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# Transplantation of Corneal Stroma Reconstructed with Gelatin and Multipotent Precursor Cells from Corneal Stroma

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## 1. Introduction

The cornea is composed of a multilayered epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium. As compared to the stroma, tissue engineering of the corneal epithelium or endothelium is relatively simple and well established, as the stroma is mainly composed of continuous cells joined by tight junctions with a few subcellular extracellular matrix (ECM) proteins. Corneal stromal engineering, however, has yet to be fully achieved due to difficulties encountered when trying to constitute an artificial corneal stroma, which consists of few fibroblast-like keratocytes, nerve fibers, and a transparent ECM of collagen fibrils and proteoglycans.

Stem cells or progenitor cells are defined by their capacity for self-renewal and the ability to generate different types of cells (multipotentiality), which leads to the formation of mature tissues. In contrast, the precursor cells are unipotent cells with a limited proliferative activity. Regenerative stem cells or precursors can be detected by the sphere-forming assay in various adult tissues, including the central nervous system (Nunes et al., 2003), bone marrow (Krause et al., 2001), skin (Kawase et al., 2004; Toma et al., 2001), retina (Coles et al., 2004), corneal epithelium (Mimura et al., 2010a), corneal stroma (Amano et al., 2006; Mimura et al., 2008a, 2008b; Uchida et al., 2005; Yamagami et al., 2007), and corneal endothelium (Amano et al., 2006; Mimura et al., 2005a, 2005b, 2005c, 2007, 2010b; Yamagami et al., 2006a, 2007; Yokoo et al., 2005).

Despite the many successes achieved in the isolation and characterization of stem cells from various tissues, relatively few studies have investigated the efficacy of stem cell transplantation therapy in animals. While the three-dimensional structure that is responsible for maintaining the cell-to-cell interactions is indispensable for tissue engineering using stem cells, the structural complexity involved in tissue engineering does not easily allow us to extend the investigations of stem cell transplantations.

In this chapter, we introduce our recent work about regenerative medicine and tissue engineering of corneal stroma using multipotent precursor cells. We isolated precursors with the propensity to develop into corneal keratocyte-like cells from the stroma of rabbit corneas and investigated the distribution and proliferative capacity of precursor cells derived from the central and peripheral regions of the cornea by the sphere-forming assay.

Additionally, we constructed corneal stroma using keratocyte precursors and porous gelatin hydrogels *in vitro* by tissue engineering and investigated the feasibility of the engineered corneal stroma with keratocyte precursors in a rabbit model.

## 2. Origin and development of corneal stroma

Corneal stromal cells (keratocytes) are derived from the neural crest (Bahn et al., 1984; Johnston et al., 1979; Mooy et al., 1990). During embryonic development, neural crest cells, from which the keratocytes originate (Bahn et al., 1984; Johnston et al., 1979), show two waves of migration and differentiation during the corneal growth (Liu et al., 1998; Meier, 1982). In the first wave, the corneal epithelium forms and then synthesizes the primary stroma (i.e., periocular mesenchymal cells of neural crest origin). Subsequently, the neural crest cells migrate to the margin of the optic cup, and to the area between the lens and the corneal epithelium. This contributes to development of the corneal stroma and the trabecular meshwork. During the second wave of migration, neural crest cells invade the primary stroma and then undergo differentiation into keratocytes.

In adults, corneal stromal cells are composed of keratocytes and bone marrow-derived cells of the monocyte lineage (Yamagami et al., 2006b). The latter cell population is continuously replaced from the bone marrow (Nakamura et al., 2005). We previously demonstrated that while the bone marrow-derived cells isolated from corneal stroma do not form spheres (Uchida et al., 2005), the keratocytes derived from the corneal stroma do form spheres (Mimura et al., 2008a). This suggests that the precursors isolated from the corneal stroma reside among the keratocytes. If so, these precursors might be able to supply new keratocytes and thus, they could potentially promote corneal wound healing. Keratocyte precursors may also have a role in the healing of corneal stromal wounds, since proliferation and migration of residual keratocytes from the peripheral part of the stroma appear to promote healing (Wilson & Kaufman, 1990).

## 3. Isolation of sphere colonies

### 3.1 Primary sphere-forming assay

Rabbits were handled in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. New Zealand white rabbits (weighing 2.0-2.4 kg) were used. The animals were anesthetized with intramuscular injections of ketamine hydrochloride (60 mg/kg) and xylazine (10 mg/kg), after which the eyes were enucleated. Subsequently, the eyes were washed three times with sterile saline and then immersed for 5 min in saline containing 10% povidone-iodine and 50 mg/ml gentamicin. After further rinsing with saline, the cornea was excised from each eye along the scleral rim. The epithelium was then carefully removed from the corneal stroma by scraping the outer surface of the cornea, while fine forceps were used to peel away the corneal endothelium and Descemet's membrane in a sheet that covered an area from the periphery to the center of the inner surface of the cornea. Using appropriate trephines and forceps, samples of the corneal stroma were excised from the periphery of the cornea (6.0-10.0 mm in diameter) and from the central region (6.0 mm in diameter) (Fig. 1A). The stromal samples were cut into small pieces that were approximately 1.0 mm in diameter. These were incubated overnight at 37°C in basal medium containing 0.02% collagenase. Subsequently, the tissue pieces were washed with phosphate-buffered saline (PBS), incubated in PBS containing 0.2%

ethylenediaminetetraacetic acid (EDTA) for 5 minutes at 37°C, and then dissociated into single cells by trituration with a fire-polished Pasteur pipette. After centrifugation at 800 g for 5 minutes, the cells were resuspended in the basal medium, which consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with B27, 20 ng/mL epidermal growth factor (EGF), and 40 ng/mL basic fibroblast growth factor (bFGF).

