

# Adipose-Derived Stem Cells on Hyaluronic Acid-Derived Scaffold

## *A New Horizon in Bioengineered Cornea*

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**Objective:** To evaluate the ability of human adipose-derived stem cells (h-ASCs) to survive and differentiate in corneal stroma.

**Methods:** Our experiment consisted of 2 phases. First, we cultured h-ASCs in different types of hyaluronic acid (HA)-derived synthetic extracellular matrixes (sECMs) to determine the capability of proliferation and survival of the cells in hydrogels. Second, h-ASCs were grown in plastic flasks, labeled with an intracytoplasmic membrane fluorescent molecule, transferred onto different types of sECMs or the native HA product, and then inserted into the corneal stroma of the rabbits. After 10 weeks, we assessed the viability of the stem cells and the expression of cornea-specific proteins.

**Results:** The in vitro study showed that the HyStem-HP hydrogel had the highest yield of cells ( $1.1 \times 10^6/\text{mL}$ ) compared with other types of HA-derived sECMs culture media, and the cells grown in the HyStem-HP hydrogel

appeared more elongated and fibroblastlike. The in vivo study demonstrated that the labeled h-ASCs could be identified in the stroma with any type of sECM. The HA-derived sECMs, particularly the HyStem-HP hydrogel, showed better survival and cell morphologic features compared with pure HA. Immunostaining of keratocan, aldehyde dehydrogenase, and type I collagen revealed that the stem cells had expressed human cornea-specific proteins.

**Conclusion:** Human adipose-derived stem cells can be successfully grown on HA-derived sECMs in vivo and can express human cornea-specific proteins.

**Clinical Relevance:** Human ASCs on an HA-derived scaffold may be used as a source of keratocytes to regenerate extracellular matrix-like material in situations where the cornea stroma has been compromised.

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**T**HE CORNEA IS A TRANSPARENT, avascular, trilaminar tissue that provides 75% of the refractive power of the eye. The stroma, the middle layer of the cornea, is approximately 500  $\mu\text{m}$  thick and relatively acellular, although corneal keratocytes constitute 3% to 10% of its volume. The stroma is composed of types I and V heterotypic collagen fibrils (15% wet weight) that are of uniform diameter (mean [SD], 32 [0.7] nm), glycosaminoglycans, keratan sulfate and dermatan sulfate (1% wet weight), various proteoglycan core proteins, and other protein constituents, including fibronectin, laminin, and type VI collagen.<sup>1</sup> The corneal stroma is extremely organized and provides most principal functions of the corneal tissue. Corneal stromal opacification can result in permanent vision loss or blindness and affects more than 10 million people worldwide, with 1.5 million new cases of blindness annually.<sup>2</sup>

The gold standard of treatment for corneal blindness is full-thickness corneal transplant or penetrating keratoplasty. However, the fundamental problem with using human donor corneas for transplant is a shortage of donor tissue caused by cultural, logistical, and technical problems.<sup>3</sup> Even under the best conditions, donor grafts can be quite variable in quality; furthermore, the risk of postoperative complications is high.

One alternative to keratoplasty is keratoprosthesis, or an artificial cornea, which was first suggested by Guillaume Pellier de Quengsy in 1771.<sup>4</sup> Most commonly used keratoprostheses consist of a functional optic core surrounded by a microporous skirt that integrates into the host tissue. However, keratoprosthesis implantation also has high rates of complication, including extrusion of the implant, epithelial downgrowth, glaucoma, and endophthalmitis.<sup>4</sup>

Because of these insufficiencies, there is substantial clinical interest in develop-

ing a suitable replacement for keratoplasty and keratoprotheses. Tissue engineering of a living cornea created by integrating biomaterials with cells could solve this problem.<sup>5</sup> Because most infectious, immune, ecstatic, and dystrophic corneal diseases involve the corneal stroma, the primary focus of this study is the repopulation and restoration of damaged corneal stroma using a cell-scaffold composite.

Keratocytes are the principal cells of the stroma that secrete the collagens and keratan sulfate proteoglycans<sup>6</sup>; however, during corneal wound healing, keratocytes transform into fibroblasts and/or myofibroblasts that can produce scar tissue and resulting in corneal opacity. On the other hand, bone marrow- or adipose-derived mesenchymal stem cells (MSCs) have been shown to have multiple lineage potential and can differentiate into a variety of cell types.<sup>7-9</sup> Recent studies showed human adipose-derived stem cells (h-ASCs) were able to differentiate into keratocytes.<sup>10,11</sup> We investigated the ability of h-ASCs to survive *in vivo* and differentiate into keratocytes when encapsulated within a hyaluronic acid (HA)-derived synthetic extracellular matrix (sECM).

