Corneal Reconstruction with Tissue-Engineered Cell Sheets Composed of Human Immature Dental Pulp Stem Cells

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Purpose. To determine the outcome of the use of a tissue-engineered cell sheet composed of human undifferentiated immature dental pulp stem cells (hIDPSC) for ocular surface reconstruction in an animal model of total limbal stem cell deficiency (LSCD).

Methods. LSCD was induced by the application of 0.5 M NaOH to the right eye of rabbits for 25 seconds (mild chemical burn [MCB]) and for 45 seconds (severe chemical burn [SCB]). After 1 month, a superficial keratectomy was performed to remove the fibrovascular pannus that covered the animals' burned corneas. A tissue-engineered hIDPSC sheet was transplanted onto the corneal bed and then covered with deepithelialized human amniotic membrane (AM). In the respective control groups, the denuded cornea was covered with AM only. After 3 months, a detailed analysis of the rabbit eyes was performed with regard to clinical aspect, histology, electron microscopy, and immunohistochemistry.

Results. Corneal transparency of the rabbit eyes that underwent hIDPSC transplantation was improved throughout the follow-up, while the control corneas developed total conjunctivalization and opacification. Rabbits from the MCB group showed clearer corneas with less neovascularization. The clinical data were confirmed by histologic analysis that showed healthy uniform corneal epithelium, especially in the MCB group. The presence of hIDPSC was detected using an anti-hIDPSC antibody. The corneal tissue also showed positive immunostaining with anti-human antibodies. In the control corneas, none of these antigens were detected.

Conclusions. Overall, these data showed that transplantation of a tissue-engineered hIDPSC sheet was successful for the reconstruction of corneal epithelium in an animal model of LSCD. (*Invest Ophthalmol Vis Sci.* 2010;51:1408–1414) DOI: 10.1167/iovs.09-4029

Total limbal stem cell deficiency (LSCD) is caused by a variety of conditions, such as primary genetic disorders (aniridia, multiple endocrine deficiency, erythrokeratodermia), chemical and thermal injury, irradiation, inflammatory diseases (Stevens-Johnson syndrome, ocular cicatricial pemphigoid, and infectious keratitis), contact lens–induced keratopathy, and iatrogenic multiple ocular surgeries.[1] It often results in persistent corneal epithelial defect or abnormal reepithelialization by conjunctival epithelial cells, which predisposes to corneal neovascularization and opacification.[2]

When LSCD is unilateral, limbal cell transplantation from the healthy contralateral eye can restore normal corneal epithelium and decrease neovascularization and inflammation, leading to improvement of corneal transparency.[3] This procedure cannot be applied to patients with bilateral LSCD. In this case, the transplant is obtained from a living related or cadaveric eye. The success of such grafts is limited by the cell delivery system, by microenvironment factors as found in severe dry eyes, and by immunologic rejection of the allogenic cell transplant.[4]

Advances in tissue culture and bioengineering have allowed the ex vivo expansion of limbal epithelial cells using different scaffolds, including amniotic membrane, fibrin gel, and collagen contact lenses. Functional reconstruction of cornea in LSCD by limbal epithelial autograft transplantation has been successfully achieved.[5] Encouraging outcome has been reported with ex vivo allogenic limbal stem cell (LSC) transplantation in LSCD eyes.[6] However, the problem of graft rejection needs to be overcome.[7]

In an attempt to prevent rejection, autologous oral mucosal epithelial cell transplantation has been proposed for the treatment of LSCD.[8] Nishida et al.[9] used an elegant technique of epithelial cell cultivation on the temperature-responsive polymer poly(N-isopropylacrylamide), which facilitates cell adhesion and growth in normal culture conditions at 37°C. After reducing the temperature below 30°C, there is complete detachment of adherent cells without the use of typical proteolytic enzymes.[9,10]

During the last years, stem cells have been tested in the treatment of LSCD.[11] Searching for a source of stem cells that have characteristics similar to those of limbal stem cells (LSC), we reported on the isolation and differentiation of stem cells

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from human deciduous teeth (hIDPSC).[12] These cells exhibit all characteristics of multipotent adult stem cells, expressing mesenchymal stem cell (MSC) and several human embryonic stem (ES) cell markers. Additionally, hIDPSC have a normal karyotype and show the capacity for multilineage differentiation into neurons, smooth and skeletal muscle, cartilage, bone, and other cell types in vitro and in vivo.[12–15] We also verified LSC gene expression profile in undifferentiated hIDPSC. Surprisingly, these undifferentiated cells continuously expressed markers of LSC such as ABCG2, ß1-integrin, vimentin, p63, connexin 43, and keratin 12, but were negative for the corneal cell marker keratin K3 when cultured in vitro.[16] The relative ease of recovery, the ex vivo expansion, and the continuous expression of LSC markers warrant further exploration of the use of hIDPSC in clinical ocular therapies.

To provide a new source of limbal epithelial stem cells, we aimed to determine the outcome of hIDPSC transplantation for ocular surface reconstruction in an animal model of LSCD after chemical injury.

METHODS

Cell culture

Human IDPSC, (2n = 46, XX) were isolated from dental pulp of deciduous teeth and previously characterized and cultured as described by Kerkis et al.[12] hIDPSC were maintained in Dulbecco's-modified Eagle's medium (DMEM)/Ham's F12 (1:1; Invitrogen, Carlsbad, CA), supplemented with 15% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 units/mL penicillin (Gibco, Grand Island, NY), 100 μg/mL streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 2 mM nonessential amino acids (Gibco). The culture medium was changed daily, and the cells were replaced every 3 days. After they reached 80% confluence, they were washed twice in sterile phosphate-buffered saline (PBS; Gibco; 0.01 M, pH 7.4), enzymatically treated with 0.25% trypsin/EDTA (Invitrogen), and seeded onto 25-cm^2 plastic flasks (Corning, New York, NY). All cultures were incubated at 37°C in 5% CO_2 in a high humidity environment. All experiments were performed using cells at the same early passages (P6 and P7).

