Original article

Stable tracheal regeneration using organotypically cultured tissue composed of autologous chondrocytes and epithelial cells in beagles

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ABSTRACT

Objective: In tracheal regeneration, the slow process of epithelialization is often a barrier to the stability and safety of the transplanted trachea. This study aimed to examine a new tracheal regeneration technique using organotypically cultured tissue composed of autologous cells.

Methods: Nine beagles were prepared. Chondrocytes from auricular cartilage and epithelial cells from buccal mucosa were isolated and cultured. Tissue-engineered cartilages were fabricated with chondrocytes at a density of 1×10^7 cells/ml (high-density group) and 1×10^6 cells/ml (low-density group). A fabricated epithelial cell sheet was laid on a poly(lactic-co-glycolic acid) block in Atelocollagen gel containing the chondrocytes, and the organotypically cultured tissues were transplanted into a partially resected trachea. The control group had only the block transplanted.

Results: The tissue-engineered cartilages in the high-density group contained many viable chondrocytes and many cartilage matrices. The low-density group had abundant collagen fibers and no chondrocytes. Tracheal endoscopy revealed no deformation or atrophy at the transplant site in the high-density group. Histologically, partially hyaline cartilages covered with epithelium and lamina propria were found in the high-density group, but not in the low-density and control groups.

Conclusion: Stable tracheal regeneration was achieved using organotypically cultured tissue fabricated with autologous high-density chondrocytes and epithelial cells.

Key words: Regeneration, Tracheal cartilage, Chondrocyte, Epithelial cell, Dedifferentiation, Epithelialization

INTRODUCTION

Patients with congenital or iatrogenic tracheal stenosis, or tracheal invasion by malignant tumors, often require extensive surgical procedures. Airway reconstruction after resection of these tracheal lesions is challenging. Due to the poor capacity of cartilage to repair itself, a tracheal defect larger than two-thirds of its circumference requires rigid reconstruction.[1,2] However, a defect larger than 6.5 cm is difficult to reconstruct using an end-to-end anastomosis procedure,[3] and standard reconstructive procedures for large tracheal defects have yet to be established.

Several approaches for tracheal replacement, including the use of autologous tissue, have been reported. Direct scaffold transplantation has been used as one approach to repair tracheal defects.[4-6] However, epithelialization from surrounding normal tissue after transplantation could take several months. Moreover, the property of scaffolds often become compromised or lost due to airway pressure and contamination. An alternative approach is to implant autologous chondrocytes subcutaneously and produce cartilage matrices in another part of the body. The fabricated cartilage can then be removed and transplanted to the tracheal defect. This method, however, requires two surgeries and may thus place a heavy burden on the patient. In addition, the epithelialization process at the transplanted site could take a long time to complete.

There are arguments for and against the use of transplanted cells in tracheal regeneration. Previous studies have shown that chondrocytes tend to dedifferentiate to fibroblast-like cells in a monolayer culture.[7] The dedifferentiated chondrocytes have little ability to produce cartilage matrices. Therefore, if these cells are used for implantation, cartilage regeneration might be delayed. Three-dimensional high-density chondrocyte cultures have been used in some studies to maintain chondrocyte

phenotype.[8,9] On the other hand, there are few reports concerning tracheal reconstruction using epithelium.[6.10] We hypothesized that in organotypically cultured tissue composed of both autologous chondrocytes and epithelial cells, rapid epithelialization could occur, creating a physical barrier that ensures the stability and safety of the regenerated trachea after transplantation.

This study aimed to examine a new tracheal regeneration technique using organotypically cultured tissue composed of autologous chondrocytes and epithelial cells, comparing high-density and low-density chondrocyte cultures.

MATERIALS AND METHODS

Experimental animals

All experimental protocols used in this study were approved by the Kurume University Animal Care and Treatment Committee. Nine female beagles (KBT Oriental, Saga, Japan), including three controls, weighing 12.0 to 13.8 kg were caged individually with free access to standard laboratory chow and tap water. The cage size was 950 mm (width) x 1000 mm (depth) x 1920 mm (height). Since tracheal structures are quite similar between dogs and humans, dogs are often used in tracheal research.[11,12]

Cell harvesting, isolation, and culture

The fabrication procedure of organotypically cultured tissue is depicted in Fig. 1. Six experimental beagles were anesthetized with intramuscular xylazine (2 mg/kg) and midazolam (0.3 mg/kg), and with intravenous pentobarbital sodium (5-10 mg/kg). Next, 5 mm × 5 mm pieces of auricular cartilage and 3 mm × 3 mm specimens of buccal mucosal tissue were surgically excised from each of the six dogs. The cartilage

excluding the perichondrium and the mucosa excluding submucosal tissue were individually washed with Dulbecco's phosphate-buffered saline (PBS) containing antibiotics.

The cartilage pieces were divided into approximately 1 mm^3 pieces and were digested in 0.1% type II collagenase (Sigma) solution at 37°C for 8 hours. Chondrocytes were then isolated and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified atmosphere of 5% carbon dioxide (Fig. 2A). The medium was replaced three times per week. On the primary culture, the chondrocytes reached confluence over a two-week period and were passaged using 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA, Gibco). The chondrocytes were collected and seeded at 3×10^6 cells/cm^2. On the second passage, the chondrocytes reached confluence in a week, and cell counts reached approximately 6×10^7 cells.