## METHODS

### CELL CULTURE

We isolated h-ASCs as described by McIntosh et al<sup>12</sup> and cultured in Dulbecco Modified Eagle Medium: Nutrient Mixture F-12 (GIBCO/Invitrogen), supplemented with 10% fetal bovine serum and a 1% combination of antibiotic and antimycotic. Media were replenished every 3 days, and cells were passaged at 80% confluency using trypsin, 0.25%, with tetrasodium EDTA (GIBCO/Invitrogen) and frozen using recovery cell culture freezing medium containing 10% dimethyl sulfoxide (GIBCO/Invitrogen).

### FIRST PHASE: 3-DIMENSIONAL CULTURE OF h-ASCs

Passage 3 of h-ASCs were cultured in hydrogels (Extracel-SS, HyStem-HP, and HyStem-CSS; Glycosan BioSystem Inc) in a 24-well plate insert system with an 8.0- $\mu$ m pore size (HTS Multiwell; BD Falcon) at a concentration of 62 000 cells per 100 mL per insert according to the manufacturer's instructions. The culture medium consisted of Minimum Essential Medium (Invitrogen), 20% fetal bovine serum (Atlanta Biologicals), 1% L-glutamine (GIBCO), and a 1% combination of penicillin and streptomycin (GIBCO). The gels were incubated in a 37°C incubator with 5% carbon dioxide. When the cells in the gels reached 90% confluence, 2 mL of 70mM *N*-acetylcysteine (Sigma-Aldrich Co) in phosphate-buffered saline solution (PBS; pH, 7.4) was added to each well to dissolve the gel. The solution was aspirated from the well plate and centrifuged at 1500 rpm to obtain cell pellets and then resuspended at a density of  $1 \times 10^6$  cells/mL in PBS. We added 5  $\mu$ L of fluorescent solution chloromethylbenzamido (CM-DiI) (Vybrant; Invitrogen) per 1 mL of cell suspension. Cells were mixed well by gentle pipetting and incubated for 16 minutes at 37°C. Cells were centrifuged at 1500 rpm for 5 minutes, at 37°C. The supernatant was removed, and the cells were gently resuspended in warm medium. The wash procedure was repeated 2 more times. The gels were again prepared following the manufacturer's instructions, with the addition of 5-ng/mL basic fibroblast growth factor (bFGF) (RD Bio-

systems) to the gels. These gels were cultured in media supplemented with 5-ng/mL bFGF for 14 days in a 37°C incubator with 5% carbon dioxide. The medium was changed every 3 days. Gels were dissolved with 70mM *N*-acetylcysteine as described in the preceding paragraph. The cells were counted and cell morphologic features were examined and compared.

### SECOND PHASE: IMPLANTATION OF h-ASCs ON DIFFERENT TYPES OF SCAFFOLDS IN RABBIT CORNEAS

#### Preparing Solid Gels

Human ASCs were cultured in cell culture flasks (T150 Corning; Sigma-Aldrich Co) with culture medium consisting of Minimum Essential Medium, 20% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin, and 5-ng/mL bFGF for 14 days in a 37°C incubator with 5% carbon dioxide. The cells were trypsinized, rinsed in PBS, and labeled with CM-DiI as described in the preceding subsection. The cells were then transferred to HyStem-HP, Extracel-SS, and HyStem-CSS with 5-ng/mL bFGF at a concentration of 400 000 cells per 100- $\mu$ L gel for 72 hours. The gels were rinsed with PBS, removed from the well insert, and floated in PBS just before implantation underneath the flap in rabbit corneas.

#### Preparing Semisolid Gels

Cells were grown and labeled with CM-DiI as described for the solid gels. The gel/cell suspensions were supplemented with 5-ng/mL bFGF and prepared according to the manufacturer's directions, with the exception that the addition of the cross-linker (disulfide-containing polyethylene glycol diacrylate or polyethylene glycol diacrylate) was modified. The cross-linker was resuspended in 1 mL of degassed water (Glycosan BioSystem Inc); the additional degassed water added to the cross-linker slowed down the gelling process for several hours and created a less stiff gel. Cells were added at a concentration of 300 000 cells per 100- $\mu$ L gel. Cross-linker solution (0.5-mL) was added to the gel/cell suspension 30 minutes before injection into the stromal pocket of rabbit corneas.

A sterile, nonpyrogenic, viscoelastic preparation of a highly purified, noninflammatory, high-molecular-weight sodium hyaluronate (Healon; AMO) was also used as a vehicle to deliver the labeled cells. The cells were added at a concentration of 300 000 per 100- $\mu$ L Healon (10 mg/mL) and injected into the stromal pocket of rabbit corneas.