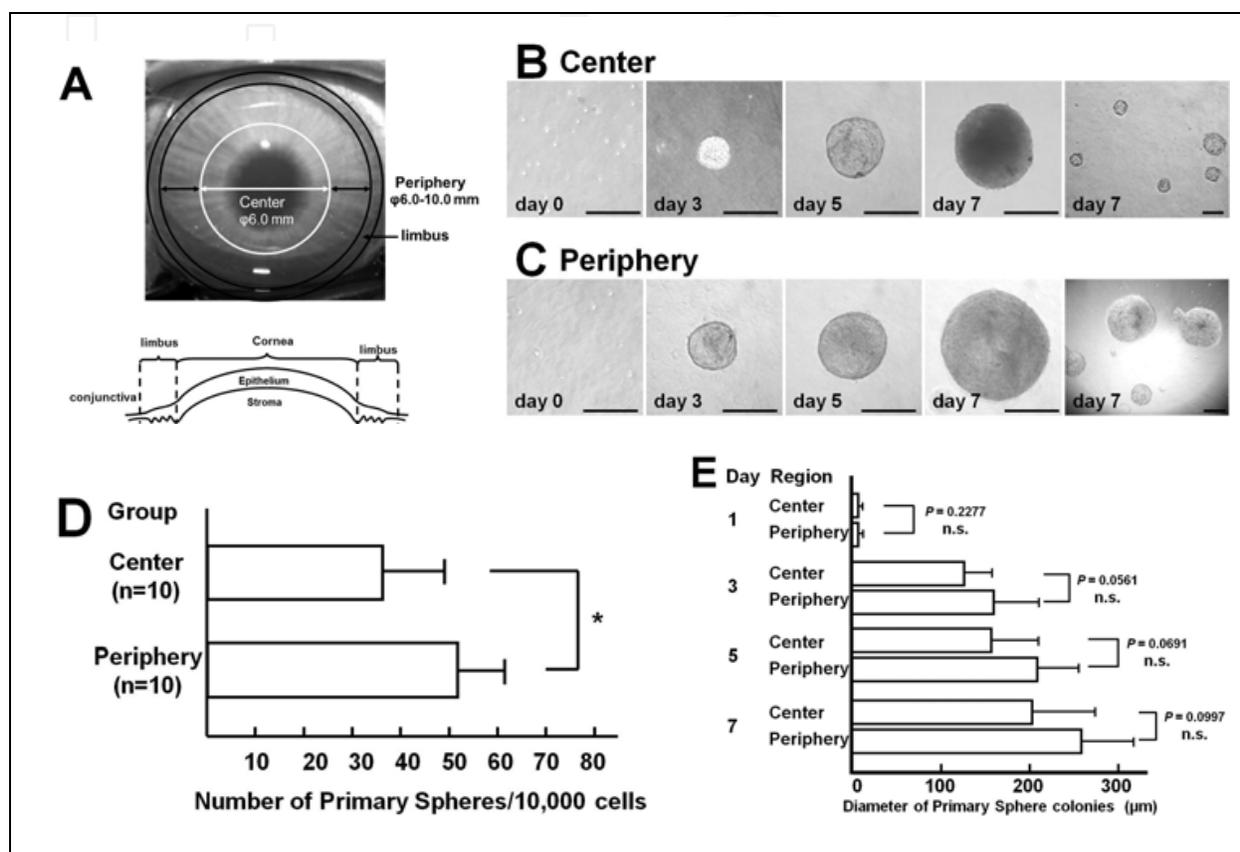


Fig. 1. (A) Anterior view of a rabbit cornea and a diagram of the corneal epithelium and stroma. Stromal keratocytes were isolated from tissue specimens obtained from both the peripheral (6.0-10.0 mm in diameter) and central regions (6.0 mm in diameter). (B, C) Primary sphere formation by the keratocytes from the peripheral and central regions of the rabbit cornea. Scale bar=200 μm. (D) The number of primary spheres derived from stromal tissue was compared between the periphery and the center of the cornea. The number of sphere colonies obtained from samples of the peripheral stroma (n=10) was significantly higher than that for the samples of the central stroma (n=10) after seven days of culture (\*  $P=0.00021$ , unpaired  $t$ -test). (E) The sizes of the primary sphere colonies derived from the samples of the peripheral (n=10) and central (n=10) corneal stroma were compared. The mean size of the spheres from both regions gradually increased during the culture period and exceeded 250 μm by day 7 (periphery:  $258 \pm 63$  μm versus center:  $203 \pm 71$  μm after seven days, mean  $\pm$  SD). n.s.= not significant

Isolated keratocytes were counted using a hemocytometer. Trypan blue staining indicated the viability of the isolated cells was greater than 90%. The sphere-forming assay was employed for the primary culture of the cells (Reynolds & Weiss, 1992). Basal medium containing a methylcellulose gel matrix (0.8%; Wako Pure Chemical Industries) was used to

prevent reaggregation of the cells, as per a previously described method (Gritti et al., 1999). Plating was done at a density of 10 viable cells/ $\mu\text{L}$  (50,000 cells/well or 2,500 cells/ $\text{cm}^2$ ) in the uncoated wells of 60 mm culture dishes. To measure the diameter of sphere colonies, culture dishes were observed under an inverted phase-contrast microscope with a 10 $\times$  objective lens, followed by analysis of the images using the NIH image program developed at the US National Institutes of Health ( $n=10$ ). The number of spheres per 10,000 cells was calculated for each well. To distinguish growing spheres from dying cell clusters, only those with a diameter of more than 50  $\mu\text{m}$  were counted.

After keratocytes were disaggregated into single cells and cultured for 7 days, the viable spheres grew larger and the non-proliferating cells were eliminated. To compare the density of the precursors between the peripheral and central regions of the cornea, primary spheres were isolated separately from the peripheral and central stroma. Representative spheres obtained from the peripheral and central regions are shown in the photographs in Figures 1B and 2C. There were significantly more spheres ( $51.4 \pm 10.1$  per 10,000 cells, mean  $\pm$  SD) obtained from the peripheral corneal stroma as compared to the central stroma ( $35.9 \pm 3.0$  per 10,000 cells) ( $P=0.00021$ , unpaired  $t$ -test; Fig. 1D). Although this result suggests that the stroma from both the peripheral and central regions of the cornea contains precursor cells, there were significantly more precursors in the peripheral stroma versus the central stroma. Additionally, there were no significant differences noted with respect to the size of the primary spheres that were derived from the two regions after culturing for 3, 5, and 7 days. This suggests that there were no differences in the proliferative capacity of the precursors obtained from either region (Fig. 1E).

