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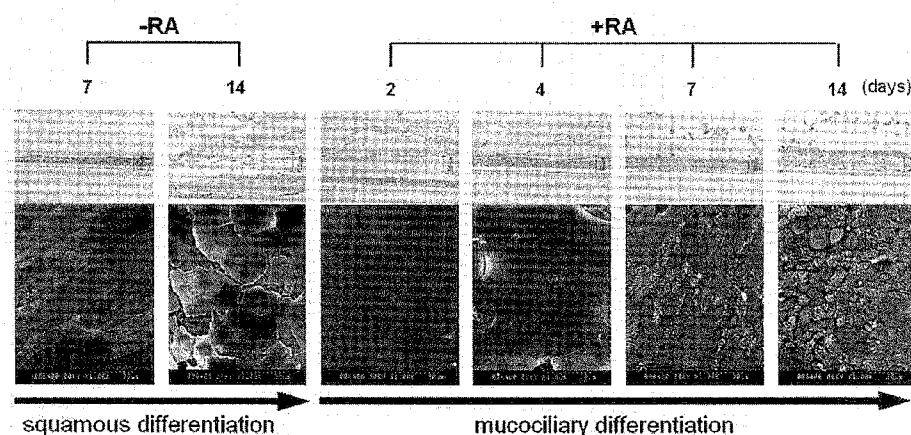
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(54) Title: **IMPLANTS FOR RECONSTRUCTING MUCOSAL LUMEN**

Fig. 1



(57) Abstract: The present invention relates to implants for reconstructing defect of mucosa which comprise squamous epithelial cells as active ingredients. The implants of the present invention, after grafted to defect site, differentiate into mucociliary epithelium, form tight junctions with surrounding cells, and regenerate mucosa with normal appearance. In addition, the present implants, after grafted to defect site, differentiate into mucociliary epithelium and induce little or no crust formation or mucus stagnation because of the ciliary movement on this epithelial cells. The implants of the present invention are especially advantageous to reconstruct the luminal side of the airway.

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IMPLANTS FOR RECONSTRUCTING MUCOSAL LUMEN

FIELD OF THE INVENTION

The present invention relates to implants for reconstructing mucosal lumen,
5 particularly to implants for reconstructing mucosal lumen of the airway.

DESCRIPTION OF THE RELATED ART

Complex reconstruction of major facial defects, especially defects of airway
mucosal lumen, is necessary in some patients with sinonasal or tracheal tumors. Such
10 surgical defects can successfully be reconstructed through the use of three-dimensional
free-flaps (1, 2), with the airway side of the free flap usually repaired with a skin graft.
A skin graft can also be applied to the denuded area of the trachea after the lesion is
removed, especially in some cases of tracheal stenosis (3).

To date, skin has been the most widely used material to reconstruct the
15 luminal side of the airway (4, 5). But there are some serious problems with the use of
skin as a substitute for the ciliated columnar epithelium that normally lines the luminal
side of the airway. These problems include constriction of the graft, desquamation, the
re-growth of hair, and an unpleasant odor (6, 7).

Because ciliated columnar epithelium is physiologically the proper tissue for the
20 reconstruction of the luminal side of the airway, the present inventors previously
attempted to use cultured nasal ciliated columnar epithelium for this purpose, but did
so by grafting it directly in the airway. However, this was unsuccessful because the
cultured nasal ciliated columnar epithelium was not tough enough to suture to the
recipient site.

25 In previous experiments of the present inventors, we identified that mucociliary
columnar epithelial cells could differentiate into squamous epithelium like skin under
retinoic acid (RA)-depleted culture conditions (8), and found that mucociliary columnar
epithelium could be restored from the squamous epithelium when RA is supplemented

to the culture medium.

Throughout this application, several patents and publications are referenced and citations are provided in parentheses. The disclosure of these patents and publications is incorporated into this application in order to more fully describe this invention and the state of the art to which this invention pertains.

DETAILED DESCRIPTION OF THIS INVENTION

The present inventors have made intensive researches to develop efficient methods of reconstructing defects of mucosal lumen, especially mucosal lumen of the airway. As a result, we have found that squamous epithelial cells-containing implants grafted to the defected areas of mucosa could ensure more efficient reconstruction of the mucosa defects with no shortcomings associated with conventional skin implantation technologies such as crust formation.

Accordingly, it is an object of this invention to provide an implant for reconstructing mucosal lumen.

It is another object of this invention to provide a method for reconstructing mucosal lumen.

It is still another object of this invention to provide a use of the implant for preparing medicament to reconstruct mucosal lumen, comprising squamous epithelial cells.

Other objects and advantages of the present invention will become apparent from the detailed description to follow taken in conjugation with the appended claims and drawings.

In one aspect of the present invention, there is provided an implant for reconstructing mucosal lumen, which comprises squamous epithelial cells as active

ingredients.

In another aspect of the present invention, there is provided a method for reconstructing mucosal lumen, which comprises administering to a subject a pharmaceutically effective amount of the implants comprising squamous epithelial cells.

5 In still another aspect of the present invention, there is provided a use of an implant for preparing medicament to reconstruct mucosal lumen, comprising squamous epithelial cells.

The present inventors have made intensive researches to develop efficient
10 methods of reconstructing defects of mucosal lumen, especially mucosal lumen of the airway. As a result, we have found that squamous epithelial cells-containing implants grafted to the defected areas of mucosa could ensure more efficient reconstruction of the mucosa defects with no shortcomings associated with conventional skin implantation technologies such as crust formation.