Epithelial cells were cultured as previously reported[13]. Briefly, submucosal sections removed from the harvested mucosal tissues were incubated in DMEM/F-12 (Gibco) containing 1.2 U/mL of dispase II (Roche) at 37°C for 1 hour. A thin epithelial layer was then peeled from the subepithelium. Collected materials were placed in trypsin and EDTA for 7 minutes at 37°C to form single-cell suspensions. Approximately 5×10^5 suspended epithelial cells were obtained per epithelial layer. Epithelial cells were then cultured in a keratinocyte culture medium composed of DMEM/F-12, supplemented with 10 ng/mL epidermal growth factor (Sino Biological), 10 μg/mL insulin (Wako), 0.5 μg/mL hydrocortisone (Wako), 0.25 μg/mL isoproterenol (Wako), 1.3 ng/mL triiodothyronine (MP Biomedicals), 100 units/mL penicillin, 100 μg/mL

streptomycin, 0.25 μg/mL amphotericin B, and 4% FBS for two weeks (Fig. 2B). Proliferated epithelial cells were harvested and temporarily stored in liquid nitrogen for two weeks.

Fabrication of tissue-engineered cartilage

Chondrocytes from the second passage were mixed with type I collagen gel (Koken) at a density of 1×10^7 cells/mL (high-density group, n=3) and 1×10^6 cells/mL (low-density group, n=3). The cell-mixed collagen gel (3 mL) was placed into a cell-culture insert 23.1 mm in diameter and 0.5 μm in pore size (Falcon). Each cartilage matrix production was promoted with 5 ng/mL transforming growth factor-β1 (TGF-β1), 5 ng/mL insulin, 50 μg/mL ascorbic acid, and 40 μg/mL L-proline, and was mixed with the collagen gel in addition to 10 times concentrated DMEM and 10% FBS. Three-dimensional cultures were performed at 37°C in a humidified atmosphere of 5% carbon dioxide to induce chondrocyte redifferentiation. After culturing for one week, each collagen gel was removed and histologically examined using Hematoxylin and Eosin (H&E) and Safranin O staining. Levels of chondrocyte proliferation were compared between the two groups.

Preparation for transplantation

A poly(lactic-co-glycolic acid) (PLGA, Funakoshi) block 15 mm in diameter and 1.5 mm in thickness (Fig. 1A and 1B) was placed into the chondrocyte suspended gel in both the high- and low-density groups as an implant carrier. The three-dimensional culture was performed for two additional weeks.

During chondrocyte culturing, the proliferated epithelial cells were seeded on

cell-culture inserts and incubated with a mitomycin C-treated 3T3 feeder layer at 37°C in a humidified atmosphere of 5% carbon dioxide for two weeks. This completed the fabrication of the epithelial cell sheet with stratified epithelial cells.

Shortly before transplantation, the membrane of the cell-culture insert was circumferentially cut out with a scalpel and the epithelial cell sheet was easily separated from the membrane with a cell scraper. The epithelial cell sheet was laid on the upper surface of the PLGA block containing chondrocytes, resulting in the organotypically cultured tissue.

Transplantation of organotypically cultured tissue to the tracheal defect

Nine dogs were anesthetized with intramuscular xylazine (2 mg/kg) and midazolam (0.3 mg/kg) and intravenous pentobarbital sodium (5-10 mg/kg), and were intubated in the supine position. The canine trachea was exposed through the anterior neck. A round tracheotomy 15 mm in diameter was performed. The prepared organotypically cultured tissues in the high- and low-density groups were transplanted into the resected part of the trachea with the epithelial surface on the luminal side and fixed with fibrin glue. In the control group, the PLGA block alone was transplanted in the same way and fixed with fibrin glue (n=3).

Evaluation of the regenerated trachea

Two months after transplantation, tracheal endoscopy was performed in all dogs under anesthesia to evaluate the tracheal lumens. Dogs were euthanized by intravenous injection of pentobarbitone solution (100 mg/kg), and their tracheas were removed. The tracheal tissues were examined histologically using H&E, Elastica van Gieson (EVG),

and Safranin O staining. Transverse sections 10 μm in thickness were obtained from the center of the reconstructed region in the whole trachea of each dog for quantitative analysis. For each dog in each group, light microscopic images of the sections stained with H&E and EVG were quantitatively analyzed using NIS-Elements D3.2 software (Nikon, Tokyo, Japan), as follows. The regenerated chondrocytes were counted from six consecutive sections of specimens by observing with a light microscope and averaged (cells/section). The thickness of the epithelium and basement membrane were measured at 10 randomly selected regions on the specimens with EVG staining. Statistical analysis was performed using StatMate III for Windows **(**ATMS, Tokyo, Japan). $P<0.05$ was considered statistically significant (nonparametric Kruskal-Wallis test).

RESULTS

Fabrication and evaluation of tissue-engineered cartilage

Tissue-engineered cartilage was successfully fabricated *in vitro* in both experimental groups. The collagen gel in the high-density group (Fig. 3A) shrank more than those in the low-density group (Fig. 3B). Phase-contrast microscopy revealed that the proliferated chondrocytes maintained a more round shape in the high-density group (Fig. 3C), compared to the flattened fibroblast-like cells in the low-density group (Fig. 3D). In the tissue-engineered cartilage in the high-density group, H&E staining showed many viable, round-shaped chondrocytes in cartilage lacunae and amorphous-shaped extracellular matrices (Fig. 3E). On the other hand, in the low-density group, fibroblast-like cells were observed in the abundant collagen fibers that originated from the collagen gel (Fig. 3F). The extracellular matrices in the high-density group were

stained with Safranin O (Fig. 3G), but those in the low-density group were not stained (Fig. 3H).