### 3.2 Secondary sphere formation

To further evaluate the proliferative capacity of the keratocytes, cells from the primary spheres were passaged under the same culture conditions that were used for the initial growth of the spheres. For passaging, primary spheres (day 7) were treated with 0.5% EDTA and after dissociation into single cells, they were then plated into the wells of 60 mm culture dishes at a density of 10 cells/ $\mu\text{L}$ . Cultures were continued for 7 days in the basal medium that contained methylcellulose gel matrix to prevent reaggregation. Experiments were performed twice and representative results are shown in Figure 2 ( $n=10$ ).

Secondary spheres were generated after dissociation of the primary spheres derived from the peripheral or central stroma. Replating to generate secondary sphere colonies was less efficient than the generation of the primary spheres, which indicates that the precursor cells appear to have a limited proliferative capacity. Photographs of representative secondary spheres are shown in Figures 2A and 2B. The number of secondary spheres per 10,000 cells was significantly higher when the primary spheres passaged were derived from the peripheral stroma versus the central stroma ( $45.6 \pm 6.4$  vs.  $33.4 \pm 2.1$ , respectively;  $P=0.000025$ ; unpaired  $t$ -test; Fig. 2C). Despite differences in the properties of the cells derived from the keratocyte spheres that were obtained from the peripheral and central regions of the cornea, there were no significant differences noted in the expression of mesenchymal and neural cell markers. These findings imply that keratocyte precursors preferentially reside in the peripheral corneal stroma and have a stronger proliferative capacity as compared to the cells from the central stroma, while the precursors from both regions demonstrate similar multipotentiality.

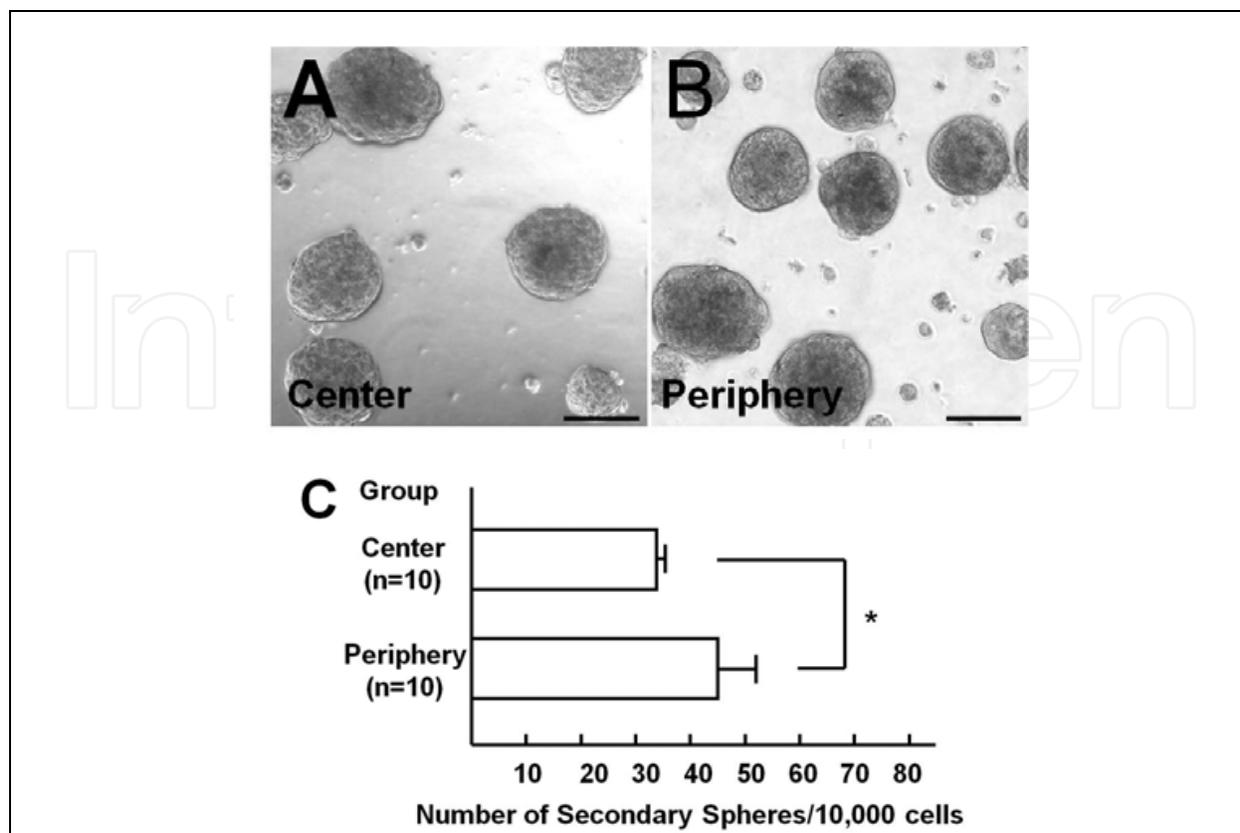


Fig. 2. Formation of secondary sphere. (A, B) Secondary spheres were generated after the dissociation of the primary spheres that were derived from the peripheral or central keratocytes. Scale bar=100 μm. (C) The replating efficiency from the primary to the secondary colonies was higher for the spheres derived from the peripheral stroma as compared to the central stroma (\*  $P=0.000025$ , unpaired  $t$ -test)

### 3.3 Differentiation of sphere colonies

Individual primary spheres (day 7) were transferred to 13 mm glass coverslips coated with 50 μg/ml poly-L-lysine (PLL) and 10 μg/ml fibronectin in separate wells, as has been previously described (Reynolds & Weiss, 1992). To promote differentiation, 1% FBS was added to the basal medium, with the culture then continued for another 7 days. Immunocytochemical examinations of the 7-day-old spheres and their progeny were performed after 7 days of adherent culture on the glass coverslips.