The h-ASCs mixed with hydrogels and h-ASCs in Hank balanced salt solution (HBSS) (Invitrogen) were categorized as the treatment group. In contrast, pure HBSS and hydrogels without cells were classified as the control group.

#### Eye Surgery on Rabbits

Animal studies were performed after approval of the Institutional Animal Care and Use Committee at Tulane University. Nine adult New Zealand White rabbits (weight, 2-3 kg) were divided in 2 groups for the experiments. All animal procedures were performed in compliance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research.

Six rabbits underwent corneal flap formation. After anesthetizing the rabbits with a combination of intramuscular ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg), a microkeratome suction ring (Carriazo-Barraquer; Moria Inc) was placed on the eyes, and suction was applied. A microkera-

**Table 1. Summary of Treatment Assignments and Complications in Rabbit Eyes**

Rabbit No./Eye	Type of Treatment	Type of Surgery	Intraoperative Complication	Postoperative Complication
1/OD	Flap only (control)	Flap formation	None	Displaced flap after 2 wk
1/OS	HBSS injection (control)	Flap formation	None	Superficial neovascularization after 8 wk
2/OD	h-ASC injection	Flap formation	None	None
2/OS	HyStem-HP hydrogel <sup>a</sup> No cells (control)	Flap formation	Free flap	None
3/OD	Extracel-SS hydrogel <sup>a</sup> No cells (control)	Flap formation	None	Displaced flap after 1 wk
3/OS	h-ASC injection	Flap formation	Free flap	Superficial neovascularization after 4 wk
4/OD	HyStem-HP hydrogel with h-ASCs	Flap formation	None	None
4/OS	Excluded from study	Flap formation	Perforation	
5/OD	Extracel-SS hydrogel with h-ASCs	Flap formation	None	None
5/OS	Excluded from study	Flap formation	Perforation	
6/OD	HyStem-CSS hydrogel <sup>a</sup> with h-ASCs	Flap formation	None	None
6/OS	HyStem-HP hydrogel with h-ASCs	Flap formation	None	None
7/OD	Healon <sup>b</sup> with h-ASCs	Stromal pocket	None	Haziness 1 to 2+
7/OS	h-ASC injection	Stromal pocket	None	Haziness 1 to 2+
8/OD	Extracel-SS hydrogel with h-ASC	Stromal pocket	None	Superficial neovascularization after 8 wk
8/OS	Healon with h-ASCs	Stromal pocket	None	Haziness 1 to 2+
9/OD	HyStem-CSS hydrogel with h-ASCs	Stromal pocket	None	Haziness 1 to 2+
9/OS	HyStem-HP hydrogel with h-ASCs	Stromal pocket	None	Haziness 1 to 2+

Abbreviations: h-ASCs, human adipose-derived stem cells; HBSS, Hank balanced salt solution.

<sup>a</sup>Indicates 1 of 3 hydrogels from Glycosan BioSystem Inc.

<sup>b</sup>Sterile, nonpyrogenic, viscoelastic preparation of a highly purified, noninflammatory, high-molecular-weight sodium hyaluronate (AMO).

tome blade (Carriazo-Barraquer; Moria Inc) was used to create a flap at an estimated depth of 120  $\mu$ m with a nasal hinge. The flap was retracted, and a second pass of the same depth was performed on the cornea, then according to a randomized assignment (**Table 1**), different types of the solid hydrogel products and control products were placed in the stromal bed and the flap was returned into its primary position. To avoid flap displacement and to maintain a relatively watertight intrastromal space, three 10-0 nylon sutures were fixed in the flap borders. In 3 other rabbits, a stromal pocket formation was performed. Rabbits anesthetized with a combination of intramuscular ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg) were placed underneath the operating microscope. A corneal incision at a depth of 250  $\mu$ m and a length of 3 mm was created by a calibrated diamond blade (DGH-KOI, Inc), then a stromal pocket dissection was performed using a Martinez dissector (Accutome Inc). Different types of semisolid hydrogel products and cells per protocol (Table 1) were injected inside the pocket, and the corneal incision was sutured with one 10-0 nylon suture.

Postoperatively, eyedrops (prednisolone acetate [Pred Forte], 1%, and moxifloxacin hydrochloride [Vigamox], 0.5%) were used twice a day as inflammation and infection prophylaxis for 4 weeks.