Transplantation of organotypically cultured tissue to the tracheal defect

The organotypically cultured tissue was successfully transplanted to the resected portion of the trachea (Fig. 4). There was no evidence of stridor, graft collapse, stenosis, or weight loss during the post-operative period. However, all dogs in the control group temporarily experienced skin redness and subcutaneous swelling in the transplanted region after transplantation. Two months after transplantation, tracheal endoscopy revealed that the tracheal luminal shape was regenerated without deformation in the high-density group (Fig. 5), but with deformation in the low-density group (not shown) and in the control group (not shown). The tracheal lumen was completely covered by epithelium in all groups.

Evaluation of the regenerated trachea

In the high-density group, whole tracheal transverse sections stained with H&E staining showed partially regenerated cartilage on the transplanted portion of the tracheal rings (Fig. 6A). With higher magnification, these were identified as hyaline cartilages (Fig. 6B). The regenerated cartilages were covered with pseudostratified ciliated columnar epithelium and lamina propria (Fig. 6C). The partially regenerated cartilages were stained with Safranin O, suggesting the production of cartilage matrices (Fig. 6D). On the other hand, the transplanted regions in the low-density and control groups were histologically atrophic and covered with thinner pseudostratified ciliated epithelium compared to that of the high-density group (Fig. 6E and 6F). The

regenerated cartilages were hardly observable in the low-density and control groups. Scar tissue formation and proliferation of collagen fibers were observed in both the low-density group (Fig. 6G) and the control group (Fig. 6H), but scar tissue was more pronounced in the control group.

In the quantitative analysis, the number of chondrocytes in the regenerated regions was significantly higher in the high-density group than in the low-density and control groups (Fig. 7A). The thickness of the epithelial layer and basement membrane in the regenerated regions were also significantly higher in the high-density group than in the low-density and control groups (Fig. 7B).

DISCUSSION

The morphological and functional characteristics of the *in vitro* tissue-engineered tracheas resembled normal tracheas in the high-density group, but not in the low-density group. During the short period after transplantation, partial regeneration was completed in the organotypically cultured tissue fabricated with autologous high-density chondrocytes and epithelial cells; the use of low-density-chondrocyte culture or PLGA alone proved to be insufficient. Our results suggest that organotypically cultured tissue could serve as an effective substitute for allografts in the reconstruction of optimally structured trachea.

Transplantation of chondrocytes in tracheal regeneration

Vacanti et al. first succeeded in regenerating the trachea using a tissue-engineering approach in 1994.[14] They achieved this by fabricating cylindrical cartilaginous tissue in the subcutis of a nude mouse using a silastic tube wrapped with chondrocytes prior to

tracheal transplantation and transplanting it into a tracheal defect. Some similar methods using cartilaginous tissue fabricated in a subcutaneous area other than the trachea have been reported.[15,16] However, these methods require a long period of time before the second transplantation. Furthermore, in clinical practice, two surgeries may be very taxing for the patient.

Previous methods using dedifferentiated chondrocytes with or without a scaffold have been reported for tracheal regeneration.[4,5,14] Direct transplantation of dedifferentiated chondrocytes to the trachea has a high risk of infection or collapse due to fluctuation in airway pressure. These complications may lead to respiratory compromise and death. Improving the strength of the implants and accelerating tissue regeneration to ensure stability and safety after transplantation are therefore important. Komura et al. reported a transplantation technique using an absorbable material made of caprolactone, polyglycolic acid mesh, and a collagen sheet with chondrocytes.[17] One disadvantage of this method was the three months it took to produce a cartilage matrix. Omori et al. reported another transplantation technique using a combination of polypropylene mesh and a collagen sponge.[4] However, transplantation using a scaffold alone cannot regenerate cartilage in the transplanted portion, and it also takes more than two months to complete epithelialization. To accelerate tissue regeneration after transplantation, the tissue-engineered trachea requires not only differentiated chondrocytes but also epithelium to serve as a physical barrier.

In the present study, autologous cultured cells and biodegradable PLGA scaffolds were directly transplanted to the tracheal defect. According to a previous report,[18] the half of PLGA scaffold was degraded 45 days after transplantation. In addition, this was the first transplantation of organotypically cultured tissue composed of autologous

chondrocytes and epithelial cells to the trachea.

Transplantation of epithelial cells in tracheal regeneration

Nomoto et al. reported that isolated rat tracheal epithelial cells were seeded on collagenous gel that was stratified on a collagenous sponge.[19] The transplanted epithelium differentiated from single- or double-stratified squamous epithelium into columnar ciliated epithelium.

In this study, even though the transplanted epithelium was stratified squamous epithelium, the regenerated regions were covered with pseudostratified ciliated columnar epithelium and lamina propria two months after transplantation in all groups. This strongly suggested that metaplasia of the epithelium and regeneration of the lamina propria had occurred. The inadequate thickness of the regenerated epithelium in the control group, however, indicated that the epithelialization was completed not by metaplasia but by ingrowth from the surrounding tracheal epithelium. On the other hand, the regenerated epithelium in the high-density group was much thicker compared to that of the control group or the low-density group. Therefore, as observed in the high-density group, the favorable regeneration of the cartilaginous tissue and lamina propria without scar tissue formation could facilitate epithelialization.

