6):429–433; doi: 10.1177/000348940511400603.
8. Teramachi M, Nakamura T, Yamamoto Y, et al. Porous-type tracheal prosthesis sealed with collagen sponge. *Ann Thorac Surg* 1997;64(4):965–969; doi: 10.1016/S0003-4975(97)00755-8.
9. Freitag J, Bates D, Boyd R, et al. Mesenchymal stem cell therapy in the treatment of osteoarthritis: reparative pathways, safety and efficacy - a review. *BMC Musculoskelet Disord* 2016;17(1); doi: 10.1186/S12891-016-1085-9.
10. Xiang XN, Zhu SY, He HC, et al. Mesenchymal stromal cell-based therapy for cartilage regeneration in knee osteoarthritis. *Stem Cell Res Ther* 2022;13(1); doi: 10.1186/S13287-021-02689-9.

11. Squillaro T, Peluso G, Galderisi U. Clinical Trials With Mesenchymal Stem Cells: An Update. *Cell Transplant* 2016;25(5):829–848; doi: 10.3727/096368915X689622.
12. de Matos BM, Robert AW, Stimamiglio MA, et al. Pluripotent-derived Mesenchymal Stem/stromal Cells: an Overview of the Derivation Protocol Efficacies and the Differences Among the Derived Cells. *Stem Cell Rev Rep* 2022;18(1):94–125; doi: 10.1007/S12015-021-10258-Z.
13. Wang T, Liu Q, Chang YT, et al. Designed peptide-grafted hydrogels for human pluripotent stem cell culture and differentiation. *J Mater Chem B* 2023;11(7):1434–1444; doi: 10.1039/D2TB02521C.
14. Tian Z, Wang CK, Lin FL, et al. Effect of extracellular matrix proteins on the differentiation of human pluripotent stem cells into mesenchymal stem cells. *J Mater Chem B* 2022;10(30):5723–5732; doi: 10.1039/D2TB01026G.
15. Kamiya D, Takenaka-Ninagawa N, Motoike S, et al. Induction of functional xeno-free MSCs from human iPSCs via a neural crest cell lineage. *NPJ Regen Med* 2022;7(1); doi: 10.1038/S41536-022-00241-8.
16. Yoshimatsu M, Ohnishi H, Zhao C, et al. In vivo regeneration of rat laryngeal cartilage with mesenchymal stem cells derived from human induced pluripotent stem cells via neural crest cells. *Stem Cell Res* 2021;52; doi: 10.1016/J.SCR.2021.102233.
17. Mizuno K, Ohnishi H, Yoshimatsu M, et al. Laryngeal Cartilage Regeneration of Nude Rats by Transplantation of Mesenchymal Stem Cells Derived from Human-Induced Pluripotent Stem Cells. *Cell Transplant* 2023;32; doi: 10.1177/09636897231178460.
18. Nakagawa M, Taniguchi Y, Senda S, et al. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. *Sci Rep* 2014;4; doi: 10.1038/SREP03594.
19. Kahveci Z, Minday FZ, Cavusoglu I. Safranin O staining using a microwave oven. *Biotech Histochem* 2000;75(6):264–268; doi: 10.3109/10520290009085130.
20. Tran D, Golick M, Rabinovitz HS, et al. Hematoxylin and safranin O staining of frozen sections. *Dermatol Surg* 2000;26(3):197–199; doi: 10.1046/J.1524-4725.2000.09220.X.
21. Yin PT, Han E, Lee KB. Engineering Stem Cells for Biomedical Applications. *Adv Healthc Mater* 2016;5(1):10–55; doi: 10.1002/ADHM.201400842.