### Clinical Observation

Each eye was examined with a portable slitlamp (model LYL-S; Wenzhou Jinzhou Group Foreign Trade Industries Co, Ltd) for corneal inflammation, opacities, or other ocular surface or anterior chamber complications on days 1 and 7 and then weekly after surgery. Haze was evaluated on a scale of 0 to 4, according to the severity of the haze, from 0 for total transparency to 4 for very severe haze that makes it impossible to observe the details of iris and pupil.

### Tissue Procurement

Rabbits were humanely killed using intravenous pentobarbital sodium, 100 mg/kg, at 10 weeks. The corneas were re-

moved and cut in half; one-half were placed in glutaraldehyde, 2.5%, for transmission electron microscopy study. Tissue samples were dissected into 1- to 1.5-mm<sup>2</sup> cubes and sent to the Core Laboratory for further processing. The other half were placed in paraformaldehyde, 4%, and fixed in a cryoprotective embedding medium (OCT; Miles Inc) for light microscopy and immunohistochemistry studies. Tissue blocks were cut at 6- $\mu$ m thickness.

### Histological Examination and Localization of h-ASCs Within the Stroma

Several sections of each cornea were stained with hematoxylin-eosin for light microscopy examination (Olympus). The CM-DiI-labeled h-ASC cells were localized using a fluorescence microscope (Olympus) before immunostaining.

### Aldehyde Dehydrogenase, Keratocan, and Type I Collagen Immunohistochemistry

To demonstrate differentiation into functional keratocytes, immunohistochemical staining for human aldehyde dehydrogenase (ALDH) and stromal cornea-specific core protein human keratocan and type I collagen was performed on OCT sections. Rabbit anti-human ALDH (Abcam) was incubated at 1:75 overnight. Goat anti-human keratocan (Santa Cruz Biotechnology) was incubated at 1:25 overnight. Mouse anti-type I collagen (Sigma-Aldrich Co) was incubated at 1:50 overnight.

Secondary antibodies, including goat antirabbit, rabbit antigoat, and goat antimouse fluorescent dyes conjugates (Alexa Fluor 488; Invitrogen), were incubated at 1:300 for 90 minutes, and then slides were mounted using Vectashield medium with 4',6-diamidino-2-phenylindole (Vector Laboratories). Keratocan, ALDH, and type I collagen fluoresced in green, and CM-DiI-labeled h-ASCs fluoresced in red. Expression of human ALDH, keratocan, and type I collagen were confirmed using a fluorescence microscopy.

## RESULTS

### THREE-DIMENSIONAL CULTURE OF h-ASCs IN HYDROGELS

The result of cell morphologic feature assessment and cell count of 3-dimensional cultures of h-ASCs after 14 days is shown in **Table 2**. The cells grown in HyStem-HP hydrogel appeared more elongated and fibroblastlike (**Figure 1A**) compared with those cultured in HyStem-CSS or Extracel-SS, which were more round and not elongated in the culture (**Figure 1B**).

The best results, based on fibroblastlike morphologic characteristics and cell viability, were obtained by growing the cells in flasks. Based on the results of the first experiment, h-ASCs were cultured in flasks for 14 days and labeled with CM-Dil (**Figure 1C**). The labeled cells were then prepared as 3-dimensional suspensions in hydrogels (solid and semisolid) for 30 minutes before insertion into the rabbit corneas. More cells were used for the solid (4000 cells/ $\mu$ L) vs semisolid (3000 cells/ $\mu$ L) hydrogels because, in the removal process from the well insert, the solid hydrogels were sheared around the edges and a number of cells were lost.

### EYE SURGERY AND CLINICAL OBSERVATION

Table 1 summarizes intraoperative and postoperative complications. The uncomplicated rabbit eyes treated with corneal flap procedure showed negligible corneal opacity or haze at 10 weeks (**Figure 2A**).

In the uncomplicated rabbit eyes treated with the stromal pocket technique, mild to moderate stromal haziness was observed (**Figure 2B**). No significant difference in the amount of haze could be observed among different materials that were used in each group.

### LIGHT AND ELECTRON MICROSCOPIC EXAMINATION OF THE CORNEAS

In light microscopic examination with hematoxylin-eosin staining, no histological differences were found between the different types of scaffolds and the control corneas, excluding the corneas with complications. No inflammatory reaction was observed at the interface, even in the vascularized cornea, and no immune rejection developed, although it was a xenograft and the rabbits were not immunosuppressed (**Figure 3**).

Human ASCs were identified in the interface of the flap or stromal pocket of the host tissue, as verified by localization of fluorescent CM-Dil labeling. Human ASCs were visible as a discontinuous layer at the flap-stromal interface or in the stromal pocket after 10 weeks (**Figure 4**). No h-ASCs were observed in the flap-displacement corneas.