15 The present invention may be applied to respiratory organs lined by mucociliary cells among human organs.

According to a preferred embodiment of the present invention, the squamous epithelial cells used in the present invention are prepared *in vitro* in accordance with a process comprising the steps of (a) culturing and proliferating epithelial cells in a
20 medium containing retinoic acid (RA); and (b) culturing the proliferated epithelial cells in a medium not containing retinoic acid to be differentiated into squamous epithelial cells.

According to a preferred embodiment of the present invention, the step (b) for obtaining the squamous epithelial cells is carried out in two substeps.

25 In the first substep, culturing is performed under submerged conditions. Under submerged conditions, epithelial cells seeded on the medium are completely immersed in the medium. Factors affecting epithelial cells act through the medium in this condition.

Afterwards, the second step is carried out under semi-dry conditions. For semi-dry conditions, epithelial cells seeded on the medium are cultured under conditions in which the cells are immersed with the lowest medium height. In this condition, the medium covers epithelial cells as thin as possible and factors affecting epithelial cells exhibit their effects through medium and air.

According to a preferred embodiment of the invention, the process for preparing squamous epithelial cells is carried out using no feeder cells (*e.g.*, 3T3 cells). This feature has advantages to eliminate adverse effects caused by heterologous cells where the present implant is applied in human body.

The preparatory process of squamous epithelial cells will be described hereinbelow in more detail with referring to illustrated examples of the present invention to produce nasal squamous epithelial cells or nasal squamous epithelium by use of nasal epithelial cells:

Nasal mucosal samples obtained from the nose are incubated with proteases to provide nasal epithelial cells dissociated. Fibroblasts, endothelial cells and red blood cells are then eliminated from the dissociated nasal epithelial cells. The nasal epithelial cells are then cultured and proliferated in a bronchial epithelial growth medium containing hydrocortisone 21-hemisuccinate, insulin, transferrin, epinephrine hydrochloride, 3,3',5-triiodothyronine, gentamycin sulfate and amphotericin B supplemented with epidermal growth factor, all-trans-retinoic acid and bovine serum albumin. Afterwards, the nasal epithelial cells are cultured in the bronchial epithelial grown medium not containing retinoic acid to prepare nasal squamous epithelial cells. Preferably, the culture for preparing squamous epithelial cells is carried out in two distinctly consecutive substeps. In the first substep, culturing is performed under submerged conditions. Under submerged conditions, epithelial cells seeded in medium are completely immersed in the medium. Afterwards, the second substep is carried out under semi-dry conditions. For semi-dry conditions, epithelial cells seeded on the medium are cultured under conditions in which the cells are immersed with the lowest

medium height. In this condition, the medium covers epithelial cells as thin as possible.

The resulting squamous epithelial cells show higher expression level of the *cornifin-a* gene and lower expression levels of the *MUC5AC* and *MUC8* genes than mucociliary epithelial cells. In particular, the *MUC5AC* and *MUC8* genes are rarely
5 expressed in the squamous epithelial cells used in the present invention.

According to a preferred embodiment of the present invention, the squamous epithelial cells used in the present invention are keratinized squamous epithelial cells.

Preferably, the squamous epithelial cells implanted grafted *in vivo* are differentiated into mucociliary epithelial cells to reconstruct mucosa defects.

10 The squamous epithelial cells used in the present invention may be heterologous or autologous, preferably autologous cells dissociated from patients themselves. The autologous squamous epithelial cells have prominent advantages in the senses that they induce no immune reactions.

According to a preferred embodiment of the present invention, the implants for
15 reconstructing the mucosa defects are applied to airway mucosal defects. For such case, nasal squamous epithelial cells are used.

The present implants may be produced in various forms according to conditions, shape and size of the defects (graft sites). Preferably, the implants have forms of squamous epithelial cell sheets. It would be known to one of skill in the art that
20 squamous epithelial cell sheets may be difficult to be handled because of their thinner thickness. Therefore, it is preferable that the squamous epithelial cell sheets are grafted using petrolatum gauze or biodegradable polymers as a support.

Where the size of the mucosa defects for graft with the implants is large (*e.g.*, in tumor-removed patients with sinonasal or tracheal tumors), the implants of the
25 present invention may be applied in the mucosa defects using three-dimensional free-flaps which are well known in the art (Piantanida, R., et al., Reconstruction of major orbital-maxillary defects with free latissimus dorsi myocutaneous flap. *Facial Plast. Surg.* 15:297(1999); Pribaz, J.J., et al., *Plast. Reconstr. Surg.* 93:285(1994)).

The implants of the present invention are developed for direct graft into mucosa defects. A suitable dosage of the present implants may vary depending on pharmaceutical formulation methods, administration methods, the patient's age, body weight, sex, pathogenic state, diet, administration time, administration route and sensitivity. The number of the grafted squamous epithelial cells is not restricted particularly. For example, the squamous epithelial cells may be grafted in the number of $1 \times 10^4 - 1 \times 10^{10}$ cells.

Following graft to defect sites, the squamous epithelial cells contained in the present implants differentiate into mucociliary epithelial cells and form tight junctions with surrounding cells to regenerate mucosa with normal appearances, finally resulting in reconstruction of mucosal lumen.

The present implants for reconstructing the mucosal lumen may be utilized in organs having a mucosal lumen structure lined by mucociliary cells. For example, they may be applied in airway, stomach, small intestine and large intestine, in order to efficiently reconstruct the mucosal lumen. Most preferably, the present implants for reconstructing the mucosal lumen are used in airway luminal reconstruction.

The implant of the present invention for reconstructing the mucosal lumen possesses advantages to induce little or no crust or mucus stagnation which is typically associated with conventional skin implants. The present implants form mucociliary epithelial cells post graft *in vivo*, and crust formation is suppressed due to ciliary movement on the epithelial cells.