However, the limitation of this experimental method is that it is still difficult to determine that the pseudostratified ciliated columnar epithelial cells covering the transplanted region were the transplanted epithelial cells. Labeling the transplanted cells with fluorescent proteins, such as GFP, might help solve this issue. Although there are challenges to overcome before clinical trials can be performed in the future, the use of tracheal epithelial cells may be more appropriate for regenerating pseudostratified

ciliated columnar epithelium on the lumen of a tissue-engineered trachea.

Recently, many investigators have reported that transplantation of epithelial cell sheets prevents mucosal stenosis and deformation.[20, 21] The present study used epithelial cell sheets on tissue-engineered tracheas, achieving partial regeneration of tracheal cartilage more quickly than in previous studies. The epithelial cell sheets on the high-density cultured chondrocytes may have played an important role in promoting the stability and safety of the regenerated trachea after transplantation. In contrast, they may have had little or no effect on the low-density cultured chondrocytes.

High-density three-dimensional chondrocyte cultures

For the purpose of preventing dedifferentiation of chondrocytes, we performed high-density three-dimensional chondrocyte cultures in a collagen gel. As a result, the high-density group regenerated round-shaped chondrocytes isolated in individual compartments or lacunae in cartilage matrices.

In auricular chondrocytes, it has been well established that a simple culturing technique using conventional monolayer passaging leads to a loss of the chondrogenic phenotype and results in an overall fibroblast-like phenotype.[22] Chondrocytes are generally considered dedifferentiated after an average of about five monolayer passages. Furthermore, the concentration of cultured chondrocytes also affects the phenotype of the cultured chondrocytes. High-density three-dimensional chondrocyte cultures are well known to maintain the chondrocyte phenotype and to support redifferentiation of dedifferentiated articular chondrocytes.[23]

In contrast, some authors in the area of orthopedics have reported a cartilage regeneration technique using a three-dimensional culture without a high-density

chondrocyte culture.[8,9] However, a low-density chondrocyte culture of less than 1×10^7 cells/mL tends to cause chondrocyte dedifferentiation.[24] In the low-density group in the present study, the dedifferentiated chondrocytes had a fibroblast-like spindle shape and did not produce cartilage matrices. On the other hand, extracellular matrices in the high-density group were amorphous and stained with Safranin O, resembling cartilage matrices. Cartilage matrices in an amorphous gel contain large amounts of proteoglycans, allowing for hydration of cartilage. Abundant cartilage matrices surrounding chondrocytes are important to enable each differentiated chondrocyte to survive in isolated cartilage lacunae. Our study revealed that organotypically cultured tissue composed of high-density chondrocytes and epithelial cells could more effectively regenerate tracheal defects.

CONCLUSION

We fabricated tissue-engineered cartilages of both autologous high- and low-density differentiated chondrocytes *in vitro*. The morphological characteristics resembled normal tracheas in the high-density group, but not in the low-density group. The partially regenerated cartilage with cartilage matrices was safely produced two months after transplantation using organotypically cultured tissue fabricated with high-density differentiated chondrocytes and epithelial cells. This reconstruction technique could offer substantial clinical advantages over using a scaffold alone for treating intractable diseases such as severe tracheal stenosis.