22. Frisbie DD, Trotter GW, Powers BE, et al. Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses. *Vet Surg* 1999;28(4):242–255; doi: 10.1053/JVET.1999.0242.
23. Outani H, Okada M, Yamashita A, et al. Direct induction of chondrogenic cells from human dermal fibroblast culture by defined factors. *PLoS One* 2013;8(10); doi: 10.1371/JOURNAL.PONE.0077365.
24. Kim IG, Park SA, Lee SH, et al. Transplantation of a 3D-printed tracheal graft combined with iPS cell-derived MSCs and chondrocytes. *Sci Rep* 2020;10(1); doi: 10.1038/S41598-020-61405-4.
25. Yang M, Chen J, Chen Y, et al. Scaffold-Free Tracheal Engineering via a Modular Strategy Based on Cartilage and Epithelium Sheets. *Adv Healthc Mater* 2023;12(6); doi: 10.1002/ADHM.202202022.
26. She Y, Fan Z, Wang L, et al. 3D Printed Biomimetic PCL Scaffold as Framework Interspersed With Collagen for Long Segment Tracheal Replacement. *Front Cell Dev Biol* 2021;9; doi: 10.3389/FCELL.2021.629796.
27. Hiwatashi S, Iwai R, Nakayama Y, et al. Successful tracheal regeneration using biofabricated autologous analogues without artificial supports. *Sci Rep* 2022;12(1); doi: 10.1038/S41598-022-24798-Y.
28. Zhao C, Ikeya M. Generation and Applications of Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cells. *Stem Cells Int* 2018;2018; doi: 10.1155/2018/9601623.
29. Madrigal M, Rao KS, Riordan NH. A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *J Transl Med* 2014;12(1); doi: 10.1186/S12967-014-0260-8.
30. Li J, Ezzelarab MB, Cooper DKC. Do mesenchymal stem cells function across species barriers? Relevance for xenotransplantation. *Xenotransplantation* 2012;19(5):273–285; doi: 10.1111/XEN.12000.
31. Shimomura K, Yasui Y, Koizumi K, et al. First-in-Human Pilot Study of Implantation of a Scaffold-Free Tissue-Engineered Construct Generated From Autologous Synovial Mesenchymal Stem Cells for Repair of Knee Chondral Lesions. *Am J Sports Med* 2018;46(10):2384–2393; doi: 10.1177/0363546518781825.

32. Carneiro D de C, Araújo LT de, Santos GC, et al. Clinical Trials with Mesenchymal Stem Cell Therapies for Osteoarthritis: Challenges in the Regeneration of Articular Cartilage. *Int J Mol Sci* 2023;24(12); doi: 10.3390/IJMS24129939.
33. Solchaga LA, Penick K, Goldberg VM, et al. Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells. *Tissue Eng Part A* 2010;16(3):1009–1019; doi: 10.1089/TEN.TEA.2009.0100.
34. Xu H, Wang B, Ono M, et al. Targeted Disruption of HLA Genes via CRISPR-Cas9 Generates iPSCs with Enhanced Immune Compatibility. *Cell Stem Cell* 2019;24(4):566–578.e7; doi: 10.1016/J.STEM.2019.02.005.
35. Mitsuzawa S, Ikeguchi R, Aoyama T, et al. Induced pluripotent stem cell-derived mesenchymal stem cells prolong hind limb survival in a rat vascularized composite allotransplantation model. *Microsurgery* 2019;39(8):737–747; doi: 10.1002/MICR.30507.
36. Xu J, Wang W, Ludeman M, et al. Chondrogenic differentiation of human mesenchymal stem cells in three-dimensional alginate gels. *Tissue Eng Part A* 2008;14(5):667–680; doi: 10.1089/TEA.2007.0272.
37. Watts AE, Ackerman-Yost JC, Nixon AJ. A comparison of three-dimensional culture systems to evaluate in vitro chondrogenesis of equine bone marrow-derived mesenchymal stem cells. *Tissue Eng Part A* 2013;19(19–20):2275–2283; doi: 10.1089/TEN.TEA.2012.0479.
38. Yang J, Yamato M, Shimizu T, et al. Reconstruction of functional tissues with cell sheet engineering. *Biomaterials* 2007;28(34):5033–5043; doi: 10.1016/J.BIOMATERIALS.2007.07.052.
39. Lu Y, Zhang W, Wang J, et al. Recent advances in cell sheet technology for bone and cartilage regeneration: from preparation to application. *Int J Oral Sci* 2019;11(2); doi: 10.1038/S41368-019-0050-5.
40. Ge Y, Gong YY, Xu Z, et al. The Application of Sheet Technology in Cartilage Tissue Engineering. *Tissue Eng Part B Rev* 2016;22(2):114–124; doi: 10.1089/TEN.TEB.2015.0189/ASSET/IMAGES/LARGE/FIGURE5.JPEG.