To date, skin and mucociliary epithelial cells have been most widely used to reconstruct the mucosal lumen. However, according to conventional technologies, skin implants have serious problems such as constriction, desquamation, the re-growth of hair and formation of crust, and implants containing mucociliary epithelial cells typically have strength not enough to handle and suture in grafting sites.

The implants of the present invention perfectly solve these aforementioned problems which conventional skin and mucociliary epithelial cell implants have. In this

regard, the implants of the present invention may be free from problems such as constriction of the graft, desquamation and crust formation and be operated more easily than conventional mucociliary epithelial cell-containing implants because of their sufficient degree of strength, as well as form tight junctions with surrounding cells
5 present at sites where they are grafted.

The features and advantages of this invention will be summarized as follows:

- (a) The implants of the present invention utilize squamous epithelial cells as an active ingredient for reconstructing the mucosal lumen.
- 10 (b) The implants of the present invention, after grafted to defect sites, differentiate into mucociliary epithelial cells, form tight junctions with surrounding cells, and regenerate mucosa with normal appearances.
- (c) The present implants, after grafted to defect sites, differentiate into mucociliary epithelial cells and induce little or no crust formation or mucus stagnation
15 because of ciliary movement on these epithelial cells.
- (d) Because squamous epithelial cells used in the present implants may be cultured and proliferated with no feeder cells, the implants may be prepared in a carrier-free form, thereby avoiding adverse effects induced by carriers.
- (e) The implants of the present invention are especially advantageous to
20 reconstruct the luminal side of the airway.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the result of histologic and scanning electron micrographic analysis of the generation of the keratinized squamous epithelium from human nasal epithelial
25 cells and the differentiation of the mucociliary epithelium from the squamous epithelium by retinoic acid.

Fig. 2 is the result of reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the markers of squamous and mucociliary epithelium differentiation during

phenotypic changes in the nasal epithelial cells.

Fig. 3 represents maxillofacial reconstruction using cultured nasal squamous epithelial cell sheets and palliative skin graft on radial forearm free flap. (A) The actual squamous epithelial cell sheets (2.4 cm diameter) to be used after elevation. (B) The result of the histological analysis of the squamous epithelial cell sheets. (C) The cultured squamous epithelium (black arrow) previously grafted onto the forearm muscle of the patients 10 days prior to the reconstructive surgery. (D) A schematic drawing of the cultured squamous epithelium (black arrow) on the forearm. The dotted oval beneath it represents the site where the skin will be grafted. (E) A schematic drawing of the reconstruction with the myocutaneous radial forearm free flap. The cultured nasal epithelial cell sheet (black arrow) and the skin graft (S) can be seen. (F) View obtained using a 70-degree endoscope 1 year after the reconstructive surgery. The site grafted with skin (white arrow) and the other site grafted with cultured nasal squamous epithelium (black arrow) can be observed. The site grafted with cultured nasal squamous epithelium appeared as healthy normal nasal mucosa. These were representative of three cases.

Fig. 4 represents phenotypic changes of the grafted squamous epithelial cell sheet. (A) Histological appearance of the squamous epithelial cell sheet before grafting. (B) p63 immunofluorescent staining of the adjacent section of which shows basal cells with a positive staining. (C) Histological appearance of the squamous epithelial cell sheet 1 month after reconstruction. (D) Histological appearance of the sheet 3 months after reconstruction. (E) p63 immunofluorescent staining of (D) which shows an increased number of basal cells with a positive staining. (F) Appearance of the ciliary epithelial cells under a scanning electron microscope 3 months after reconstruction. These were representative of three cases.

The present invention will now be described in further detail by examples. It would be obvious to those skilled in the art that these examples are intended to be

more concretely illustrative and the scope of the present invention as set forth in the appended claims is not limited to or by the examples.

EXAMPLES

5 MATERIALS AND METHODS

Isolation and expansion of nasal epithelial cells from patients

Once the procedure described in this study received approval from the Institutional Review Board of the Yonsei University College of Medicine, we proceeded with using it in our three maxillary cancer patients with their informed consent. For the

10 isolation and expansion of nasal epithelial cells, first, the samples of nasal mucosa were obtained from the middle turbinate of opposite side of maxillary cancer (one patient with adenoid cystic carcinoma and two patients with squamous cell carcinoma). To dissociate the epithelial cells, the sample of nasal mucosa was treated with 1.0% Pronase (type XIV protease; Sigma-Aldrich, St. Louis, MO) in a 1:1 mixture of

15 Dulbecco's modified Eagle's medium and Ham's nutrient F12 (DMEM/F12) supplemented with penicillin G sodium (50 IU/ml) and streptomycin sulfate (50 µg/ml) for 16 to 20 hours at 4°C. The dissociated epithelial cells were washed three times in DMEM/F12 containing antibiotics and suspended in DMEM/F12 supplemented with antibiotics and 10% fetal bovine serum. The cells were then plated onto a plastic dish

20 at 37°C for 1 hour to eliminate fibroblasts, endothelial cells, and red blood cells. The suspended epithelial cells were plated and reseeded at a density of 3×10^4 cells per dish (500 cells per square centimeter). The culture medium used was bronchial epithelial growth medium (BEGM; Clonetics Corp., Walkersville, MD), which contains hydrocortisone 21-hemisuccinate (0.5 µg/ml), insulin (5 µg/ml), transferrin (10 µg/ml),

25 epinephrine hydrochloride (0.5 µg/ml), 3,3',5-triiodothyronine (6.5 ng/ml), gentamycin sulfate (50 µg/ml), and amphotericin B (50 µg/ml), and this was supplemented with epidermal growth factor (EGF; 25 ng/ml; Collaborative Research, Bedford, MA), *all-trans*-retinoic acid (10^{-7} mol/L; Sigma-Aldrich), and bovine serum albumin (1.5 µg/mL;

Sigma-Aldrich). The cultures were maintained at 37°C in an atmosphere of 5% carbon dioxide in air. This culture medium was changed on the first day after seeding and then every other day until the cultures were 50% to 60% confluent. The cells were then dissociated by treatment with trypsin-ethylenediaminetetraacetic acid (EDTA) (Clonetics Corp., San Diego, CA) according to the methods of the manufacturer. Cell numbers were determined by hemocytometry, and cells were plated a second time. When these passage 2 cell cultures reached 50% to 60% confluence, the cells were again dissociated and stored frozen in liquid nitrogen at 1.8×10^6 cells/vial (9).

