Sphere Therapy for Corneal Endothelium Deficiency in a Rabbit Model

Tatsuya Mimura,1 Satoru Yamagami,2 Seiichi Yokoo,2 Yasuo Yanagi,1 Tomobiko Usui,1 Kyoko Ono,1 Makoto Araie,1 and Shiro Amano1

PURPOSE. To isolate precursor cells derived from rabbit corneal endothelium (CE) and to use them for the treatment of CE deficiency in a rabbit model.

METHODS. A sphere-forming assay was performed to isolate precursor cells from rabbit CE. Immunocytochemistry was used to examine marker expressions of neural and mesenchymal cells in the sphere colonies and their progenies. The pump function of the CE sheet was evaluated by measurement of the potential difference and short circuit current. Precursors obtained from rabbit CE by a sphere-forming assay were injected into the anterior chamber of the eye, after which an eye-down (i.e., CE up) position was maintained for 24 hours to allow attachment by gravitation (sphere eye-down group). The sphere eye-down and control groups, observed for 28 days after surgery, underwent histologic and fluorescence microscopic examinations.

RESULTS. Rabbit CE formed primary and secondary sphere colonies. The progeny expressed α-smooth muscle actin, nestin, and neural markers and showed a CE-like hexagonal shape and adequate transport activity. Mean corneal thickness in the sphere eye-down group was significantly less than in the other control groups 3, 7, 14, 21, and 28 days (P < 0.05) after surgery. CE-like hexagonal cells were detected on Descemet’s membrane, and corneal edema was substantially suppressed. Dil-labeled cells were spread over the rear corneal surface in the sphere eye-down group only.

CONCLUSIONS. Precursors from rabbit CE were isolated by a sphere-forming assay. Rabbit CE-derived sphere therapy is an effective treatment in a rabbit CE deficiency model. (Invest Ophthalmol Vis Sci. 2005;46:3128–3135) DOI:10.1167/iovs.05-0251

Ethical problems related to the clinical use of human embryonic stem cells have recently encouraged research on adult stem cells or precursors. Use of such cells offers new and powerful strategies for tissue regeneration and engineering.1 The sphere-forming assay is widely used to isolate adult stem cells or precursors from various tissues, including the central nervous system,2–3 bone marrow,4 skin,5 inner ear,6 retina,7,8 pancreas,9 corneal limbal explants,10 human corneal stroma,11 and human corneal endothelium (CE).12 The CE derived from the neural crest13 forms a single layer of hexagonal cells that is located between the corneal stroma and the aqueous humor of the anterior chamber. This barrier and its pump function maintain corneal transparency by regulating stromal hydration. Deficiency of the CE occurs in conditions such as pseudophakic and aphakic bullous keratopathy, as well as Fuchs dystrophy, leading to corneal stromal edema and decreased visual acuity. Full-thickness corneal transplantation has been performed to treat these conditions. In fact, more than half of the patients who require full-thickness corneal transplantation have decreased visual acuity due to CE deficiency alone.14,15 Therefore, the feasibility of cultured CE transplantation has been tested in CE-deficiency models in vivo.16–20 Transplantation of spheres derived from neural tissue or from bone marrow stromal cells by a sphere-forming assay has already been used therapeutically to regenerate parts of the nervous system, including the spinal cord and the brain.21–25 but sphere therapy has not yet been tried for tissues outside the nervous system. In this study, we isolated precursors of rabbit CE by a sphere-forming assay and tested a treatment strategy that used these precursors for CE deficiency in a rabbit model.