41. Mashimo T, Takizawa A, Voigt B, et al. Generation of knockout rats with X-linked severe combined immunodeficiency (X-SCID) using zinc-finger nucleases. *PLoS One* 2010;5(1); doi: 10.1371/JOURNAL.PONE.0008870.
42. Rolstad B. The athymic nude rat: an animal experimental model to reveal novel aspects of innate immune responses? *Immunol Rev* 2001;184:136–144; doi: 10.1034/J.1600-065X.2001.1840113.X.
43. Miyasaka Y, Wang J, Hattori K, et al. A high-quality severe combined immunodeficiency (SCID) rat bioresource. *PLoS One* 2022;17(8); doi: 10.1371/JOURNAL.PONE.0272950.
44. Labet M, Thielemans W. Synthesis of polycaprolactone: a review. *Chem Soc Rev* 2009;38(12):3484–3504; doi: 10.1039/B820162P.
45. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;8(4):315–317; doi: 10.1080/14653240600855905.
46. Okuyama H, Ohnishi H, Nakamura R, et al. Transplantation of multiciliated airway cells derived from human iPS cells using an artificial tracheal patch into rat trachea. *J Tissue Eng Regen Med* 2019;13(6):1019–1030; doi: 10.1002/TERM.2849.
47. Kobayashi K, Suzuki T, Nomoto Y, et al. A tissue-engineered trachea derived from a framed collagen scaffold, gingival fibroblasts and adipose-derived stem cells. *Biomaterials* 2010;31(18):4855–4863; doi: 10.1016/J.BIOMATERIALS.2010.02.027.
48. Suzuki T, Kobayashi K, Tada Y, et al. Regeneration of the trachea using a bioengineered scaffold with adipose-derived stem cells. *Ann Otol Rhinol Laryngol* 2008;117(6):453–463; doi: 10.1177/000348940811700609. 45.

Table 1. The number of rats showing HNA-positive cells and cartilage-like regeneration

Period	HNA-positive cells	Cartilage-like regeneration
4 weeks post-transplantation	6/6 (100%)	4/6 (67%)
12 weeks post-transplantation	6/7 (86%)	3/7 (43%)

Figure Legends

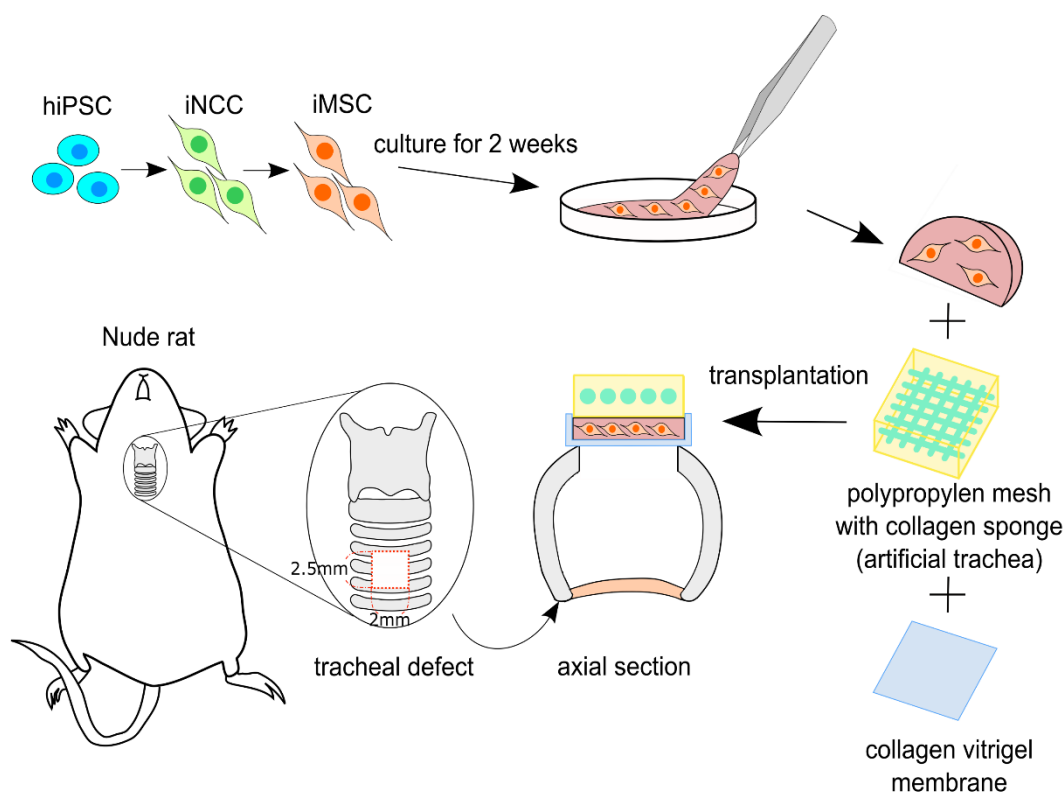


Figure 1. Schematic representation of iMSC transplantation into the tracheal defects of nude rats