10 ***Induction of squamous differentiation in vitro***

The passage 2 nasal epithelial cells (10^5 cells per culture, 2×10^4 cells per square centimeter) from the patient were seeded in 0.5 ml of culture medium onto the surface of 24.5-mm Transwell clear culture inserts with a 0.45- μ m pore size (Costar Co., Cambridge, MA).

15 The cells were cultured in a 1:1 mixture of BEGM:DMEM containing the same concentration of supplements as described above, except that the mixture also contained 0.5 ng/mL EGF but no retinoic acid. The cultures were grown in submersion for the first nine days; the culture medium was changed on day 1 and every other day thereafter. The air-liquid interface was created on day 9 by removing the apical medium and feeding the cultures only from the basal compartment. To obtain the squamous epithelium, the RA-deleted culture medium was changed daily after the creation of the air-liquid interface until 14 days after confluence.

25 ***Prefabrication of carrier-free airway epithelial cell sheet on the radial forearm free flap and reconstruction***

On day 14 after cell confluence had been reached, the cultured nasal squamous epithelium was elevated from each culture insert with an elevator, placed onto petrolatum gauzes, and then sutured to the inner aspect of the right

myocutaneous radial forearm free flap (Fig. 3c, 3d), which was to be applied to the side of the nose as part of the reconstructive surgery 10 days later. To compare the clinical results of the cultured nasal squamous epithelium graft and split-thickness skin graft, which was usually used to reconstruct the luminal side of the airway (10), skin
5 from the right thigh was grafted beneath the cultured nasal epithelium. Ten days later, maxillofacial reconstruction surgery was performed. The reason for the staged operation was to determine whether cultured nasal squamous epithelium could be taken from the muscle. Once this was ascertained, we proceeded with the complete removal of the tumor, including maxilla and skin. Finally, the surgical defect was
10 reconstructed with a prefabricated myocutaneous radial forearm free flap with the cultured nasal squamous epithelium (Fig. 3e).

Reverse transcriptase-polymerase chain reactions for MUC5AC, MUC8 and cornifin- α

15 The oligonucleotide primers were constructed according to the published sequence for the mucous differentiation marker (9), the mucin gene 5AC (MUC5AC; GenBank accession No. U06711, 680 bp, 5' primer: TCCGGCTCCATCTTCTCC, 3' primer: ACTTGGGCACTGGTGCTG), the ciliary differentiation marker (11), mucin gene 8 (MUC8; GenBank accession No. U14383, 239 bp, 5' primer: ACAGGGTTTCTCCTCATTG, 3'
20 primer: CGTTTATTCCAGCACTGTTC), and the squamous epithelial marker, cornifin- α (GenBank accession No. BC056240, 172 bp, 5' primer: CATTCTGTCTCCCCCAAAA, 3' primer: ATGGGGGTATAAGGGAGCTG). The oligonucleotide amplimers for β 2 microglobulin (β 2M; Clontech Laboratories Inc., Palo Alto, CA) were used as the control gene for reverse transcriptase-polymerase chain reactions (RT-PCR); they generated a
25 335 bp PCR fragment. RT-PCRs were performed using a Perkin Elmer Cetus DNA Thermal Cycler (Perkin Elmer, Wellesley, MA) according to the manufacturer's recommendations. Annealing was performed for 1 minute at 55°C for MUC8 and cornifin- α and at 60°C for MUC5AC and β 2M. Extension was performed at 72°C for 1

minute. A comparative kinetic analysis was performed to determine the mRNA levels for each gene under each set of culture conditions. The PCR products were separated by electrophoresis on a 2% Seakem agarose gel (FMC; Rockland, ME) containing 50 ng/ml ethidium bromide and were photographed on Polaroid Type 55 film. The experiments were performed in triplicate.

Histological examination, immunofluorescent staining and scanning electron microscopy

At one- and three-month intervals after the reconstructive surgery, intranasal punch biopsies were performed under endoscopic guidance to obtain material for determinations of the histological phenotype. Specimens were fixed in 10% neutral buffered formalin, sectioned at a thickness of 5 μ m, and stained with hematoxylin-eosin. For immunofluorescent staining, the adjacent sections were stained with monoclonal anti-p63 antibody (4A4, Santa Cruz Biotechnology, Santa Cruz, CA) and fluorescein isothiocyanate-labeled secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The same concentration of corresponding normal nonspecific IgG provided negative controls. For the scanning electron microscopic studies, the specimen was fixed by exposure to chilled 2.5% glutaraldehyde for 4 to 6 hours and then washed with 0.1 M phosphate-buffered saline. The cells were post-fixed by exposure to 1% osmium tetroxide for 2 hours. Specimens were then examined under a scanning electron microscope (H-800, Hitachi, Ibaraki, Japan).