Nestin has been used as a marker for the detection of immature neural progenitor cells within the multipotential sphere colonies derived from the brain (Gage, 2000), skin (Toma et al., 2001), inner ear (Li et al., 2003), retina (Tropepe et al., 2000), corneal stroma (Uchida et al., 2005), and endothelium (Mimura et al., 2005b, 2005c; Yokoo et al., 2005). In addition, it was recently suggested that the stem cell marker CD34 could be a useful cell surface marker for human keratocytes (Toti et al., 2002). Most cells in the spheres were immunopositive for nestin, CD34, and 5-bromo-2'-deoxyuridine (BrdU) (Fig. 3A). Subsequently, we then examined whether the sphere colonies could give rise to cells expressing neural lineage markers. Some cells in the sphere colonies along with their progeny expressed microtubule-associated protein 2 (MAP2: a neural cell marker) and neuron-specific enolase (NSE: a marker of neural differentiation) (Fig. 3A). Most of the cells in the spheres and their progeny

were immunoreactive for vimentin (which is a marker of mesenchymal cells) or for alpha-smooth muscle actin ( $\alpha$ SMA: a marker of fibroblasts). However, all of the cells were negative for staining by the control IgG and the differentiated epithelial cell marker cytokeratin 3 (Figs. 3A and 3B). Expression of nestin and vimentin by the spheres and their progeny was confirmed using RT-PCR (Fig. 3C). Both spheres were derived from the peripheral and central regions of the cornea and their progeny displayed the same patterns of immunostaining (data not shown) and mRNA expression (Fig. 3C).

Spheres derived from both the peripheral and central regions of the rabbit cornea expressed a stem cell marker (CD34) and a neural stem cell marker (nestin), while their progeny expressed mesenchymal markers (vimentin and  $\alpha$ -SMA) and neural lineage markers (MAP2 and NSE). These findings indicate that spheres isolated from the corneal stroma of rabbits contained bi-potential precursors, with their progeny able to display the morphologic characteristics of keratocytes. Taken together, these results suggest that precursors from the corneal stroma remain close in nature to the tissue of origin and that they undergo differentiation into corneal keratocytes. Thus, since precursors need to be able to efficiently differentiate and produce their tissue of origin, precursors obtained from the corneal stroma may be more appropriate for tissue regeneration or cell transplantation than those that are derived from the multipotential stem cells.

## 4. Tissue engineering of corneal stroma

### 4.1 The organization of the corneal stromal matrix

Adult vertebrate corneal stroma is primarily composed of collagen type 1 fibrils, smaller amounts of other extracellular matrix (ECM) proteins, and a few keratocytes. Therefore, both the cells and the stratified complex of the ECM, which forms a scaffold for the precursors, are necessary in order to be able to reconstruct a three-dimensional corneal stroma. The porous scaffolds that are used in tissue engineering contribute to cell proliferation and differentiation in suitable environments, as well as taking part in the maintenance of the structure and composition of injured tissues. Furthermore, three-dimensional porous scaffolds provide a larger surface for cell attachment, migration and proliferation in contrast to the two-dimensional scaffolds, which facilitate contact inhibition in confluent cells. Because corneal keratocyte proliferation is substrate-dependent, increases in the surface area of the culture substrate are preferable. Several three-dimensional substrates that use collagen have been designed and tested for their capacity for proliferative enhancement (Takahashi et al., 2004, 2005; Yasuda et al., 2004). While their long-term safety, stability, and efficacy *in vivo* have been adequately established in humans, there are significant drawback of using collagen due to its poor biodegradation and bioabsorption. Biodegradation and biocompatibility are probably the two most important properties that have to be examined when considering the use of insoluble biomaterials. Unfortunately, biological reactions can develop when using these materials *in vivo*, resulting in tissue opacity. Gelatin, which is a denatured type of collagen, possesses most of the properties of an ideal scaffold and thus, has been clinically applied as an implant material (Waldrop & Semba, 1993). We have also used a biodegradable porous gelatin hydrogel as a carrier of the corneal keratocyte precursors, and found the material to be effective in facilitating cell migration and delivering oxygen and nutrients to the migrated cells.