MATERIALS AND METHODS

Isolation of Sphere Colonies from CEs

The rabbits were treated in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. The CE and Descemet’s membrane of the New Zealand White rabbit (Saitama Experimental Animals, Inc., Saitama, Japan), were peeled off from periphery to center as a sheet with fine forceps. To avoid the inclusion of posterior stromal tissue, smoothly peeled off CE with Descemet’s membrane only was used.20 The separated CE was incubated at 37°C for 3 hours in basal medium containing 0.02% collagenase (Sigma-Aldrich, St. Louis, MO). The tissues next were incubated in 0.2% EDTA for 3 hours in basal medium containing 0.02% collagenase (Sigma-Aldrich, St. Louis, MO). The tissues next were incubated in 0.2% EDTA at 37°C for 5 minutes, then dissociated into single cells by trituration with a fire-polished Pasteur pipette. Viability of the isolated CEs was >90%, as shown by trypan blue staining (Wako Pure Chemical Industries, Osaka, Japan). The sphere-forming assay was the primary cell culture technique.2 Basal medium containing a methylcellulose gel matrix (1.5%, Wako Pure Chemical Industries) was used as described previously27–29 to prevent cell reaggregation. Cells were plated at the density of 1.0 viable cell/μL (5000 cells/well; 250 cells/cm²) in uncoated wells of 60-mm culture dishes. The basal medium was Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with B27, epidermal growth factor (EGF, 20 ng/mL), and basic fibroblast growth factor (bFGF, 20 ng/mL).

After 7 days, the spheres that formed were incubated with 10 μM/mL bromodeoxyuridine (BrdU; Sigma-Aldrich) overnight before fixation. Cells within the sphere colonies were stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Ab) at room temperature for 60 minutes in the dark. To distinguish growing
spheres from dying clusters, only those clusters having a diameter of more than 50 μm were counted. For passaging, primary spheres (day 7) were treated with 0.5% EDTA, dissociated into single cells, and the cells plated in 24-well culture plates at a density of 1 cell/mL. Culture was continued for 7 days in basal medium containing a methycellulose gel matrix to prevent reaggregation. To measure sphere colony diameters, cultures were observed under an inverted phase-contrast microscope (ELWD 0.3; Nikon, Tokyo, Japan) with a 10× objective lens, and the images analyzed by NIH Image (available by ftp at zippy.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

**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; Sigma-Aldrich) and 10-μg/mL fibronectin (BD Biosciences, Billerica, MA) placed in separate wells, as described elsewhere. To promote differentiation, 1% FBS was added to the basal medium with EGF (20 ng/mL) and bFGF (20 ng/mL) to create the differentiation medium, and culture continued for another 7 days.

**Immunocytochemistry**

Immunocytochemical analysis was performed on the 7-day spheres and their progenies in the adherent cultures on the glass coverslips after 7 days. Cells were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries) in phosphate-buffered saline (PBS) for 10 minutes. After being washed in PBS to block nonspecific binding, the cells were incubated for 30 minutes with 5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS containing 0.3% Triton X-100 (BSA/PBST; Rohm & Haas, Philadelphia, PA). They next were incubated for 2 hours at room temperature with specific primary Abs diluted in BSA/PBST: mouse anti-cytokeratin 3 monoclonal Ab (mAb, AE-5, Progen Biotechnik GmbH, Heidelberg, Germany), Cy3-conjugated mouse anti-α-smooth muscle actin (SMA) mAb (1:400; Sigma-Aldrich), mouse anti-nestin mAb (1:400; BD Biosciences), mouse anti-microtubule-associated protein (MAP)-2 mAb (1:400; Chemicon, Temecula, CA), mouse anti-neuron–specific enolase mAb (NSE, 1:400; Dako, Glostrup, Denmark), or FITC-conjugated mouse anti-BrdU mAb (1:100; Roche Diagnostics, Basel, Switzerland). As the control, mouse IgG replaced the primary Ab. After being washed in PBS, the cells were left to react for 1 hour at room temperature with fluorescein-labeled goat anti-mouse IgG (Alexa Fluor 488, 1:2000; Molecular Probes, Eugene, OR) as the secondary Ab. Nuclei were counterstained with Hoechst 33342 (1:2000; Roche Diagnostics). Immunocytochemistry was viewed under a fluorescence microscope (model BH2-RFL-T3 and BX50; Olympus, Tokyo, Japan).