The corneas inserted with h-ASCs and scaffolds, particularly HyStem-HP, demonstrated a longer layer of CM-Dil fluorescence and in more sections of tissue cut (average, 7 of 9 sections in each eye) than the group with h-ASCs alone (average, 3 of 9 sections in each eye). When the scaffolds were compared, the HA-derived sECM hydrogels gave a higher number of cells in each section (average, 10 cells in 7 of 9 sections in each eye) than the

**Table 2. Cell Count and Morphologic Features After 3- and 2-Dimensional Culture of Human Adipose-Derived Stem Cells**

Type of Culture	Cell Count	Cell Morphologic Feature
HyStem-HP hydrogel <sup>a</sup>	$1.1 \times 10^6/\text{mL}$	Fibroblastlike
HyStem-CSS hydrogel <sup>a</sup>	$5.6 \times 10^5/\text{mL}$	Round
Extracel-SS hydrogel <sup>a</sup>	$5.2 \times 10^5/\text{mL}$	Round
T150 tissue culture flasks <sup>b</sup>	$5.5 \times 10^6/\text{mm}^2$	Fibroblastlike

<sup>a</sup>From Glycosan BioSystem Inc.

<sup>b</sup>From Sigma-Aldrich Co.

unmodified viscous HA solution, Healon (average, 4 cells in 5 of 9 sections in each eye).

Using transmission electron microscopic examination (Hitachi), the h-ASCs were large, spindle-shaped cells compared with the very thin, elongated normal keratocytes (**Figure 5A**). **Figure 5B** shows the h-ASCs at the interface. There was no difference in the stromal structure of the cornea when the treated corneas were compared with the controls (**Figure 5C**).

### COLLAGEN PRODUCTION OF TRANSPLANTED h-ASCs

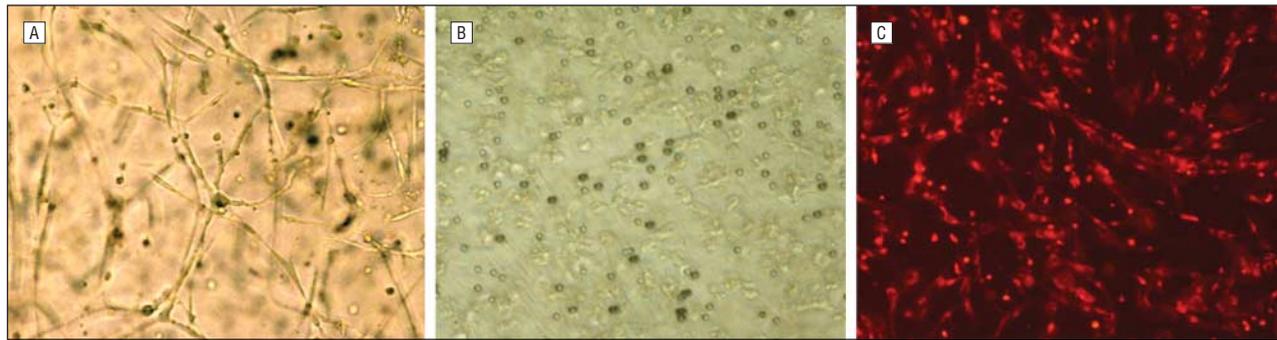
To illustrate the collagen production by the h-ASCs after transplant, immunohistochemical analysis for type I collagen was performed on the corneas. Type I collagen was expressed in the normal rabbits' corneal stroma and in the cleft created from stromal pockets enclosing transplanted h-ASC cells (**Figure 6A**).

### ALDH AND KERATOCAN PRODUCTION BY TRANSPLANTED ASCs

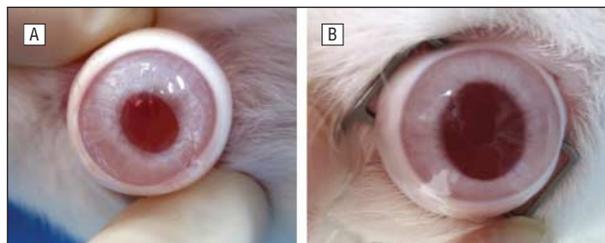
The expression of the keratocyte marker human ALDH and the corneal stroma-specific core protein human keratocan by h-ASCs was used to confirm differentiation into functional keratocytes. The presence of these proteins was studied in OCT sections from corneas 10 weeks after surgery. Expression of ALDH was detected in some of the CM-Dil-labeled human cells (**Figure 6B**) by fluorescence microscopy. Moreover, human keratocan was expressed by the transplanted human cells (**Figure 6C**). Human ALDH and keratocan protein could not be detected in the control corneas. As shown in **Figure 6B** and **C**, ALDH and keratocan expression was exclusively found in the CM-Dil-labeled transplanted human cells, and HyStem-HP scaffold seems to be a superior scaffold for transplanted cells as far as showing positive results in more sections (average, 7 of 9 sections in each eye).