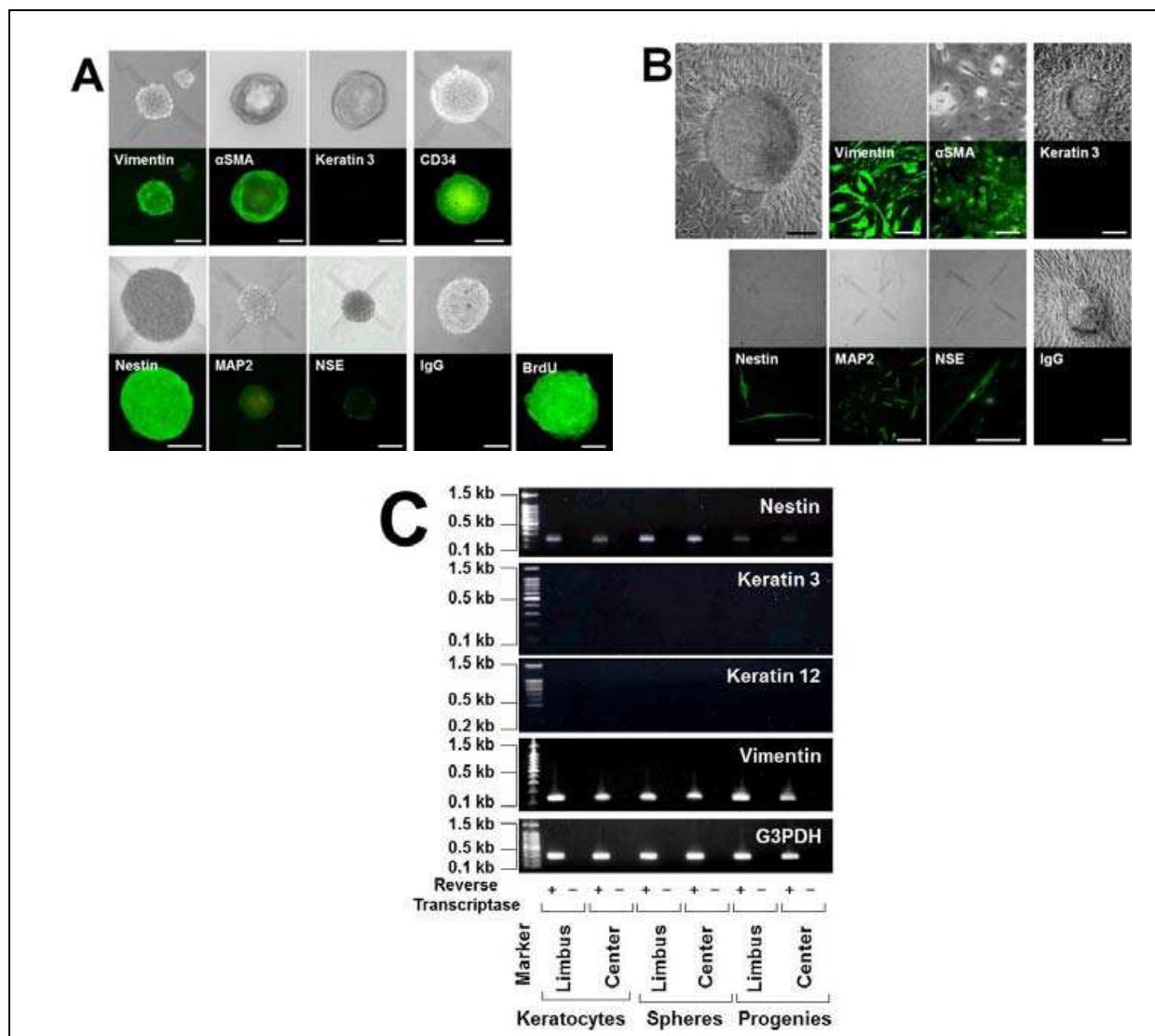


Fig. 3. (A) Immunocytochemical analysis of sphere colonies from the peripheral stroma on day 7. Bright-field images and immunostaining of the spheres are shown. The spheres were stained for vimentin, alpha-smooth muscle actin ( $\alpha$ -SMA), cytokeratin 3, nestin, microtubule-associated protein 2 (MAP2), neuron-specific enolase (NSE), and CD34. Each colony was also labeled by BrdU. As a negative control, IgG was used instead of the primary antibody. Scale bar=100  $\mu$ m. (B) Immunocytochemical analysis of differentiated cells from spheres derived from the peripheral cornea. The cells that migrated out from the spheres expressed  $\alpha$ -SMA, MAP2, and NSE, indicating that the colonies contain differentiated mesenchymal and neuronal cells. No staining with the control IgG was noted. Scale bar=100  $\mu$ m. (C) Reverse-transcription polymerase chain reaction analysis of the corneal stromal tissue, spheres, and sphere progeny. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene expression was detected in all samples except those processed without reverse-transcriptase (RT). Vimentin was expressed by the corneal stromal tissues and the spheres derived from the peripheral or central regions and their progeny. However, it was not detected by PCR of the total RNA without RT. Expression of nestin by the progeny was lower than that seen for the spheres from both the peripheral and central regions of the cornea. No expression of keratin 3 or 12 was detected in any of these samples

#### 4.2 Preparation of gelatin hydrogels

Porous gelatin hydrogels were prepared through the chemical crosslinking of an aqueous gelatin solution with glutaraldehyde, using a method described elsewhere (Tabata et al., 1999; Yamamoto et al., 2003). Briefly, an aqueous gelatin solution mixed with glutaraldehyde was cast into a polypropylene dish (138x138 mm<sup>2</sup>, BIO-BIK), followed by the crosslinking reaction, which was performed at 4°C for 12 h. The hydrogel samples were added to a 100 mM aqueous glycine solution at 37°C and stirred for 1 h to block the residual glutaraldehyde. After washing three times with double-distilled water, the hydrogel samples were freeze-dried and sterilized with ethylene oxide gas and ultraviolet for 2 hours. Before use, the desiccated sheets were trephined with a 5.0 mm diameter trephine and then immersed in sterilized saline for 10 minutes.

#### 4.3 Sphere or keratocyte seeding onto gelatin hydrogels and cell culture

The 5.0 mm gelatin hydrogel disks were placed into wells of a 24-well culture dish. Subsequently, 10 primary rabbit corneal keratocyte spheres that had been cultured for 7 days or 100,000 primary cultured rabbit corneal keratocytes were applied to the porous gelatin hydrogels. The reconstructed corneal stroma was then placed in the 24-well plates and centrifuged at 1000 rpm (176 g) for 10 min to promote the attachment of the cells to the porous gelatin hydrogels. To trace the localization in some of the experiments, primary rabbit corneal keratocyte spheres (cultured for 7 days) were labeled with a fluorescent cell tracker, as has been described elsewhere (Mimura et al., 2004). The corneal keratocytes or the corneal keratocyte spheres were cultured in the basal medium containing 10% FBS for 7 days, with the medium changed every 2 to 3 days.

#### 4.4 Immunocytochemical analysis of ECM in gelatin hydrogels

Corneal keratocytes or keratocyte precursors were cultured on porous gelatin hydrogels for 7 days, with the degree of ECM production by the cells then evaluated. Vimentin expression was more frequent in the porous gelatin hydrogels that contained keratocyte precursors versus those with only the corneal keratocytes (Fig. 4). Weak expression of laminin was seen in the gelatin hydrogels with keratocyte precursors, while no expression was observed in those with the corneal keratocyte. There was little or no expression of type I and IV collagen found in either the porous gelatin hydrogels with corneal keratocytes or the keratocyte precursors (Fig. 4).

Despite having a total cell number less than 100,000 corneal keratocytes within the 10 corneal keratocyte spheres, when the porous gelatin hydrogels that incorporated the spheres were cultured *ex vivo* for 7 days, there was intense immunostaining for vimentin, a marker of mesenchymal cells. This staining was much more intense than that which was seen for the incorporated 100,000 corneal keratocytes that were also cultured for 7 days. This indicates that the corneal keratocyte precursors have a superior proliferative potential on the gelatin hydrogels as compared to the corneal keratocytes. While weak expression of laminin and collagens was detected in the gelatin hydrogels that incorporated the corneal keratocytes precursors, these ECM components were barely detected in the gelatin that incorporated the corneal keratocytes *ex vivo* before transplantation. These results indicate that the gelatin hydrogel, itself, does not have the ability to induce tissue regeneration either *in vitro* or *ex vivo*.