**Total RNA Extraction and RT-PCR**

Total RNA was isolated from primary sphere colonies with a kit (Iso- gen; Nippon Gene, Tokyo, Japan). The total RNA isolated was treated with RNase-free Dnase I (Stratagene, La Jolla, CA) for 30 minutes, and cDNA was made with a commercial reverse transcriptions (Super Script II; Invitrogen-Gibco, Carlsbad, CA). T12VN primer at the concentration of 25 ng/μL was used to make the first strand. As the negative control, an RT-PCR was performed in the absence of reverse transcriptase. The PCR primers, based on the nestin, keratin-3, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) sequences, were 5′-TTG AGA CA(T/C) CTG TTG(C/A) CAG CCT-3′ (nestin, sense), 5′-CTTTCAT AC (T/C) CAG(T/C) GATC ATTC T-3′ (nestin, antisense), 5′-GCA GCA GCA GGA GTA GCT G-3′ (keratin-3, sense), 5′-GTT GAG GGT CTG CAT CGT-3′ (keratin-3, antisense), 5′-CAT CAC CAT CTT GCA GGA GC-3′ (G3PDH, sense), and 5′-ACA ATG CCG AAG TGG TG TG TG-3′ (G3PDH, antisense). Products were separated on 1% agarose gels and made visible with ethidium bromide.

**Measurement of the Pump Function of Cells Derived from CE Spheres**

Pump functions of four collagen sheets seeded with cells derived from CE spheres were measured in a Ussing chamber as reported previously but with some modification. The collagen sheen cell carriers were obtained from the Nippi Research Institute of Biomatrix (Tokyo, Japan). Cells derived from a CE sphere suspension of 5.0 × 10^5 cells in 1.5 mL of culture medium were transferred to the individual circular collagen sheets, each 10 mm in diameter, and each sheet placed in separate wells of 24-well plates. The plates next were centrifuged at 1000 rpm (176g) for 10 minutes to enhance cell attachment. They were then kept in the cell culture medium for 2 days, after which nonadhering cells and debris were removed. Rabbit corneas with epithelia scraped off mechanically (n = 4), collagen sheets only (n = 4), and CE sphere collagen sheets (n = 4) were mounted in the Ussing chamber.

**Migration and Proliferation of CE Spheres on Rabbit Descemet’s Membrane Ex Vivo**

Primary CE spheres cultured for 7 days were labeled with a fluorescent cell tracker (CM-Dil; C-7000; Molecular Probes), as described elsewhere, to trace CE localization. CEs were gently scraped off four freshly excised rabbit corneas with a sterile cotton swab, and CE spheres were applied to the posterior surface of each cornea, after which the corneas were placed in 24-well plates. The plated corneas were maintained in culture medium for 7 days. Fluorescence of CE spheres that migrated was viewed under a fluorescence microscope, and the area occupied by cells that had migrated from the spheres was measured by the NIH Image program (n = 10).