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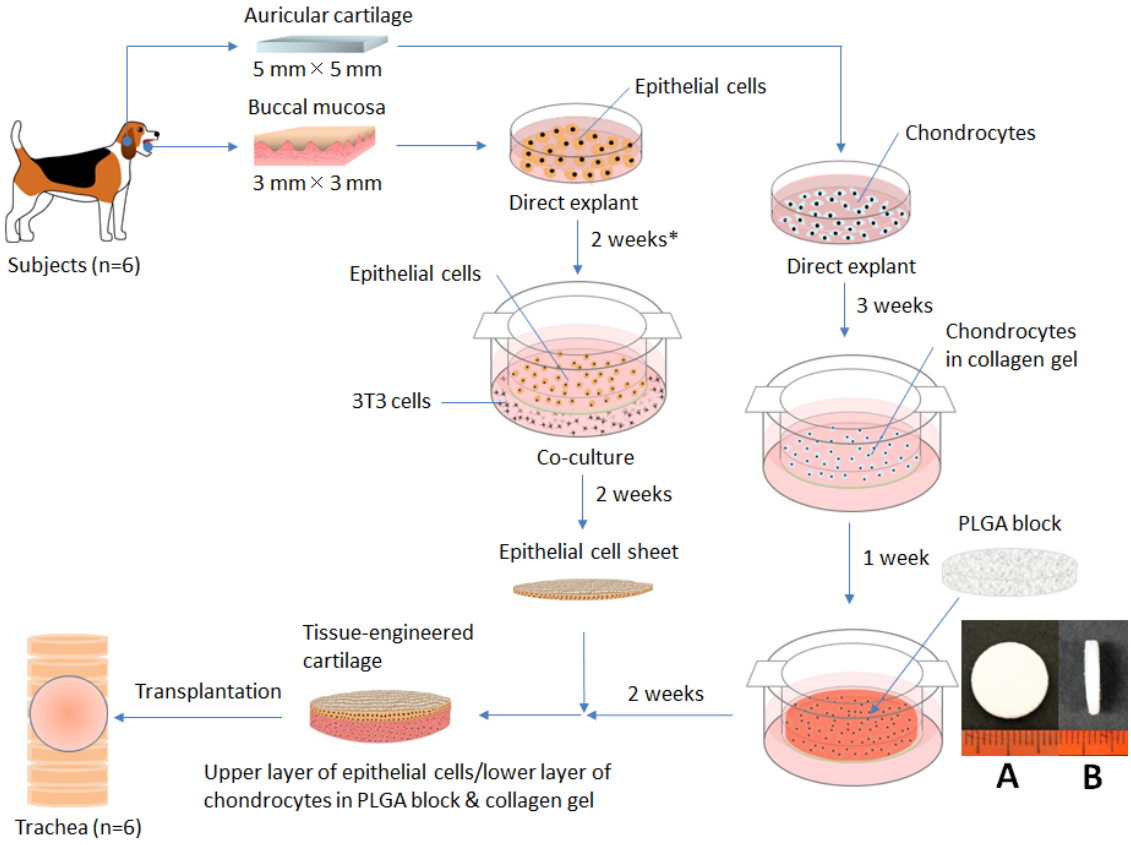
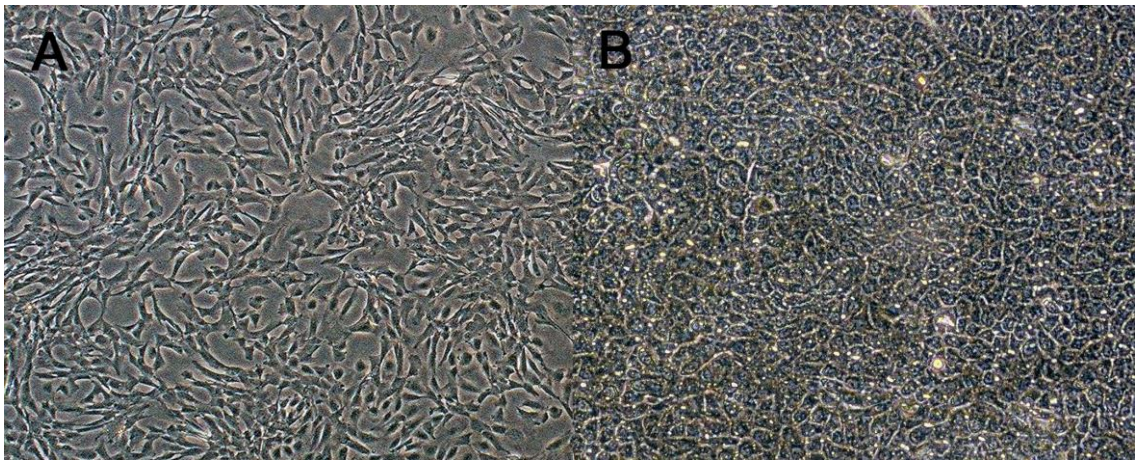
Fig. 1. Cell culturing protocol for tissue-engineered organotypic tissue and transplantation to tracheal defect



A 5 mm × 5 mm piece of auricular cartilage and a 3 mm × 3 mm specimen of buccal mucosal tissue were surgically excised. Chondrocytes were isolated from the cartilage piece and cultured for two weeks. Chondrocytes of the second passage were mixed into type I collagen gel. A poly(lactic-co-glycolic acid) (PLGA) block was placed into the chondrocyte suspended gel. A three-dimensional culture was performed for an additional two weeks. Isolated epithelial cells from the epithelium were separately cultured for two weeks. The proliferated epithelial cells were cultured on cell-culture inserts with a mitomycin C-treated 3T3 feeder layer for two weeks. This completed the fabrication of the epithelial cell sheet with stratified epithelial cells. Just before transplantation, the epithelial cell sheet was laid on the upper surface of the PLGA block