An artificial trachea covered with a cell sheet and a collagen vitrigel membrane was positioned over the tracheal defect, with the collagen vitrigel membrane facing the tracheal lumen. hiPSCs: human induced pluripotent stem cells, iNCC: hiPSCs-derived neural crest stem cell, iMSCs: hiPSCs-derived mesenchymal stem cells.

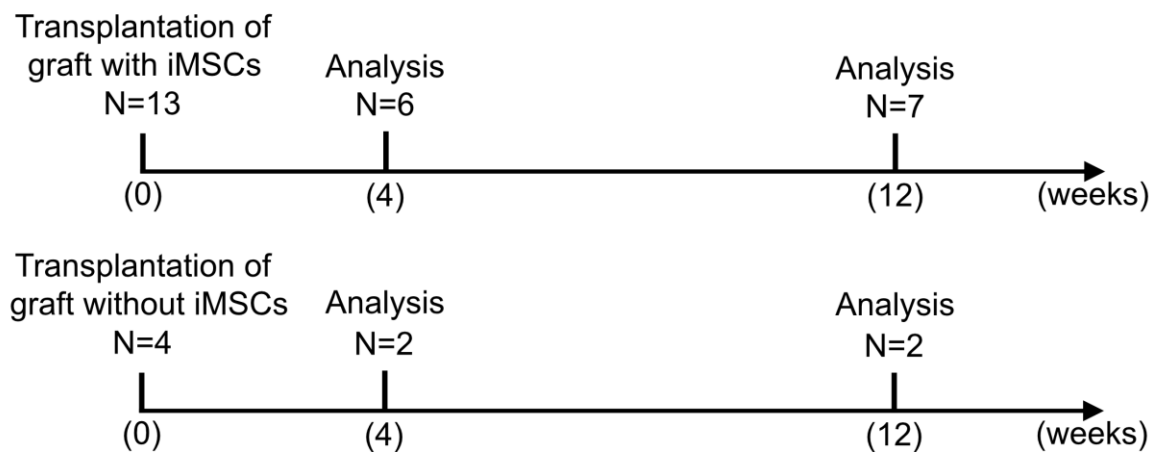


Figure 2. Timeline after the transplantation

Six rats were euthanized at 4 weeks post-transplantation, and seven rats were euthanized at 12 weeks post-transplantation for evaluation. Additionally, grafts without iMSCs were transplanted into four rats as a control. Two rats from the control group were euthanized at 4 weeks post-transplantation, and another two rats were euthanized at 12 weeks post-transplantation for evaluation. iMSCs: human induced pluripotent stem cells-derived mesenchymal stem cells.