**Cryoinjury and Labeled CE Sphere Injection into the Anterior Chamber**

New Zealand White rabbits, weight 2.0 to 2.4 kg, were anesthetized with intramuscular ketamine hydrochloride (60 mg/kg; Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany). To detach the CE from Descemet’s membrane, a brass dowel that had been cooled in liquid nitrogen was touched to a rabbit’s cornea nine times. At the center and eight peripheral regions on the corneal epithelium. This procedure was repeated twice to prevent CE prolif- eration after cryoinjury. Bullous keratopathy was maintained for 2 months with this procedure as described elsewhere. The anterior chamber was washed with PBS three times, and 150 Dil-labeled CE spheres (1.0 × 10^7) were injected into the anterior chamber of the right eye. Thereafter, rabbits in the cryo (cryoinjury alone), CE eye-down (cryo injury and CE injection), and sphere eye-down (cryo injury and sphere injection) groups were kept in the eye-down position for 24 hours to be attached by gravitation, and rabbits in the sphere eye-up group were kept in the eye-up position for 24 hours under deep anesthesia. Each surgical eye was checked twice or three times a week by external examination, and photographs were taken on days 7, 14, and 28 after surgery. Central corneal thickness was measured with an ultrasound pachymeter (Tomey, Nagoya, Japan) and intraocular pressure with a pneumotonometer (model 30 Classic; Mentor O & O, Norwell, MA) on days 0.5, 1, 3, 7, 14, 21, and 28 after surgery. An average of three readings was taken. A one-way ANOVA and Scheffe’s multiple comparison were used to compare the mean results. P < 0.05 was considered significant.

**Histologic Examination**

One month after transplantation the rabbits’ eyes were removed and viewed as wholemounts under a fluorescence microscope, to examine Dil fluorescence. The corneas next were excised and bisected. Post- operative endothelial morphology was evaluated on one side of the divided corneas, after staining with 3% trypan blue and 5% alizarin red, to determine CE cell density, as described elsewhere. The number of cells in 0.1 × 0.1 mm² was counted at four different sites in the six reconstructed corneas. Next, the corneas were immersed in a fixative composed of 4% paraformaldehyde (Wako Pure Chemical Industries) in 0.1 M PBS at pH 7.4 and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Laboratories, Naperville, IL) at −20°C. Frozen 8-μm-thick sections were cut. The other half of the
cornea was fixed immediately in 10% formalin (Wako Pure Chemical Industries) and used for hematoxylin and eosin (HE) staining.

RESULTS

Isolation of Sphere Colonies from CE

We adapted a sphere-forming assay,\(^2\) devised to enrich neural stem cells and other precursors,\(^2\text{"}^{12}\) to isolate precursors from rabbit CEs. The CE monolayer is removed without contamination as a sheet with Descemet’s membrane.\(^2\text{"}\) To investigate whether isolated CEs were contaminated by corneal epithelial cells, the expression of the epithelial marker, keratin,\(^3\text{"}^{37,38}\) assessed by an RT-PCR before culture, was negative (data not shown). The CE was disaggregated into single cells and plated at a density of 1.0 viable cell/µL in basal medium containing a methylcellulose gel matrix to prevent reaggregation. Sphere colonies were derived by proliferation as 10 viable cells/µL, but not by reaggregation of dissociated cells as described elsewhere.\(^2\text{"}^{27,28}\) Nearly complete dissociation into single cells, confirmed by counting the percentage of single, double, and triple cells, showed that more than 99% of the cells were single ones (Fig. 1a). After 5 days of culture, small floating spheres formed that grew larger after 7 days, as shown by representative single sphere growth (Figs. 1b–d) and mean sphere size (Fig. 1e), whereas nonproliferating cells died and were eliminated. To verify that the increase in colony size actually was due to proliferation, BrdU was added 24 hours before fixation. BrdU labeled most cells in each colony by day 7 (Fig. 1f), indicative that the colonies contained proliferating cells. These findings suggest that sphere colonies are produced from single isolated CEs and that sphere-forming cells are highly proliferative. The number of sphere colonies obtained from the CEs, counted after 7 days of culture, showed that \(35\pm11\) spheres per 10,000 cells (mean ± SD) were generated (Fig. 1g). Replating efficiency is maintained from the primary to secondary sphere colonies (Fig. 1h) and the passage cells from the secondary spheres did not form spheres (not shown).

Immunocytochemistry and RT-PCR in Sphere Colonies

The spheres were immunostained with nestin, a marker for immature cells,\(^3\text{"}^{39}\) since this marker is expressed by immature cells within sphere colonies derived from the brain, skin, retina, inner ear, and pancreas.\(^3\text{"}^{12}\) Most cells in the spheres were immunoreactive for nestin (Fig. 2a), as confirmed by the presence of nestin-mRNA (Fig. 2b).