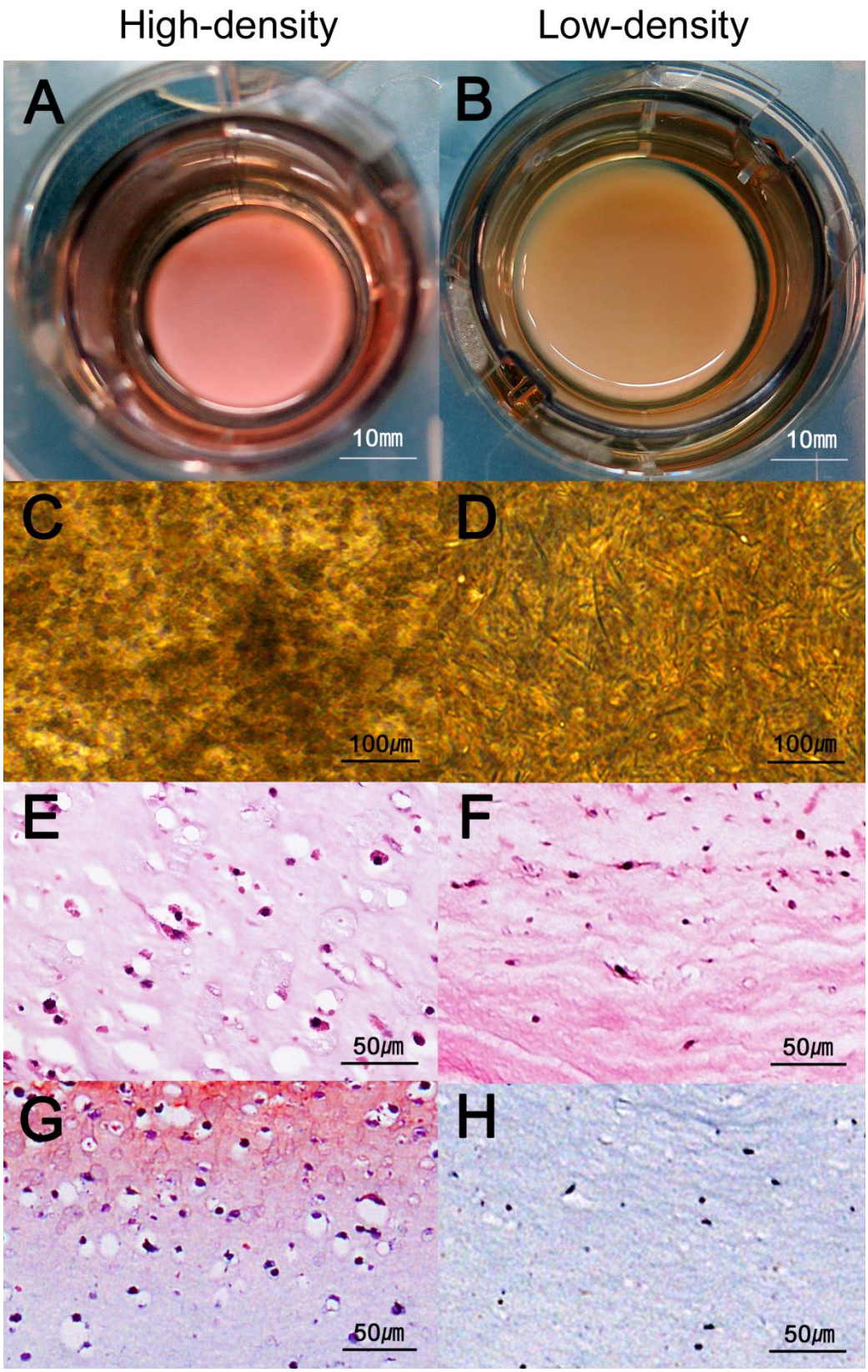
including chondrocytes. The organotypically cultured tissues were transplanted into the resected part of the trachea with the epithelial surface on the luminal side. *: The proliferated epithelial cells were harvested and temporarily stored in liquid nitrogen for two weeks additionally. Photographs of PLGA block 15 mm in diameter (A) and 1.5 mm in thickness (B).

Fig. 2. Phase-contrast microscopy of the cultured chondrocytes



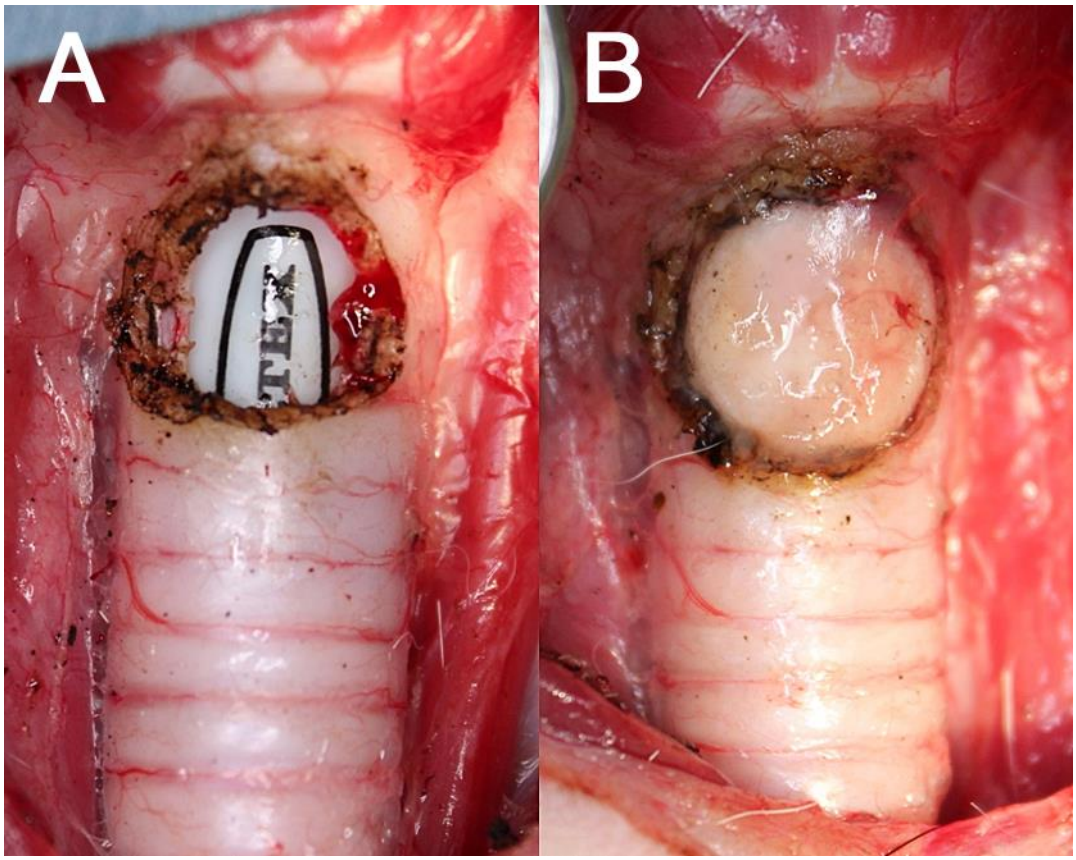
The isolated chondrocytes (A) and epithelial cells (B) were cultured and proliferated in each culture medium.