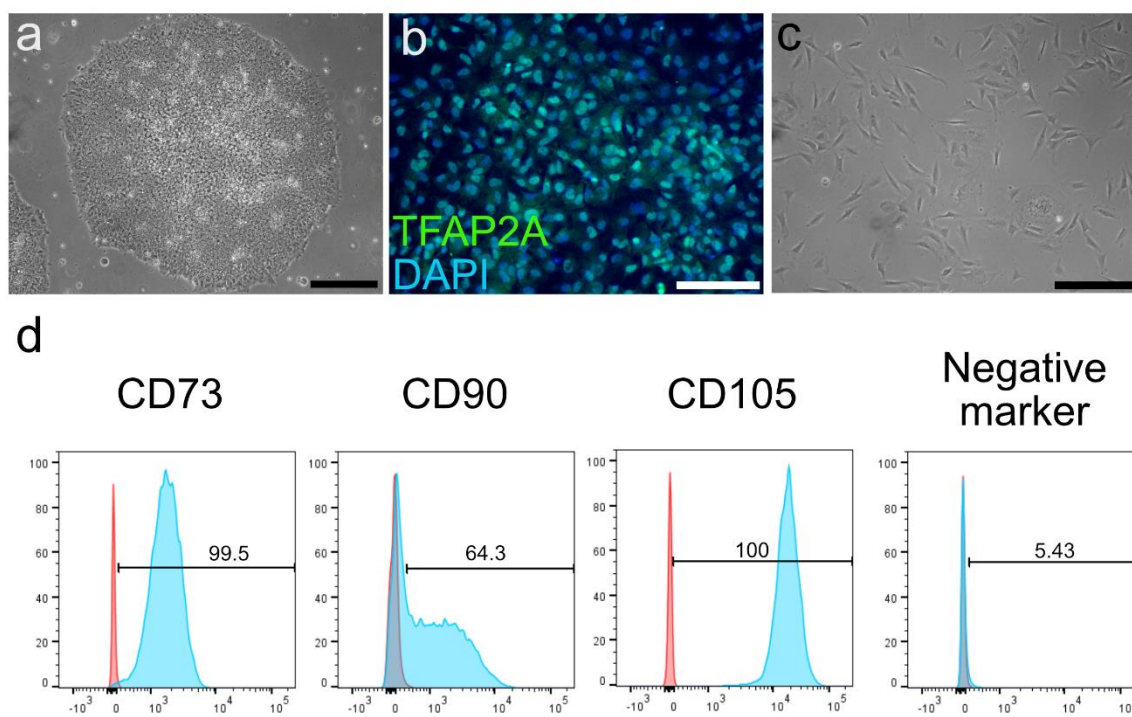


Figure 3. Induction of iMSCs

(a): Phase-contrast image of 1231A3 line undifferentiated hiPSCs. Scale bar = 200 μ m. (b): Immunocytochemistry images of iNCCs using anti-TFAP2A antibody. Scale bar = 100 μ m. (c): Phase-contrast image of induced iMSCs. Scale bar = 200 μ m. (d): Flow cytometry analysis of iMSCs. Induced MSCs show MSC marker expression (CD73, CD90 and CD105), while negative for CD11b, CD19, CD34, CD45, and HLA-DR (blue). Histograms of the isotype control shown in red. hiPSCs: human induced pluripotent stem cells, iNCC: hiPSC-derived neural crest stem cell, iMSCs: hiPSC-derived mesenchymal stem cells.

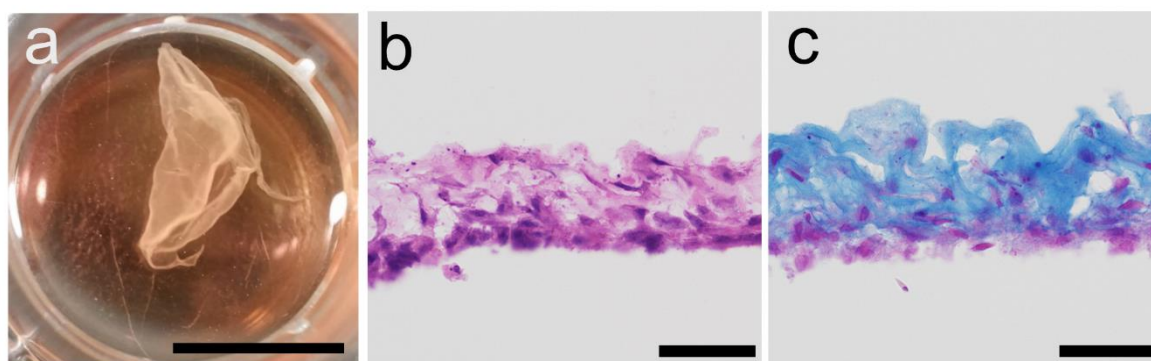


Figure 4. Evaluation iMSC sheets

(a): Macroscopic images of the cell sheet. Scale bar =1 cm. (b, c): Vertical sections of the cell sheet. (b): HE staining, (c): Alcian blue staining. Scale bar = 50 μ m. iMSCs: human induced pluripotent stem cells-derived mesenchymal stem cells.