Immunocytochemistry in the Progeny of Individual Sphere Colonies

To test whether sphere progeny expresses marker proteins of neural and mesenchymal cells, on day 7 the spheres were

\[\text{FIGURE 1. Sphere-forming rabbit CEs. CE s were disaggregated into single cells, and plated at a density of 1.0 viable cell/µL in basal medium containing a methylcellulose gel matrix to prevent reaggregation. (a) More than 99% are single cells on day 0. (b, c, d) Growth stages of a representative single sphere up to day 7. (e) Mean sphere size was 210±32 µm on day 7. (f) Each colony was labeled by BrdU on day 7. (g) 35±11 primary spheres per 10,000 cells (mean ± SD) were generated. Replating efficiency is maintained from the primary to secondary sphere colonies. (h) Secondary spheres generated from dissociated primary spheres. Scale bar, 50 µm.}\]
transferred to poly-L-lysine/fibronectin-coated glass coverslips placed in 24-well plates and then were cultured in differentiation medium containing 1% FBS, EGF (20 ng/mL), and bFGF (20 ng/mL). After 7 days, many cells had migrated from the colonies. A small percentage of those migrating from the spheres were nestin-positive, but were negative for the differentiated epithelial cell marker, cytokeratin-3 (Fig. 3). These cells were immunostained by the mesenchymal cell marker α-SMA and by the neuronal markers, MAP 2 and NSE (Fig. 3), and did not show coexpression of these markers (not shown), evidence that a single sphere colony in a methylcellulose gel matrix to prevent reaggregation completely can give rise to mesenchymal and neuronal cells. CEs therefore can generate nestin-positive precursor cells that, when differentiated, produce mesenchymal and neuronal cell marker proteins, indicating that CE-derived spheres have at least bipotent capabilities.

**CE Morphology and Pump Function of the CE Sheet Reconstituted with CE Spheres**

We differentiated spheres with DMEM containing 10% FBS. Then we determined whether the differentiated cells had a CE-like hexagonal form and tested the essential CE pump function, potential differences, and short circuit current in the standard Ussing chamber system, as CEs should have significant transport activities. Confluent cells derived from CE spheres had the characteristic hexagonal CE-like form (Fig. 4a). Time-course changes are shown as means plus SD of the potential differences (Fig. 4b) and short circuit current (Fig. 4c) in normal rabbit corneas and in the CE sheet reconstituted with CE-derived spheres. Average potential differences and short circuit current in the sphere-derived CE sheets 1, 5, and 10 minutes after measurement varied from 79% to 92%, the range for normal rabbit corneas deprived of their epithelia. These morphologic and functional findings suggest that CE-derived spheres mainly generate CE-like cells that have adequate transport activity.

**Clinical Observation after Surgery**

Our findings suggested that rabbit CEs contain precursor cells that have tripotency including differentiation into CE-like cells and self-renewal ability. Precursors derived from the CE were next used as therapy for CE deficiency. To estimate the number of spheres needed to cover the rear surface of the cornea (Descemet’s membrane), Dil-labeled spheres were prepared and seeded on denuded Descemet’s membrane in cultures. Figure 5a shows that Dil-labeled spheres migrated and that the mean area covered per sphere was 0.62 ± 0.22 mm² after day 7 (Fig. 5b). The required number of spheres per cornea was calculated to be 150 spheres/cornea; therefore, 150 spheres were injected to the anterior chamber of CE-deficient rabbit eyes by cryoinjury, and the animals were kept in the eye-down position for 24 hours after treatment so that the spheres would be attached by gravitation (sphere eye-down group, n = 6). Cryo injury alone (cryo group, n = 6), CE injection and the eye-down position for 24 hours (CE eye-down group, n = 6), and sphere injection and the eye-up position (sphere eye-up group, n = 6) were the controls. CE-injection and the eye-up position did not decrease corneal edema, as reported previously. In the cryo, CE eye-down, and sphere eye-up groups, mean corneal thickness was approximately 1000 μm throughout the 28 days of observation. In contrast, it decreased rapidly in the sphere eye-down group and was significantly less than in the other three groups 3, 7, 14, 21, and 28 days (P < 0.0001) after treatment (Fig. 6a). As shown in the representative anterior segment photographs from the three control groups (Figs. 6b–d), corneas were opaque with epithelial and stromal edema, whereas in the sphere eye-down group corneas became clear, and the iris was clearly visible (Fig. 6e). There was no intraocular pressure increase, a possible side effect, in any group during the observation period (data not shown).