Fig. 3. Two types of tissue-engineered cartilage fabricated *in vitro*



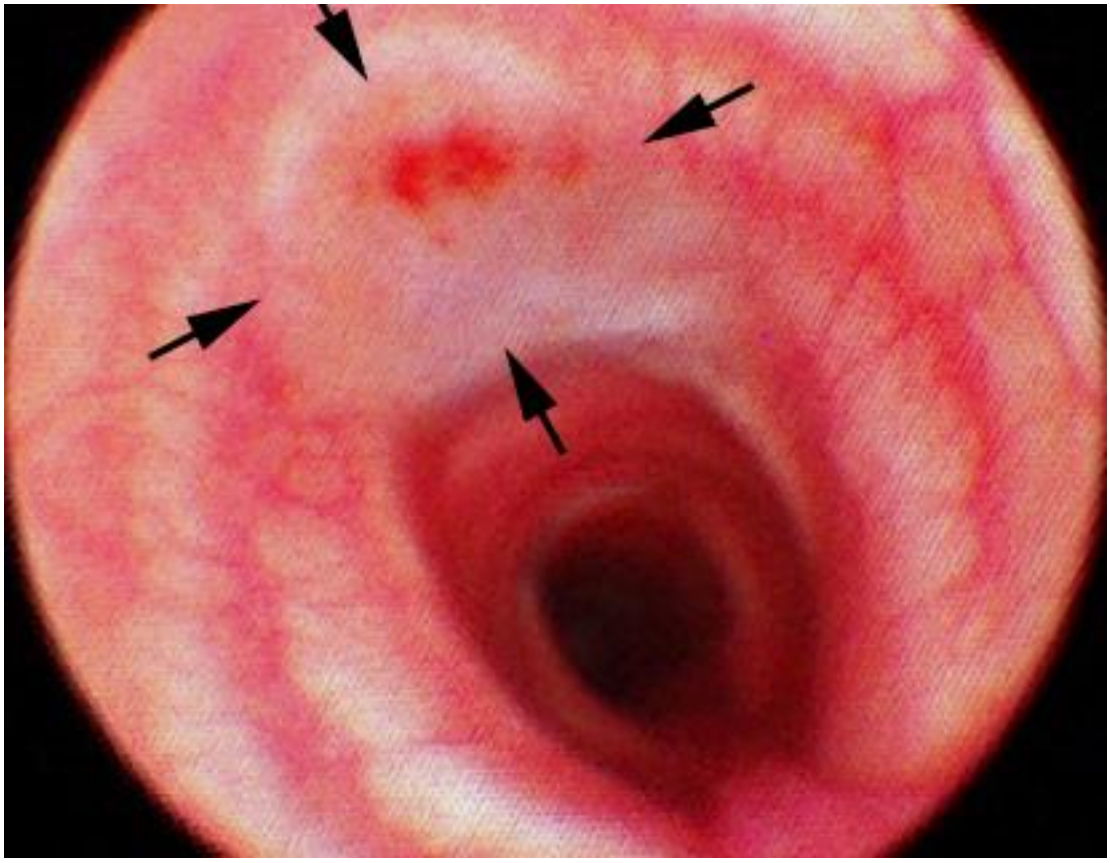
The collagen gel in the high-density group (A) shrank more than those in the low-density group (B) in each cell culture insert. Phase-contrast microscopy revealed that the proliferated chondrocytes maintained a rounder shape in the high-density group (C) as compared with the flattened fibroblast-like cells in the low-density group (D). Hematoxylin and Eosin staining showed many viable round chondrocytes with cartilage lacunae and amorphous extracellular matrices in the tissue-engineered cartilages in the high-density group (E). The engineered tissues in the low-density group were composed of fibroblast-like cells and abundant collagen fibers (F). The extracellular matrices in the high-density group were stained with Safranin O (G), but those in the low-density-group were not stained (H).