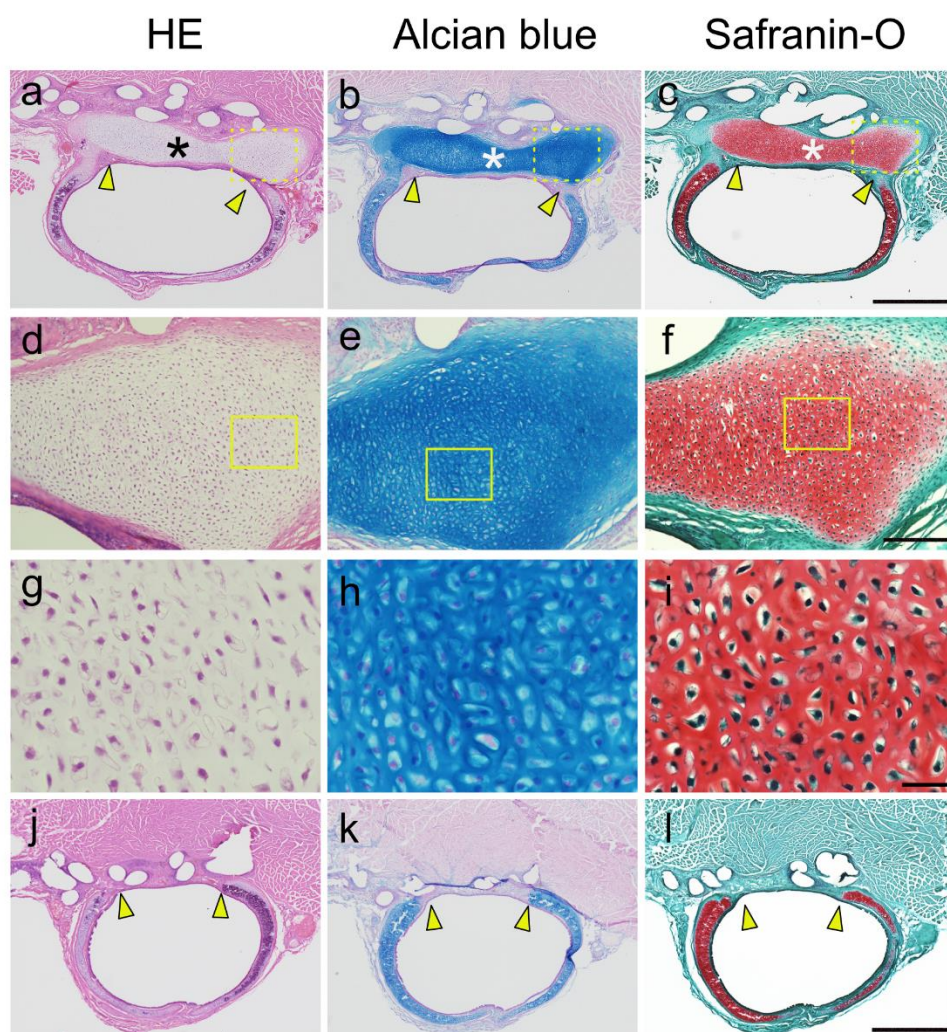


Figure 5. Histological evaluation of tracheal axial sections at 4 weeks postoperatively

(a, d, g, j): HE staining, (b, e, h, k): Alcian blue staining, (c, f, i, l): Safranin-O staining. (a-c) Histological evaluation of the iMSCs graft transplanted trachea. The area between the yellow arrow heads indicates the defect area in the tracheal cartilage. Regenerated tissue was indicated by an asterisk. Regenerated tissue was stained with Alcian blue (b) and Safranin-O staining (c). (d-f): A higher magnification image of the yellow dot box in (a-c). (g-i): A higher magnification image of the yellow box in (d-f). Cartilage-like regenerated tissue was observed in the transplanted area. (j-l): Histological evaluation of the no-cell graft transplanted trachea (control group). Cartilage-like regenerated tissue was not observed. (a-c): Scale bar; (a-c): 1 mm, (d-f): 200 μ m, (g-i): 50 μ m (j-l): 1 mm.