**Histologic Examination and Evaluation of CEs**

In the cryo (Fig. 7a, c), CE (Fig. 7b, f), and sphere eye-up (Figs. 7c, g) groups, no CEs were present on Descemet’s membrane of the central cornea in the flatmounts (Figs. 7a–c) nor in cross-section HE stainings viewed under a light microscope (Figs. 7e–g). In contrast, CE-like hexagonal cells were present in the sphere eye-down group and had formed a monolayer, as seen by HE staining, 28 days after transplantation (Figs. 7d, 7h). These cells were Dil-positive both in the flatmounts (Fig. 7i) and cross-section (Fig. 7j) under a fluorescence microscope, indicative that they were injected sphere-derived cells and not host derived-cells. Dil-positive cells were detected on the lower angle in the CE eye-down, sphere eye-up, and sphere eye-down groups (not shown). CE densities of the six grafts in the sphere eye-down group 28 days after surgery ranged from 2550 to 3375 cells/mm² (mean ± SD: 2963 ± 302 cells/mm²).

**DISCUSSION**

Spheres derived from rabbit CE were capable of high proliferation as shown by BrdU-positive staining. Limited self-renewal potential is indicated by the inability of the progeny of individual spheres to form secondary spheres, but not third-passage
spheres. Moreover, the individual spheres and their progeny expressed mesenchymal and neuronal lineage marker proteins. Although we cannot show direct evidence that isolated spheres give rise to CEs, the characteristic hexagonal morphology and the essential CE transport activity, seen with the Ussing chamber system, suggest that the spheres mainly give rise to CE-like cells that have adequate transport activities, which are required for corneal hydration. These findings indicate that isolated spheres have the character of tripotent precursors and that their progenies have essential CE functions.

Corneal transplantation has become the most common form of solid tissue transplantation throughout the world.40

![Figure 4](image1.png)

**Figure 4.** Morphology and function of CE sphere-derived cells. (a) Confluent cells in DMEM containing 10% FBS had the characteristic CE-like hexagonal form. Time-course changes in the means ± SD of the potential differences (b) and short circuit currents (c) in rabbit corneas and in CE collagen sheets reconstituted with CE-derived spheres. Mean potential differences and short circuit current in the CE sheets 1, 5, and 10 minutes after measurement were 79% to 92%, the range in rabbit corneas deprived of their epithelia, indicating that CE-like cells have satisfactory transport activities. After the Na⁺-K⁺ ATPase inhibitor ouabain was added to the chambers, the potential difference became 0 mV and the short circuit current 0 μA in all the test samples. (●) Rabbit corneas; (○) CE collagen sheets; and (■) collagen sheets alone.