## COMMENT

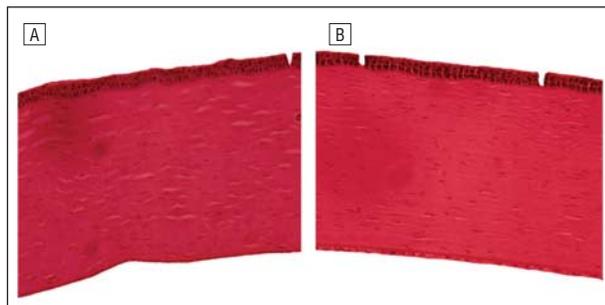
The corneal stroma contains several types of multipotent stem cells, such as bone marrow-derived<sup>13,14</sup> and neural crest-derived stem cells.<sup>15</sup> Previous studies have shown that it is possible to culture and differentiate these cells into keratocytes to produce full-thickness corneal



**Figure 1.** Human adipose-derived stem cells (h-ASCs) cultured in hydrogels (phase-contrast microscopy) and chloromethylbenzamido (CM-Dil) (fluorescent microscopy). A, HyStem-HP hydrogel (Glycosan BioSystem Inc). B, Extracel-SS hydrogel (Glycosan Biosystem Inc). C, Cell culture flasks (T150; Corning) labeled with CM-Dil for 14 days (original magnification  $\times 100$ ).



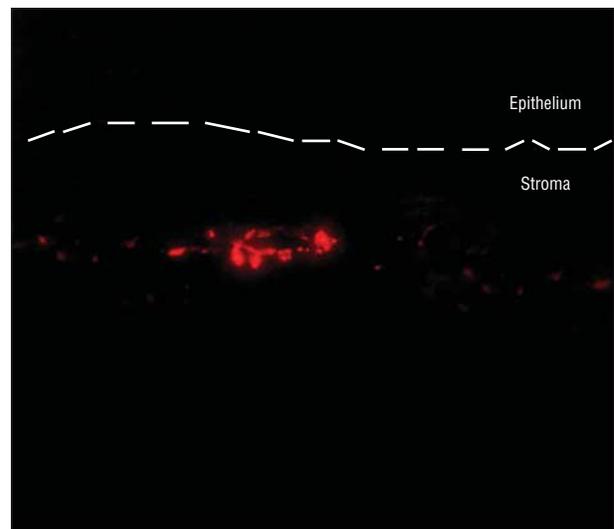
**Figure 2.** Clinical observation of cultured human adipose-derived stem cells (h-ASCs) at 10 weeks after eye operations. A, Negligible corneal opacity in an eye with flap formation and h-ASCs with solid HyStem-HP hydrogel (Glycosan BioSystem Inc) (rabbit 4, right eye). B, Corneal haziness (grade, 1+) in an eye with stromal pocket formation and h-ASCs with semisolid HyStem-HP hydrogel (Glycosan BioSystem Inc) (rabbit 9, left eye).



**Figure 3.** Hematoxylin-eosin staining of histological cornea sections reveal no differences between groups in inflammatory reaction (original magnification,  $\times 100$ ). A, Control cornea. B, Treated cornea with human adipose-derived stem cells on HyStem-HP hydrogel (Glycosan BioSystem Inc). Images are electronically enhanced.

stroma.<sup>16,17</sup> However, obtaining sufficient numbers of stromal stem cells by subculturing has been challenging. Moreover, keratocyte stem cells are not sufficiently numerous or easy to obtain, and their extraction requires several donor corneas.<sup>10</sup>