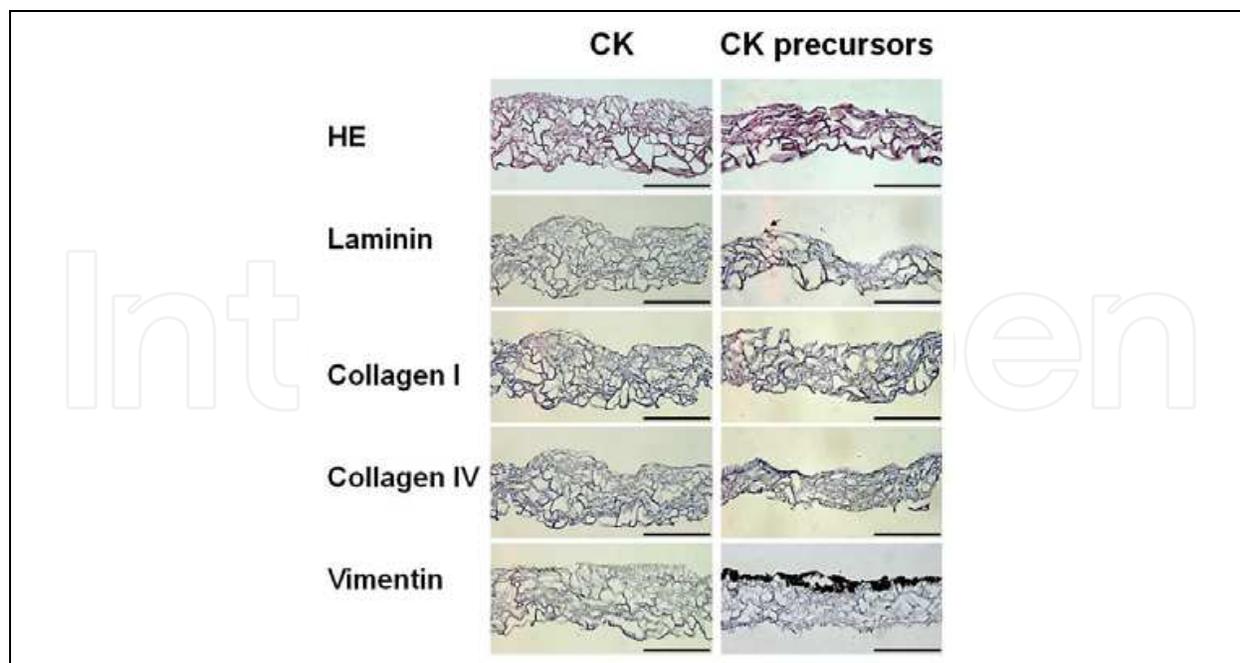


Fig. 4. Immunohistochemical analysis of the extracellular matrix in the porous gelatin hydrogel with corneal keratocytes or keratocyte precursors. Corneal keratocytes or keratocyte precursors were seeded onto porous gelatin hydrogels and cultured for one week. Vimentin staining was more intense in the gelatin hydrogels with corneal fibroblast precursors than that seen with the corneal fibroblasts (arrows). Except for a weak expression of laminin in the gelatin hydrogels with corneal keratocyte precursors (arrow), there was no expression of the other ECM components such as laminin, type I collagen, and type IV collagen in the gelatin hydrogel with the corneal keratocytes or keratocyte precursors before transplantation. Scale bar=200  $\mu\text{m}$ . HE=hematoxylin and eosin staining

## 5. Transplantation of gelatin hydrogels with corneal keratocyte precursors

### 5.1 Surgical procedure

New Zealand white rabbits (weighing 2.0-2.4 kg, n=24) were anesthetized with an intramuscular injection of ketamine hydrochloride (60 mg/kg) and xylazine (10 mg/kg). A diamond knife was used to make a 6 mm incision 1.0 mm inside the superior limbus, at a depth of 150-200  $\mu\text{m}$  (Fig. 5A). A lamellar dissection of the cornea was then performed with a bevel-up crescent knife. The reconstructed corneal stroma with corneal keratocyte precursors and porous gelatin was then implanted into a mid-stroma corneal pocket with a jeweler's forceps (Fig. 5B). No sutures were used for the corneal incision after the implantation (Fig. 5C). An antibiotic ointment (0.3% ofloxacin) was applied to the eye. The 24 rabbits were divided into three groups: the gelatin group (n=8) for which porous gelatin hydrogels alone were transplanted, the keratocyte/gelatin group (n=8) for which corneal keratocytes cultured on porous gelatin hydrogels for 7 days were transplanted, and the precursor/gelatin group (n=8) for which the corneal keratocyte precursors cultured on porous gelatin hydrogels for 7 days were transplanted. Histological examinations and immunohistochemistry were performed in the animals killed at 1 week (n=2 in each group) and at 4 weeks after surgery (n=6 in each group). None of the animals received any immunosuppressive treatment after the transplantation.