RESULTS

Induction of squamous epithelium in vitro and phenotypic changes in mucociliary epithelium by RA

The nasal epithelial cells cultured in the RA-deficient culture medium were fully differentiated into squamous epithelium that included keratin layers (Fig. 1). In contrast, the keratin layers began to scale off the keratinized squamous epithelium

treated with RA starting 2 days after the treatment, with differentiated squamous epithelial cells exfoliated by 4 days after the treatment. Within 7 days, the epithelium differentiated into cuboidal epithelium with some cilia, which then ultimately differentiated into tall, ciliated columnar epithelium with abundant cilia (Fig. 1).

5

Expression of squamous and mucociliary differentiation markers during phenotypic changes in the nasal epithelium

In the RA-deficient culture, *MUC5AC* and *MUC8* gene expressions were decreased and the *cornifin-a* mRNA level was increased as a function of time. In
10 keratinized squamous epithelium, the *MUC5AC* and *MUC8* mRNA levels were minimally expressed and the *cornifin-a* mRNA level was high. However, after the treatment with RA, the *cornifin-a* mRNA level abruptly decreased and the levels of the *MUC5AC* and *MUC8* mRNAs were increased as a function of time. In the mucociliary epithelium, *cornifin-a* mRNA was minimally expressed and *MUC5AC* and *MUC8* mRNA was
15 expressed at high levels (Fig. 2).

The construction of squamous epithelial cell sheets and graft uptake and phenotype changes in squamous epithelial cell sheet after placement of the graft *in vivo*

20 The present inventors previously used mucociliary epithelial cell sheets for graft but the cell sheet was not strong enough to suture for graft, In contrast, the cultured squamous epithelial cell sheets (Fig. 3a, 3b) were found to be well-attached to the donor muscle on the day of the reconstructive surgery when examined under the operating microscope (Fig. 3c, 3d, 3e for the location of the epithelial cell sheets and
25 skin graft).

The epithelial cell sheet showed typical squamous epithelium containing keratin layers at the time of reconstruction surgery (Fig. 4a). By one month after surgery, the cultured squamous epithelium had differentiated into cuboidal epithelium

(Fig. 4c). At three months after surgery, the cuboidal epithelium had further differentiated into ciliated columnar epithelium (Fig. 4d, 4f), the typical phenotype of human airway epithelium. Three months after surgery, there was no crust or mucus stagnation that is usually observed in cases where skin graft is used for airway luminal reconstruction. In addition, the number of basal cells expressing p63 had increased so that most of the basal cells were p63-positive (Fig. 4b, 4e).

An endoscopic view obtained with a 70-degree endoscope one year after the reconstructive surgery (Fig. 3f) showed that the site had grafted with cultured nasal squamous epithelium (black arrow) and the other site had grafted with skin (white arrow) in the nasal cavity. It is clearly showed that the site grafted with squamous cell sheet regenerated nasal mucosa with the normal appearance. Since autologous nasal epithelial cells were used for the cell sheet graft, no obvious immune response or host rejection was visible at the mucosa. Most importantly, there were no clinical complications during a follow-up period of 12 months in all three cases.

As described previously, the implants of the present invention, after grafted to defect site, differentiate into mucociliary epithelium, form tight junctions with surrounding cells, and regenerate mucosa with normal appearance. In addition, the present implants, after grafted to defect site, differentiate into mucociliary epithelium and induce little or no crust formation or mucus stagnation because of the ciliary movement on this epithelial cells. The implants of the present invention are especially advantageous to reconstruct the luminal side of the airway.

Having described a preferred embodiment of the present invention, it is to be understood that variants and modifications thereof falling within the spirit of the invention may become apparent to those skilled in this art, and the scope of this invention is to be determined by appended claims and their equivalents.

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What is claimed is:

1. An implant for reconstructing mucosal lumen, which comprises squamous epithelial cells as active ingredients.
- 5 2. The implant according to claim 1, wherein the squamous epithelial cells are prepared *in vitro* in accordance with a process comprising the steps of (a) culturing and proliferating epithelial cells in a medium containing retinoic acid (RA); and (b) culturing the proliferated epithelial cells in a medium not containing retinoic acid to be differentiated into squamous epithelial cells.
- 10 3. The implant according to claim 2, wherein the process for preparing squamous epithelial cells is carried out using no feeder cells.
4. The implant according to claim 1, wherein the squamous epithelial cells show
15 higher expression level of the *cornifin-a* gene and lower expression levels of the *MUC5AC* and *MUC8* genes than mucociliary epithelial cells.
5. The implant according to claim 1, wherein the squamous epithelial cells are differentiated into mucociliary epithelial cells when the squamous epithelial cells are
20 grafted *in vivo*.
6. The implant according to claim 1, wherein the squamous epithelial cells are autologous cells.
- 25 7. The implant according to claim 1, wherein the squamous epithelial cells are nasal squamous epithelial cells.
8. The implant according to claim 1, wherein the mucosal lumen is in airway.

9. The implant according to claim 1, wherein the implant has the form of squamous epithelial cell sheet.

5 10. A method for reconstructing mucosal lumen, which comprises administering to a subject a pharmaceutically effective amount of an implant comprising squamous epithelial cells.

10 11. The method according to claim 10, wherein the squamous epithelial cells are prepared *in vitro* in accordance with a process comprising the steps of (a) culturing and proliferating epithelial cells in a medium containing retinoic acid (RA); and (b) culturing the proliferated epithelial cells in a medium not containing retinoic acid to be differentiated into squamous epithelial cells.

15 12. The method according to claim 11, wherein the process for preparing squamous epithelial cells is carried out using no feeder cells.

20 13. The method according to claim 10, wherein the squamous epithelial cells show higher expression level of the *cornifin-a* gene and lower expression levels of the *MUC5AC* and *MUC8* genes than mucociliary epithelial cells.

25 14. The method according to claim 10, wherein the squamous epithelial cells are differentiated into mucociliary epithelial cells when the squamous epithelial cells are grafted *in vivo*.