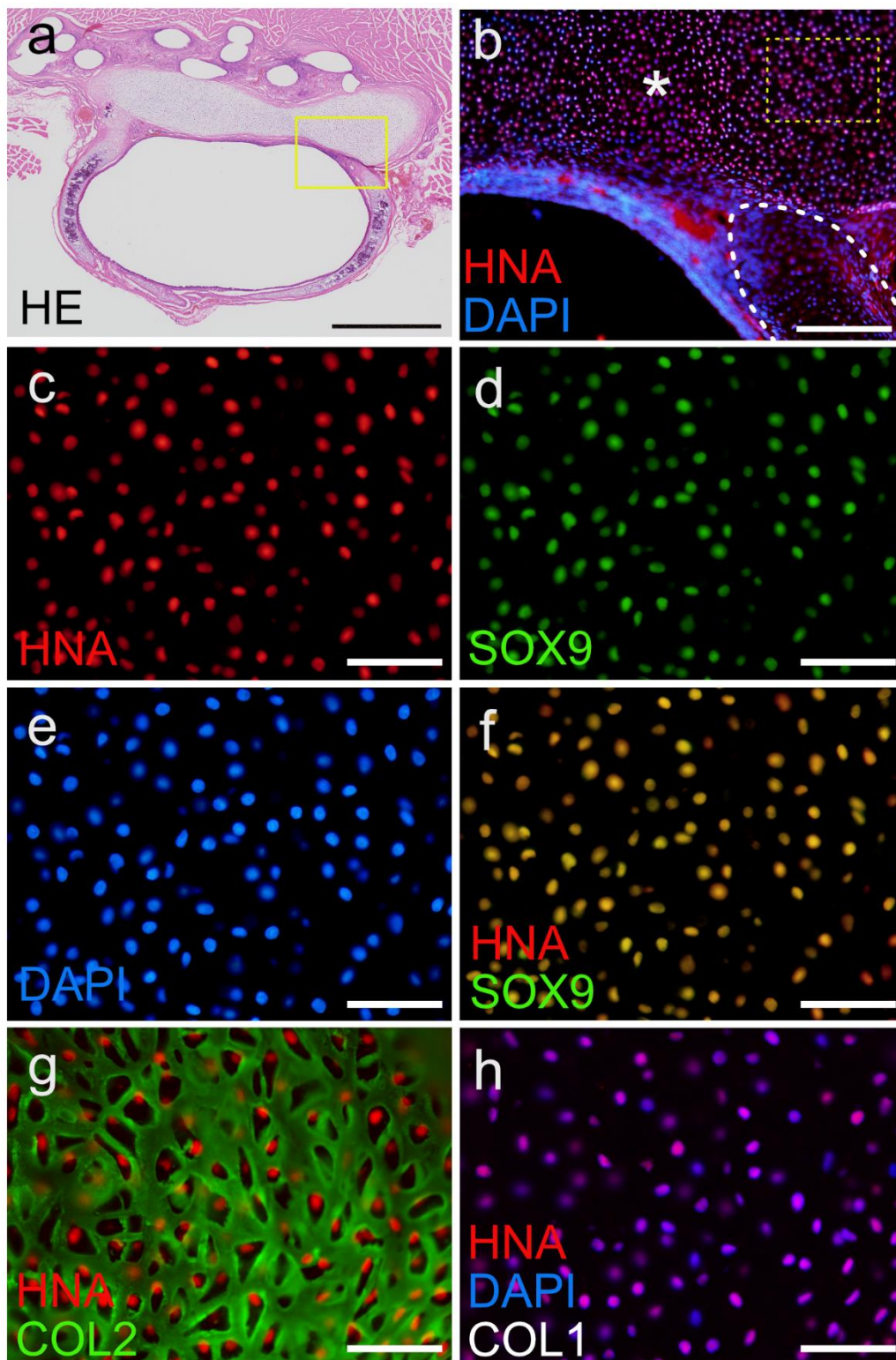


Figure 6. Immunofluorescence staining of cartilage-like regeneration tissue

Immunofluorescence staining of cartilage-like regeneration tissue using, anti-SOX9, anti-human nuclear antigen (HNA), type II collagen (COL2), type I collagen (COL1) antibody.

(a): HE staining. (b): A higher magnification image and immunofluorescence staining of the yellow box in (a). Merge of HNA (red) and DAPI (blue). The regenerated tissue is indicated by an asterisk. The rat trachea is outlined with white dashed lines. (c-h): A higher magnification of the yellow dot box in (b). (c): HNA. (d): SOX9. (e): DAPI. (f): Merge of HNA (red) and SOX9 (green). (g): Merge of HNA (red) and COL2 (green). (h): Merge of COL1 (white), HNA (red), and DAPI (blue). Scale bar; (a): 1 mm, (b): 200 μ m, (c-h): 50 μ m.