![Figure 5](image2.png)

**Figure 5.** Measurement of migration areas of sphere-derived differentiated cells during 1 week of culture. A Dil-labeled sphere was seeded on denuded Descemet’s membrane in a humidified incubator with an atmosphere of 5% CO₂ and cultured for 1 week. Each migration area was photographed and calculated by NIH Image. (a) Representative photographs of time-course changes of an adhering Dil-labeled sphere. Scale bar, 100 μm. (b) The mean area per sphere increased gradually to 0.62 ± 0.22 mm² on day 7 (n = 10).
More than 45,000 corneal transplants are performed each year in the United States, to improve vision and increase the quality of life of patients with damaged or diseased corneas. In our study, sphere injection and the eye-down position, but not differentiated cultured CE injection nor sphere injection in the eye-up position, restored CE function and markedly decreased corneal edema in a rabbit bullous keratopathy model. Cells adhering to the corneal rear surface, Descemet’s membrane, were all DiI positive in the sphere eye-down group, evidence that CEs are derived from injected spheres, not from residual host CEs. The fact that only in the sphere eye-down group did injected spheres attach to and spread over the corneal CE suggests that precursor injection and the eye-down position may provide useful treatment with minimum trauma for CE

**FIGURE 6.** Clinical findings after surgery. (a) Corneal thickness was measured with an ultrasound pachymeter. Mean corneal thickness decreased rapidly in the sphere eye-down group (●, n = 6), being significantly less (‡P < 0.001) than in the cryo (○, n = 6), CE eye-down (△, n = 6), and sphere eye-up (□, n = 6) groups on days 5, 7, 14, 21, and 28 after treatment. (b–e) Representative corneal photographs. Corneas in the cryo (b), CE eye-down (c), and sphere eye-up (d) groups were opaque, and the iris was not clearly visible. In contrast, there was no corneal opacity in the sphere eye-down group (e).

**FIGURE 7.** Histologic examination and assessment of CEs. In the cryo (a), CE eye-down (b), and sphere eye-up (c) groups, no CEs are present on Descemet’s membrane from the central cornea in the flatmounts under a phase-contrast microscope. HE-stained cross section showing similar results under a light microscope (e–g). CE-like hexagonal cells were present in the sphere eye-down group (d) and formed a monolayer, as shown by HE staining 28 days after transplantation (h). These cells were Dil-positive in flatmounts (i) and cross section (j) under a fluorescence microscope.
deficiency in place of conventional full-thickness corneal transplantation. Endless proliferation of transplanted precursors in the anterior chamber may cause glaucoma, because excessive numbers of CEs would obstruct the trabecular meshwork and increase intraocular pressure. In vivo, CEs are inhibited in the G1-phase of the cell cycle,41 and TGF-β2 in the aqueous humor suppresses entry into the S-phase.42 Cell–cell contact inhibition also may be an important mechanism for inducing cell cycle arrest to maintain the mature monolayer in a nonproliferative state.43 Moreover, the number of highly proliferative, cycle arrest to maintain the mature monolayer in a nonproliferative state.43 Moreover, the number of highly proliferative, cultured CEs in vitro decreased after the CE sheet was transplanted to the anterior chamber,44 indicative that transplanted precursors may not proliferate continuously in the anterior chamber, because sphere progenies are exposed to aqueous humor containing TGF-β2 and reach cell confluence on Descemet’s membrane for a short while. Spheres from human CEs that express neuronal and mesenchymal cell marker proteins have a strong propensity to differ- entiate into CE-like cells.12 Fibroblast-like human corneal stromal cells also express neuronal cell marker proteins and give rise mainly to the mesenchymal cell marker vimentin and α-SMA-positive myofibroblast-like cells.13 Pancreatic precursor population for cell-based therapeutic strategies was identified from mouse pancreas by a sphere-forming assay.9 These find- ings and our data on spheres from rabbit CE may imply that spheres can provide a therapeutic source for original tissue-committed cells as well as neural cells.16–20

We isolated precursors from rabbit CE using a sphere-forming assay. Sphere transplantation to the anterior chamber and short-term maintenance of the eye-down position to allow attachment by gravitation provide a simple, ideal strategy for treating CE deficiency in a rabbit model and may have the possibility of replacing full-thickness corneal transplantation for reduced visual acuity due to CE deficiency.

References