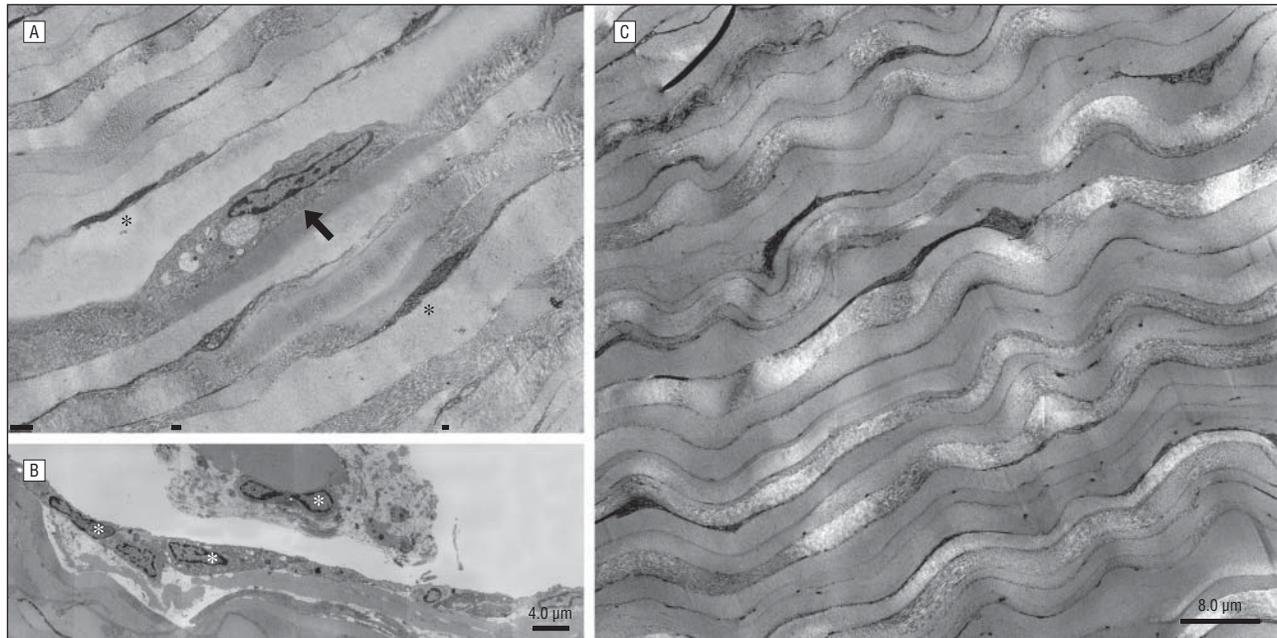
We selected h-ASCs as a cell source of keratocytes for several reasons. First, adipose tissue is readily harvested in relatively large quantities with minimal risk. In addition, the yield of mesenchymal stem cells from adipose tissue is 100- to 500-fold greater than that from bone marrow,<sup>7-9</sup> and the h-ASCs have immunosuppressive properties that are similar to bone marrow-derived mesenchymal stem cells, as previously described.<sup>18-20</sup> Furthermore, Arnalich-Montiel et al<sup>10</sup> demonstrated differentiation of h-ASCs in rabbit corneal stroma, and Du et al<sup>11</sup> showed that h-ASCs



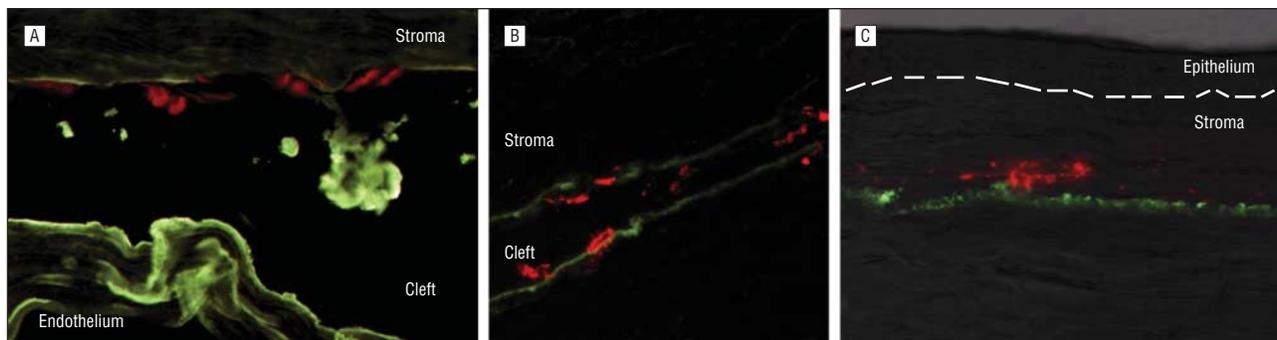
**Figure 4.** Fluorescent chloromethylbenzamido-labeled human adipose-derived stem cells at the interface before immunostaining (original magnification  $\times 200$ ). Image is electronically enhanced.