15. The method according to claim 10, wherein the squamous epithelial cells are autologous cells.

16. The method according to claim 10, wherein the squamous epithelial cells are nasal squamous epithelial cells.

17. The method according to claim 10, wherein the mucosal lumen is in airway.

5

18. The method according to claim 10, wherein the implant has the form of squamous epithelial cell sheet.

19. A use of an implant for preparing medicament to reconstruct mucosal lumen,
10 comprising squamous epithelial cells.

20. The use according to claim 19, wherein the squamous epithelial cells are prepared *in vitro* in accordance with a process comprising the steps of (a) culturing and proliferating epithelial cells in a medium containing retinoic acid (RA); and (b)
15 culturing the proliferated epithelial cells in a medium not containing retinoic acid to be differentiated into squamous epithelial cells.

21. The use according to claim 20, wherein the process for preparing squamous epithelial cells is carried out using no feeder cells.

20

22. The use according to claim 19, wherein the squamous epithelial cells show higher expression level of the *cornifin-a* gene and lower expression levels of *MUC5AC* and *MUC8* genes than mucociliary epithelial cells.

23. The use according to claim 19, wherein the squamous epithelial cells are differentiated into mucociliary epithelial cells when the squamous epithelial cells are grafted *in vivo*.
25

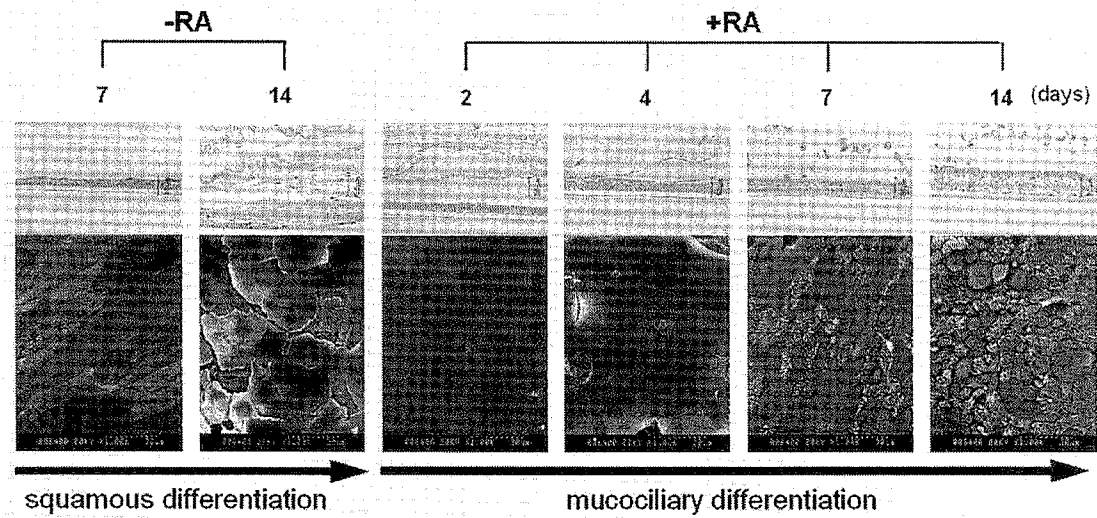
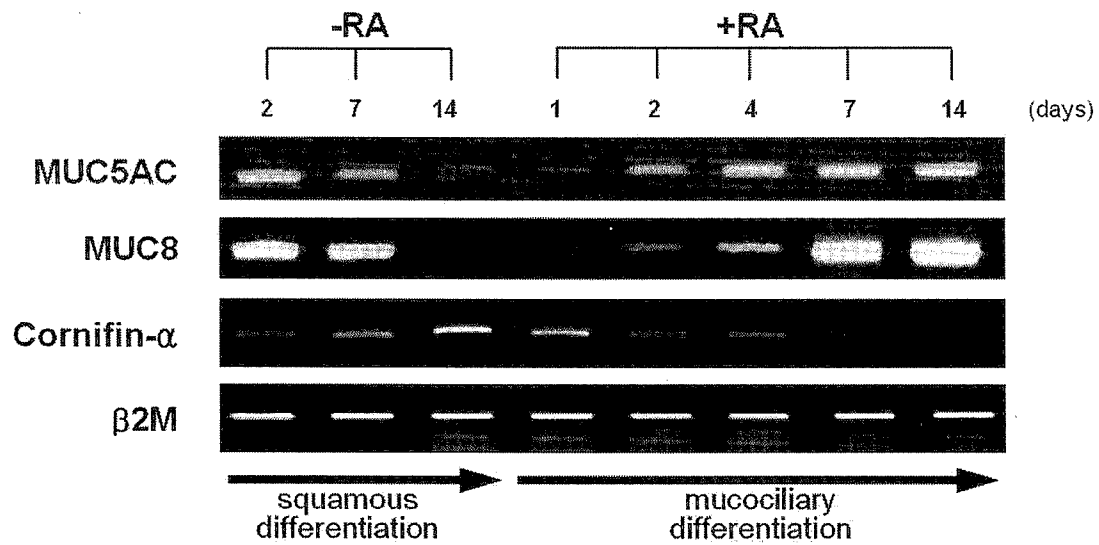
24. The use according to claim 19, wherein the squamous epithelial cells are autologous cells.