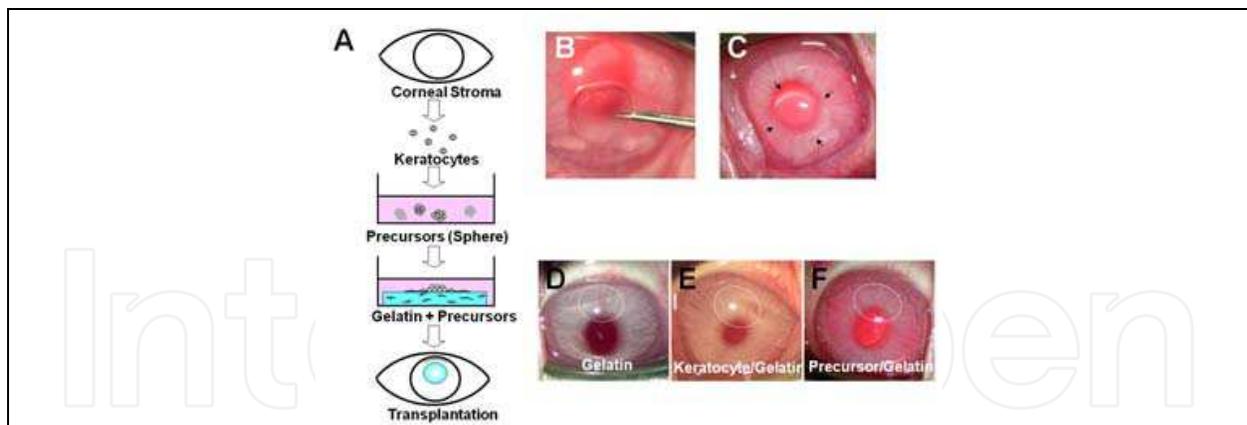


Fig. 5. Schematic illustration and clinical findings. A: Keratocyte precursors were isolated from the rabbit corneal stroma using a sphereforming assay. Corneal stroma was engineered by cultivating precursors in porous gelatin for one week. The engineered corneal stromal sheet with precursors was transplanted in a pocket of rabbit corneal stroma. B,C: Gelatin hydrogels (gelatin group), gelatin hydrogels with corneal keratocyte (keratocyte/gelatin group), or gelatin hydrogels with corneal keratocyte precursors (precursor/gelatin group) were implanted into the corneal stroma (indicated by arrows in C). D-F: Representative photographs of corneas four weeks after transplantation in each group are shown. No corneal opacity and no rejection were observed in any group four weeks after transplantation (indicated by the white circles in D-F)

## 5.2 Clinical observation after surgery

Each eye that underwent the surgical procedure was checked two to three times a week by external examination, in addition to being photographed on postoperative days 7, 14, 21, and 28. As seen in the representative anterior segment photographs from the gelatin group (transplanted with gelatin hydrogels alone, Fig. 5D), the keratocyte/gelatin group (transplanted with gelatin hydrogels with corneal keratocytes, Fig. 5E), and the precursor/gelatin group (transplanted with gelatin hydrogels with corneal keratocyte precursors, Fig. 5F), the corneas were clear, and there was no edema or mononuclear cell infiltration of the stroma in any of the groups. There were also no apparent inflammatory reactions suggestive of immunological rejection observed with a slit lamp microscope nor were there any increases of intraocular pressure, or possible side effects noted in any of the groups during the follow-up period.

## 5.3 Histological examination and analysis of ECM production with immunohistochemistry

The corneas were excised from the eyes 1 and 4 weeks after the transplantations and subjected to histological examinations and immunohistochemical staining. In the gelatin and keratocyte/gelatin groups, the few cells that did migrate into the thick transplanted gelatin hydrogels exhibited no changes in shape. In contrast, large numbers of cells migrated into the gelatin hydrogels of the precursor/gelatin group, which became thinner than the other two groups at 1 and 4 weeks after transplantation. The expressions of vimentin and ECM, such as laminin and type I and type IV collagens were more intense in the precursor/gelatin group versus the other groups after 1 week (data not shown), with their expressions significantly increased at 4 weeks (Fig. 6A). These results suggest that the gelatin hydrogel

by itself can induce ECM production to some extent *in vivo*, although the efficacy is not as high as that seen for the gelatin hydrogels with precursors. These findings clearly indicate that transplanted corneal keratocyte precursors possess the ability to generate new corneal stromal tissues *in vivo* by cell proliferation and differentiation properties, and ECM production.