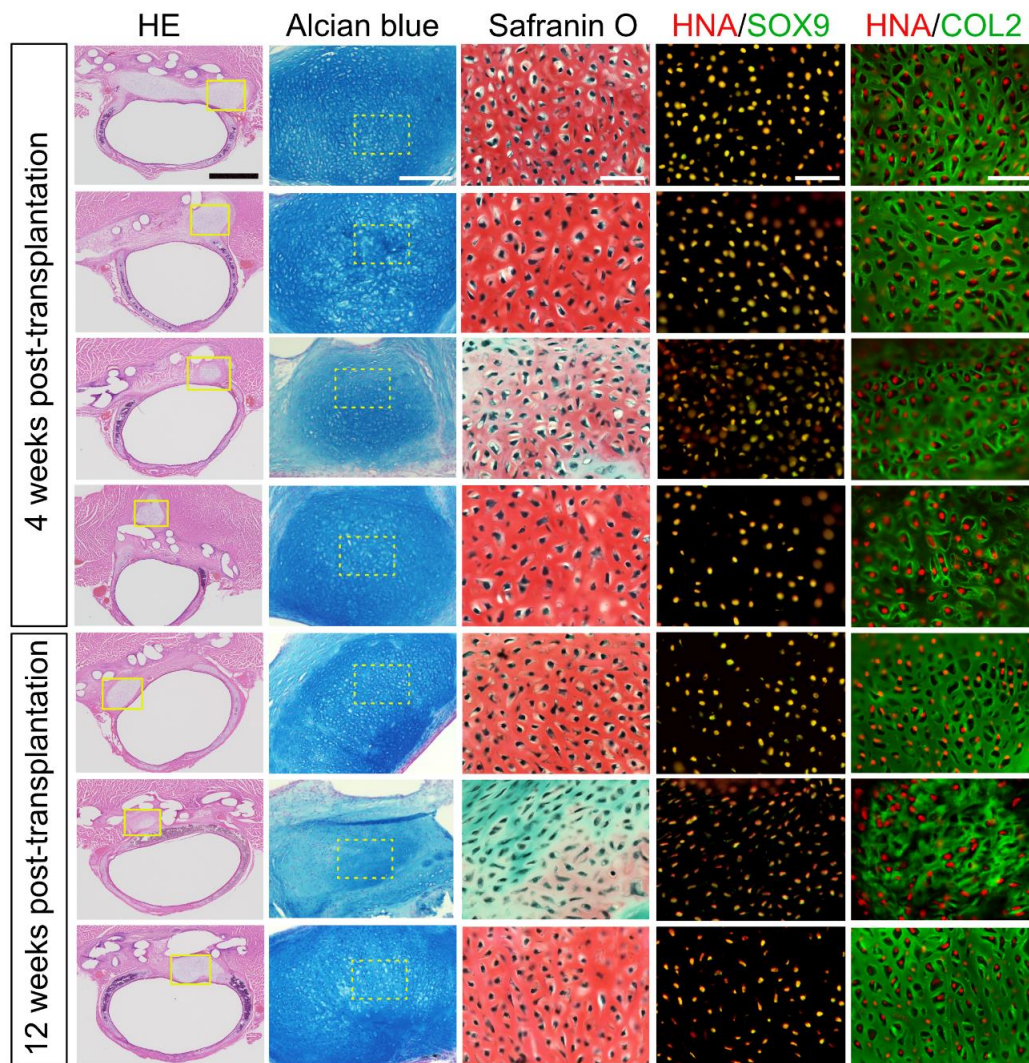


Figure 7. Regenerated cartilage-like tissue at 4 and 12 weeks postoperatively in all rats

The first columns indicate HE staining, the second columns indicate Alcian blue staining, and the third columns show Safranin O staining. The second column shows a higher magnification of the yellow box in the first column. The third to fifth column shows a higher magnification of the yellow dot box in the second column. The fourth column shows immunofluorescence staining, using anti-HNA (red) and anti-SOX9 (green) in regenerated tissue. The fifth column shows immunofluorescence staining, using anti-HNA (red) and anti-COL2 (green). HNA: human nuclear antigen. COL2: type II collagen, COL1: type I collagen. Scale bar; first column: 1 mm, second column: 200 μm , third to fifth columns: 50 μm .