25. The use according to claim 19, wherein the squamous epithelial cells are nasal
5 squamous epithelial cells.

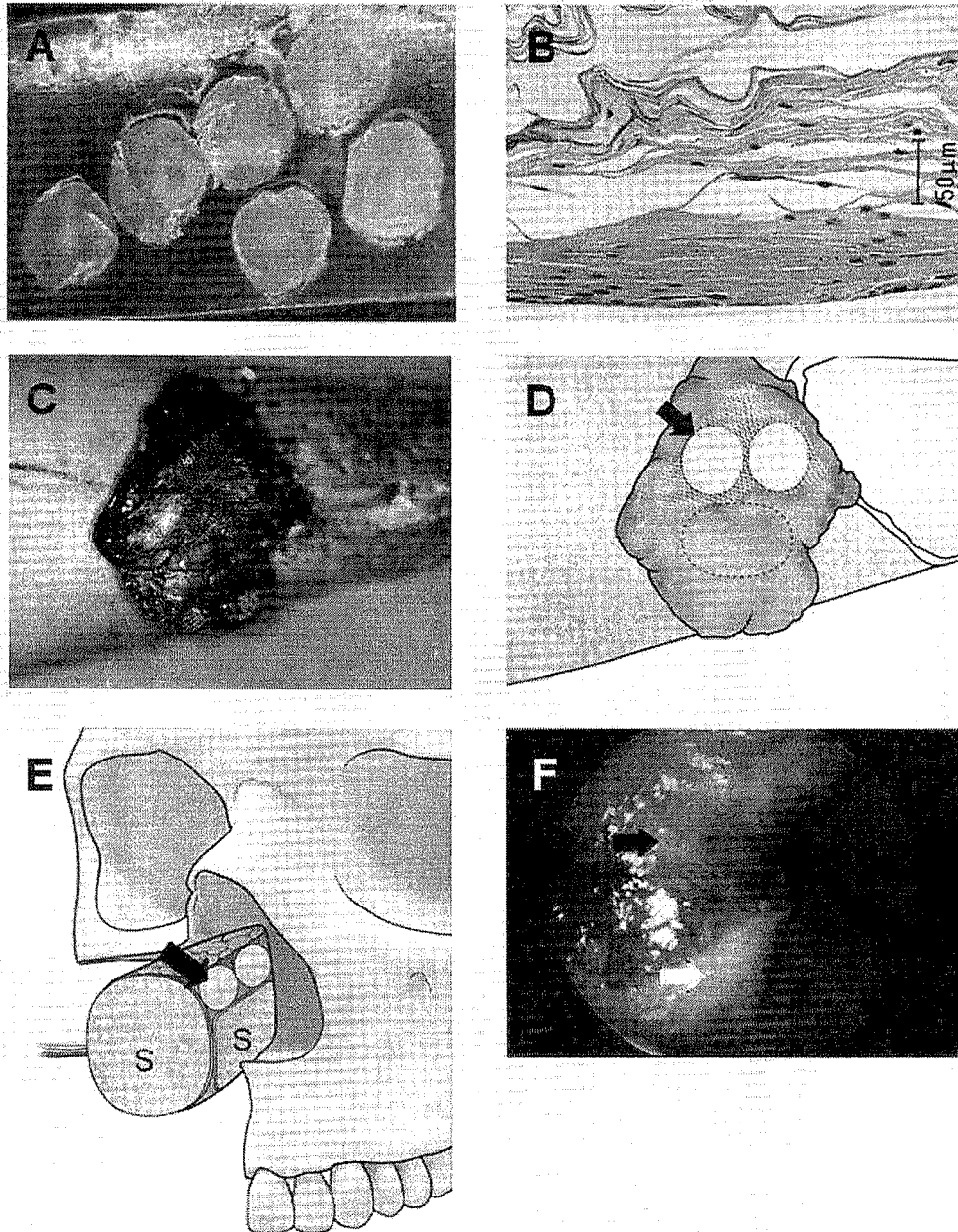
26. The use according to claim 19, wherein the mucosal lumen is in airway.

27. The use according to claim 19, wherein the implant has the form of squamous
10 epithelial cell sheet.

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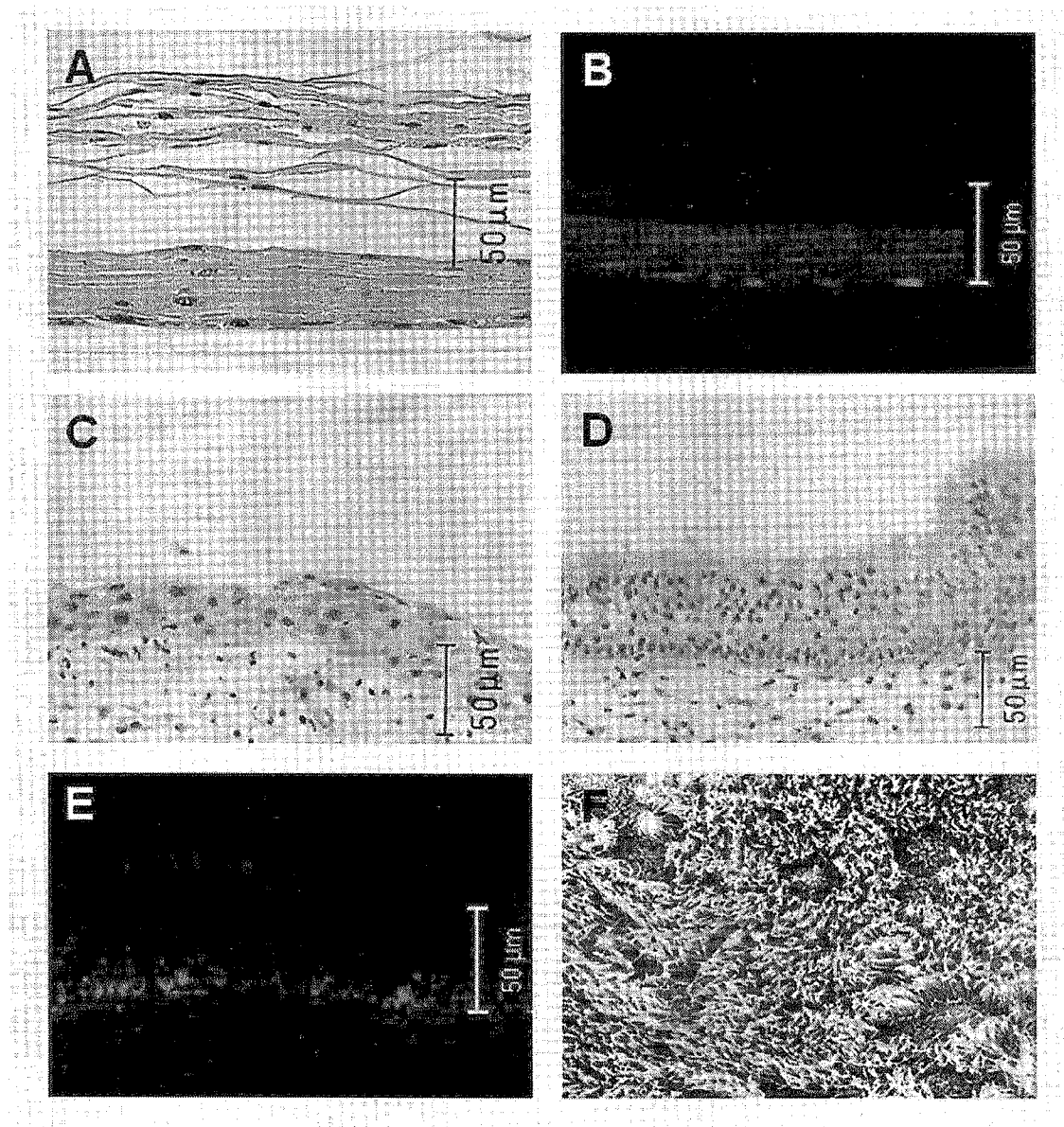
Fig. 1**Fig. 2**