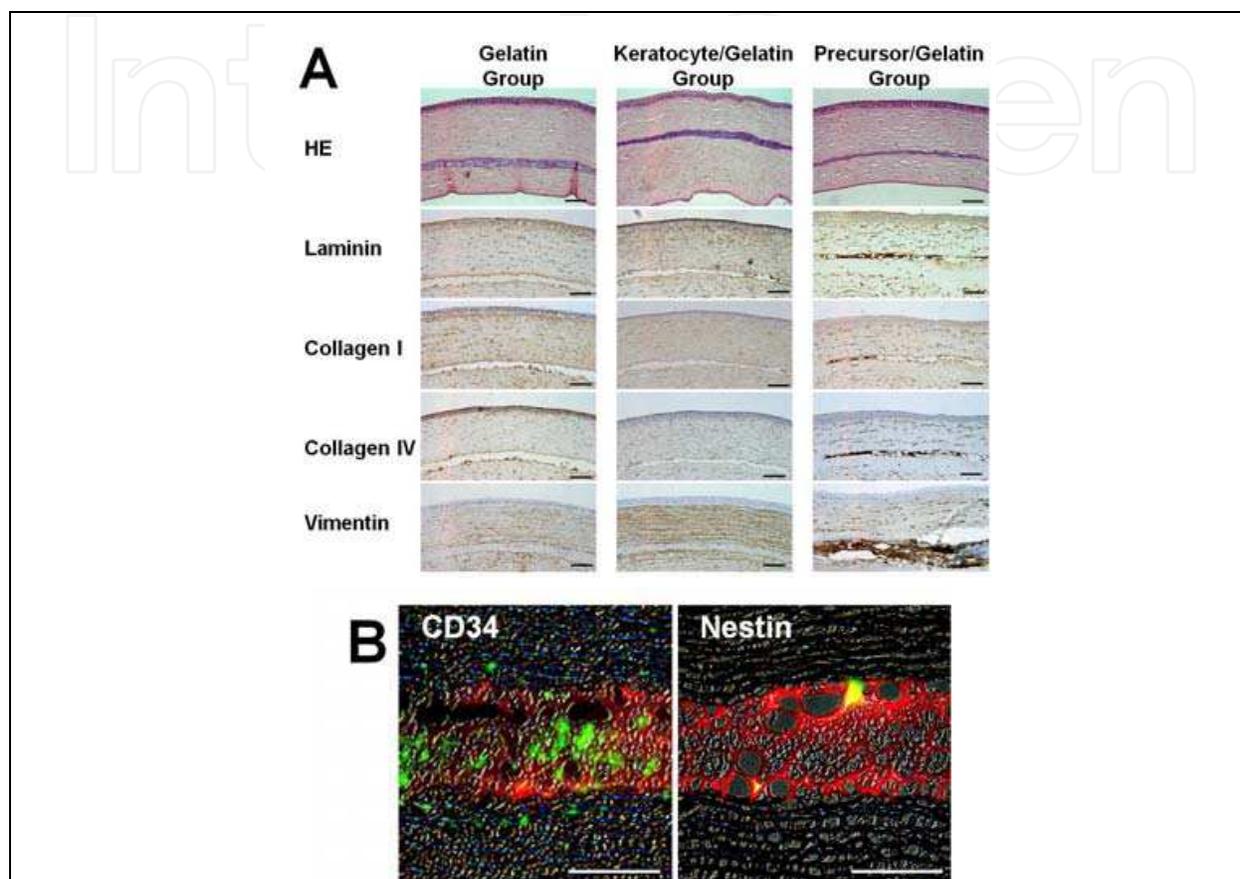


Fig. 6. (A) Histological findings and immunocytochemical analysis of the extracellular matrix at 4 weeks after transplantation. Transplanted gelatin hydrogels were found in the corneal stroma in all groups. HE staining revealed no mononuclear cell infiltration around the gelatin hydrogels in any of the groups. The precursor/gelatin group showed more intense staining for laminin, type I collagen, type IV collagen, and vimentin in the transplanted gelatin as compared to that seen in the gelatin and keratocyte/gelatin groups at 4 weeks after transplantation. Scale bar=100  $\mu\text{m}$ . (B) Immunolocalization of CD34- or nestin-positive cells within the transplanted DiI-positive precursors in the precursor/gelatin group 4 weeks after transplantation of gelatin hydrogels with corneal keratocyte precursors. A computer software program (Adobe Photoshop) was used to superimpose the findings in a single image. In this image, bright light represents the background (black and white), while rhodamine (red color) shows the transplanted DiI-labeled corneal keratocyte precursors in the gelatin hydrogels, and FITC (green color) shows the CD34- or nestin-positive cells. The light red color associated with the many DiI-positive corneal keratocyte precursors indicates the whole transplanted gelatin hydrogel. A few CD34- positive cells or nestin-positive spindle cells are seen scattered within the gelatin hydrogels. Scale bar=100  $\mu\text{m}$

#### **5.4 Expression of stem or progenitor cell markers in the transplanted gelatin hydrogels**

We next examined the expressions of the stem cell marker, CD34, and the neural progenitor cell marker, nestin, in the precursor/gelatin group 4 weeks after transplantation. Many CD34-positive cells were detected in and around the transplanted gelatin hydrogels (Fig. 6B). In addition, a few nestin-positive cells were also seen in the transplanted gelatin hydrogels, whereas nestin-positive cells were barely detected in the corneal stroma around the gelatin hydrogels (Fig. 6B). This indicates that corneal keratocyte precursors with a greater self-renewal potential continue to proliferate even after the transplantation, and thus, are able to supply the keratocytes that are necessary for regeneration of the host stroma. The nestin-positive cells were also present in the transplanted gelatin hydrogels, which indicates that they can contribute to the induction of the nerve regeneration. Large corneal nerves penetrate from the pericorneal nerve plexus into the anterior corneal stroma as thick nerve bundles, which provides sensory innervation of the cornea (MacIver & Tanelian, 1993). Since the transplanted corneal keratocyte precursors would be expected to consistently promote nerve regeneration *in vivo*, they may consequently be able to produce sufficient corneal sensation.

#### **5.5 Advantage of transplantation of keratocyte precursors with gelatin hydrogels**

The combined approach of transplanting the corneal keratocyte precursors along with the gelatin hydrogels into a corneal stromal pocket has several advantages over the use of penetrating keratoplasty in a full-thickness donor cornea. For example, complications associated with open-sky surgery that involve expulsive hemorrhage and risks of wound dehiscence are essentially eliminated. In addition, several postoperative complications, such as postoperative corneal irregular astigmatism, wound leakage, corneal infection, vascularization, and persistent epithelial defect can be avoided when using the combined approach. In conventional full-thickness human corneal allografting with local and/or systemic immunosuppressants, the leading cause of the failure is allograft rejection (Price et al., 1991; Wilson & Kaufman, 1990). Since histologically there was no apparent inflammatory reaction observed, this suggests that there was no immunological rejection detected in the current corneal keratocyte precursor allotransplantation. Thus, this finding indicates that the transplanted precursors can survive in the corneal stroma without rejection.

### **6. Conclusion**

We demonstrated that as compared to the stroma from the central cornea, the stroma from the peripheral region of the rabbit cornea contains a higher density of precursors with a strong proliferative capacity. These keratocyte precursors are able to differentiate into both mesenchymal fibroblasts and neural cells. Furthermore, by using corneal keratocyte precursors and gelatin hydrogels, we were able to establish a new method of three-dimensional reconstruction of the corneal stroma. This new approach that uses corneal keratocyte precursor-based corneal stromal regeneration combined with gelatin hydrogel is a potentially promising new therapy that can be used to attract keratocytes and ECM after the transplantation of the corneal keratocyte precursors. Additionally, the current findings have important implications with regard to the field of regenerative medicine, as this therapeutic tissue engineering approach appears to be applicable to any type of cell. The transplantation of corneal keratocyte precursors into a corneal stromal pocket proved to be a

simple and an effective treatment strategy. By being able to use precursors to initiate corneal regeneration, and ECM production to improve wound healing, this new method could potentially be used to replace conventional full-thickness corneal grafting, thereby helping to alleviate the worldwide shortage of donor corneas that exists at the present time.

## 7. Acknowledgment

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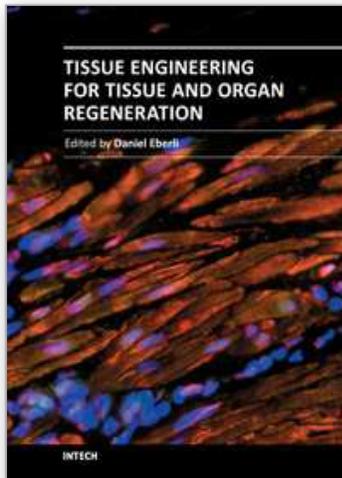
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