can differentiate to keratocytes in vitro. In contrast to the study by Arnalich-Montiel et al,<sup>10</sup> we evaluated several hydrogels that had the composition and compliance of the native extracellular matrix.<sup>21-24</sup> These physiological sECMs provide support and guidance for stem cell growth and development.<sup>16,17,21-33</sup> As prior experiments showed, the scaffolds used in the bioengineered corneas may consist of collagen, collagenlike material,<sup>17,25,28,33,34</sup> or glycosaminoglycan derivatives.<sup>21-24,35-37</sup> We investigated survival and differentiation of the h-ASCs in damaged corneas by encapsulation in a biodegradable, biocompatible, cross-linked HA-derived sECM biomaterial. This sECM contains a thiol-modified derivative of HA<sup>38-44</sup> and is cross-linked with poly(ethyleneglycol) diacrylate. This material was chosen to accelerate partial-thickness corneal repair. Hyaluronic acid is an unsulfated glycosaminoglycan and the primary glycosaminoglycan component in the interfibrillar space of a cross-linked collagen matrix in the cornea. Hyaluronic acid contributes to the natural arrangement and microarchitecture of the natural corneal matrix.<sup>45</sup>

Based on the results of our in vitro cell culture experiments, we decided to culture the stem cells on cell culture flasks and subsequently transfer the cells suspended in the sECM hydrogels or in Healon just before



**Figure 5.** Transmission electron microscopy examination of treatment group. A, Spindle-shaped, larger human adipose-derived stem cells (h-ASCs) (black arrow) relative to thinner natural keratocyte (black stars) in the interface (original magnification  $\times 2500$ ). B, The h-ASCs (white stars) in the cleft (original magnification  $\times 5000$ ). C, Normal corneal stromal structure beyond the interface and cleft (original magnification  $\times 2000$ ). Images are electronically enhanced.



**Figure 6.** Immunostaining under fluorescence and phase-contrast microscopy. A, Staining for type I collagen and fluorescence microscopy reveal collagen expression in host rabbit corneal stroma and in the cleft created where the human adipose-derived stem cells (h-ASCs; labeled with chloromethylbenzamide [CM-Dil]) were enclosed by the HyStem-HP scaffold. B, Immunostaining for human aldehyde dehydrogenase detected in CM-Dil-labeled human cells by fluorescence microscopy. C, Immunostaining for keratocan by fluorescence and phase-contrast microscopy. Aldehyde dehydrogenase and keratocan are expressed only next to transplanted h-ASC cells (labeled with CM-Dil) and HyStem-HP scaffold in the cleft (shown in B) or interface (shown in C) (original magnification  $\times 200$ ). Images are electronically enhanced.

insertion into the cornea. We observed that the h-ASCs grown on the hydrogel sECMs survived; furthermore, among the hydrogels, HyStem-HP was the best environment for h-ASCs in the cornea. Quantitative measurement of the number of surviving cells was very difficult because tissue cuts were variable in different eyes and also the cells could be lost during surgery, follow-up procedures, and tissue processing.

When h-ASCs combined with hydrogel scaffolds were transplanted into the corneal stroma, they survived as long as 10 weeks and differentiated into functional keratocytes, as assessed by the expression of the cornea-specific proteins, keratocan, and ALDH. However, we observed mild to moderate haze in the treated corneas, particularly in the stromal pocket group, regardless of the type of hydrogel scaffold used. This finding could be owing in part to surgical trauma and dissection effect. Furthermore, h-ASCs that were transferred with a hy-

drogel scaffold, particularly HyStem-HP, survived better and differentiated into keratocytes more than injections of h-ASCs in HBSS as the vehicle.

We observed that h-ASCs did not migrate from the flap interface or the stromal pocket where the cells had been placed. However, a longer observation period is needed to characterize the differentiation and construction of a normal, well-organized corneal stroma.

Although immune-competent animals and xenograft were used in this study, the human-derived cells did not generate an immune response in the rabbit corneal stroma. This can be explained by the immunomodulatory properties of ASCs<sup>18-20</sup> in addition to immune privilege characteristic of the cornea.

Based on our result and those of Arnalich-Montiel et al<sup>10</sup> and Du and coworkers,<sup>11</sup> h-ASCs can be used as a cell source of keratocytes. Human ASCs might be able to repopulate keratocytes and regenerate extracellular matrix-like ma-

terial in situations where the stroma has been compromised, such as ecstastic diseases, trauma, infectious keratitis, or immune-related keratitis. The intrinsic immunomodulatory properties of h-ASCs make them particularly appropriate for inflammatory and immunologic diseases.

These results suggest that stem cell therapy of the cornea shows considerable potential for clinical translation. Moreover, one can conceive of the use of genetically manipulated stem cells for corneal diseases such as corneal dystrophies. In addition, sECM-delivered stem cells could be used in combination with a drug delivery system to treat infectious keratitis or immune-related keratolysis.

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