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Fig. 3

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Fig. 4



A. CLASSIFICATION OF SUBJECT MATTER**A61F 2/10(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 : A61F 2/10, C12N 15/00, A61L 27/38, A61L 27/56

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Utility Models and Applications for Utility Models since 1975

Japanese Utility Models and Applications for Utility Models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS, WPI, USPTO, PAJ, PUBMED "squamous epithelial cells, implant, airway, mucosal lumen "

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US20060198865 A1 (Toby M. Freyman), 07 September 2006(07.09.2006)	1, 19
Y	See abstract, claims 1, 23, column 1[0014], [0077]	2, 4-8, 20, 22-26
Y	Yoon JH, Koo JS, Norford D, Guzman K, Gray T, Nettesheim P., Lysozyme expression during metaplastic squamous differentiation of retinoic acid-deficient human tracheobronchial epithelial cells., Am J Respir Cell Mol Biol. 1999 Apr;20(4):573-81. See abstract, Figs. 2-4,	2, 5, 7, 8, 20, 23, 25, 26
Y	Choi JY, Cho KN, Yoo KH, Shin JH, Yoon JH, Retinoic acid depletion induces keratinizing squamous differentiation in human middle ear epithelial cell cultures.Acta Otolaryngol. 2003 May;123(4):466-70. See abstract	4, 22
Y	US05980888 A(Nikolaos Dimoudis et. al.), 09 November 1999(09.11.1999) See abstract, claims 1, 11, 21	6, 24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

14 MARCH 2008 (14.03.2008)

Date of mailing of the international search report

14 MARCH 2008 (14.03.2008)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2007/005734

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US20030216811 A1(Stephen F. Badylak), 20 November 2003(20.11.2003) See abstract, claims 1, 11	1, 2, 4-8, 19, 20, 22-26
A	Gray TE, Guzman K, Davis CW, Abdullah LH, Nettesheim P., Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells., Am J Respir Cell Mol Biol. 1996 Jan;14(1):104-12. See abstract	1, 2, 4-8, 19, 20, 22-26

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2007/005734**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 10-18
because they relate to subject matter not required to be searched by this Authority, namely:
The subject matter of claim 10 comprises a step of administering to an implant containing squamous epithelial cells, which is a therapeutic method to treat human body. These steps fall into the category of methods for treatment of the human body or animal body by surgery or therapy as well as diagnostic methods. The same reasoning applies to claims 11-18, which are dependent on claim 10. [Article 17(2)(a)(i), Rule 39.1(iv) PCT]
2. ☒ Claims Nos.: 3, 9, 21, 27
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 3, 9, 21, 27 are so unclear that no opinion could be formulated as to novelty or inventive step. The problems are that the expression "... not use a feeder cell layer" (claim 3&21) and "... squamous epithelial cell plate shape" (claim 9&27) are too broad to make meaningful search possible.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2007/005734

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US20060198865 A1	07.09.2006	EP01855733A2 EP1855733A2 US2006198865AA W02006096688A2 W02006096688A3	21.11.2007 21.11.2007 07.09.2006 14.09.2006 31.05.2007
US05980888 A	09.11.1999	AT237373E AU3806695A1 CA2201099AA DE4438015A1 DE69530409C0 DE69530409T2 EP00788381B1 EP0788381A1 EP788381A1 EP788381B1 IL115728A0 JP09511673 JP9511673T2 US5980888A W09612510A1 ZA9508982A	15.05.2003 15.05.1996 02.05.1996 02.05.1996 22.05.2003 24.12.2003 16.04.2003 13.08.1997 13.08.1997 16.04.2003 19.01.1996 25.11.1997 25.11.1997 09.11.1999 02.05.1996 24.04.1997
US20030216811 A1	20.11.2003	NONE	