Fig. 4. Transplantation of organotypically cultured tissue



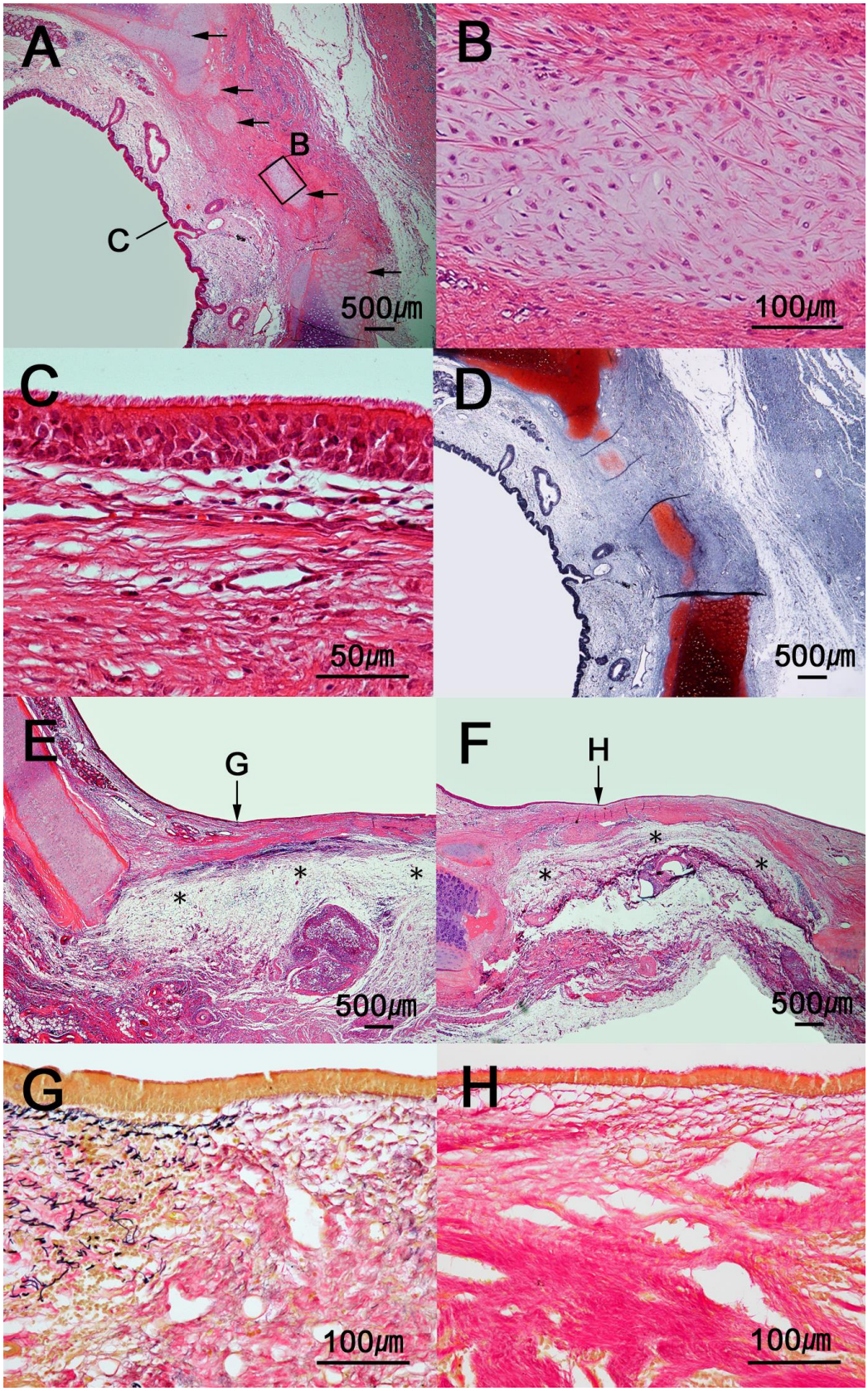
The tracheal wall was partially resected (A). The organotypically cultured tissue was successfully transplanted to the resected portion of the trachea (B).

Fig. 5. Tracheal endoscopic findings two months after transplantation



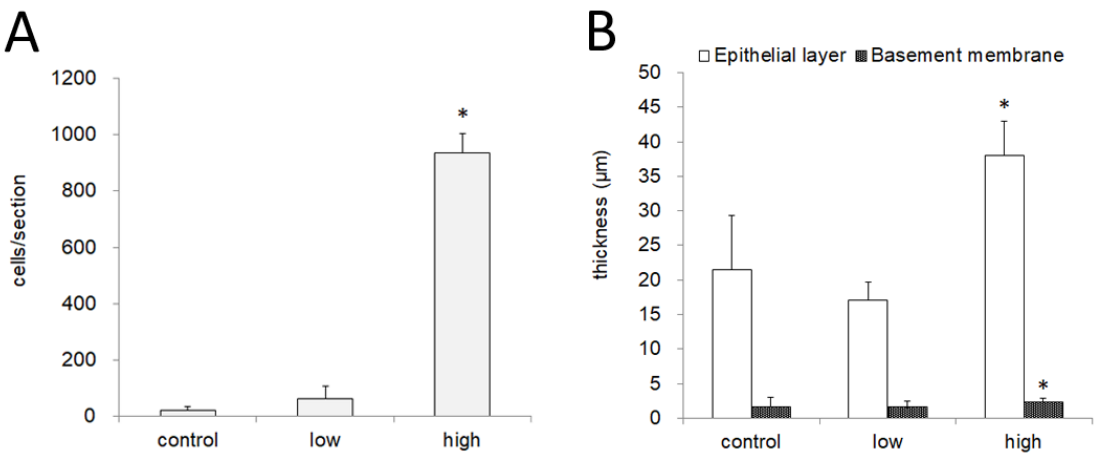
Tracheal endoscopy in the high-density group showed that the tracheal luminal shape was regenerated without deformation at the resected part of the trachea (arrows) and the lumen of the trachea was completely covered with epithelium.

Fig. 6. Evaluation of the regenerated trachea



In the high-density group, Hematoxylin and Eosin staining revealed the partially regenerated cartilages (arrows) on the transplanted portion of the tracheal ring (A). Many of these were identified as hyaline cartilage (B). The regenerated cartilages were covered with pseudostratified ciliated columnar epithelium (respiratory epithelium) and lamina propria (C). The partially regenerated cartilages were stained with Safranin O (D). The transplanted regions in the low-density (E) and control (F) groups were histologically atrophic and had no regenerated cartilages (asterisks). Scar tissue formation and proliferation of collagen fibers were observed in both the low-density group (G) and the control group (H), but scar tissue was more pronounced in the control group.

Fig. 7. Measurement results in each regenerated region



The number of chondrocytes in the regenerated regions was significantly higher in the high-density group than in the low-density and control groups (A). The thickness of the epithelial layer and basement membrane in the regenerated regions were also significantly higher in the high-density group than in the low-density and control groups (B). Statistical significance was determined by the nonparametric Kruskal-Wallis test.

Dunn’s post-tests were used to determine statistical significance between the high–density group and low-density group, and between the high-density group and control group. * is $p<0.05$.