Preparation of hIDPSC for Transplantation

Three days before the surgery, hIDPSC (Fig. 1A) were seeded directly, without any feeder cell layer, at a density of 2×10^6 cells per temperature-responsive cell culture dish (35-mm diameter; Cell Seed Inc., Tokyo, Japan), which were kindly donated by Masayuki Yamato of Tokyo Women's Medical University.[17,18] The cells were maintained for 3 days in basal medium, which was changed daily, at 37°C in 5% CO_2 in a high humidity environment until they reached confluence and formed a cell sheet (Fig. 1B). On the day of transplantation, the viable cell sheet was harvested by reducing the temperature of the culture to 20°C for 30 minutes following a previously described protocol[7] (Figs. 1C, 1D).

Animal LSCD Model

All procedures were performed according to the Institutional Ethics Committee requirements (Butantan Institute; protocol number 06/205) and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male New Zealand White rabbits, weighing 2 to 2.5 kg, were anesthetized with an intramuscular injection of ketamine (10 mg/kg) and xylazine (2 mg/kg) and topical proparacaine. LSCD were induced by chemical burn. NaOH, 0.5 M, was applied to the right eye of male rabbits for 25 seconds (mild chemical burn [MCB]) and for 45 seconds (severe chemical burn [SCB]). After injury, the eye was irrigated with PBS. Dexamethasone-gentamicin solution was applied to each treated eye twice daily for 7 days, and pain was managed with midazolan (0.3 mg/kg) once a day for 1 week.

Transplantation of hIDPSC

One month after injury, a 360° peritomy followed by a superficial keratectomy was performed under general anesthesia to remove the fibrovascular pannus covering the animals' burned corneas (Fig. 1E). Subsequently, the harvested sheet of hIDPSC was placed directly onto the exposed transparent stromal bed as described previously[9,17–19] (Figs. 1F, 1G). Afterward, it was covered with a patch of acellular human amniotic membrane (AM), which was sutured using 10.0 nylon with the epithelial side down to the episclera to avoid casual damage by the animal (Fig. 1H). In control animals, the denuded cornea was covered only with AM.[20,21] To prevent pain that can be caused by the surgery, the animals were given a subcutaneous injection of buprenorphine (0.05 mg/kg) once a day for 1 week. No other drugs were used, allowing the evaluation of cell contribution without interference by other factors. It was not necessary to use an immunosupressor because these cells are not rejected by the animal.[13,14] After 3 months, the animals were killed according to a protocol approved by the Butantan Institute, and their corneas were collected and processed for further analysis.

Histology

For histologic analysis, the corneal tissues were fixed in 4% paraformaldehyde overnight and embedded in paraffin (Sigma Aldrich, St. Louis, MO) in accordance with Du et al.[7] The cornea specimens were then sectioned (5 μm), and slides were prepared. The paraffin was removed from slides with xylene treatment; the samples were rehydrated with an ethanol series and stained with hematoxylin and eosin. The histologic images were captured (Axio Imager A1 and AxioCAM MRc5; Carl Zeiss, Jena, Germany) and processed with commercial software (AxioVision; Carl Zeiss).

Antibodies

Mouse anti-human cell surface monoclonal antibodies used were: anti-$ß_1$-integrin and anti-K12 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ABCG2 (Chemicon, Temecula, CA), anti-vimentin (NeoMarkers, Fremont, CA), and cytokeratin 18 (CK 18; Sigma). Other monoclonal antibodies used were mouse anti-cytokeratin 3 (K3), which reacts with human and rabbit, mouse anti-human p63 (Chemicon). Mouse anti-hIDPSC antibody was obtained as described previously[12] and successfully used by us in previous studies.[14–16]

Immunohistochemical Analysis

The corneal tissue samples were obtained from experimental animals that received transplants of hIDPSC and of AM, were fixed with 4% paraformaldehyde (Sigma), and embedded (Tissue-Tek OCT [optimal cutting temperature]; Sakura, Torrance, CA). Frozen sections (5 μm) were obtained using a cryomicrotome (Cryostat CM1100; Leica, Wetzlar, Germany) and placed on poly-L-lysine-coated slides, which were incubated in cold methanol (Sigma) for 15 minutes to decrease tissue autofluorescence. Afterward, they were washed three times in rinse buffer (TBS) containing: 20 mM Tris-HCl (Vetec, Duque de Caxias, Brazil), pH 7.4; 0.15 M NaCl (Dinâmica Reagent, Sao Paulo, Brazil); and 0.05% Tween-20 (Sigma). Permeabilization was performed using 0.1% Triton X-100 (Santa Cruz Biotechnology) for 30 minutes. The slides were washed three times and incubated for 1 hour with 5% bovine serum albumin (BSA; Sigma) in PBS (Gibco), pH 7.4. Primary antibodies were diluted in PBS and the slides were incubated for 1 hour at indicated dilution: anti–β1-integrin, anti-CK18, anti-K3, anti-K12, anti-vimentin, anti-ABCG2 (1:100), anti-p63 (1:200), and anti-hIDPSC (1:500) at room temperature. After washing in TBS (three times), the slides were incubated in the dark for 1 hour with secondary anti-mouse antibody conjugated with fluorescein isothiocyanate (FITC) or cianine dye 3 (Cy3) both at a dilution of 1:500. The same concentration of corresponding normal non-specific IgG provided the negative control. Frozen sections obtained from rabbits that received only AM and native rabbit corneal tissue were also used as controls. After washing with

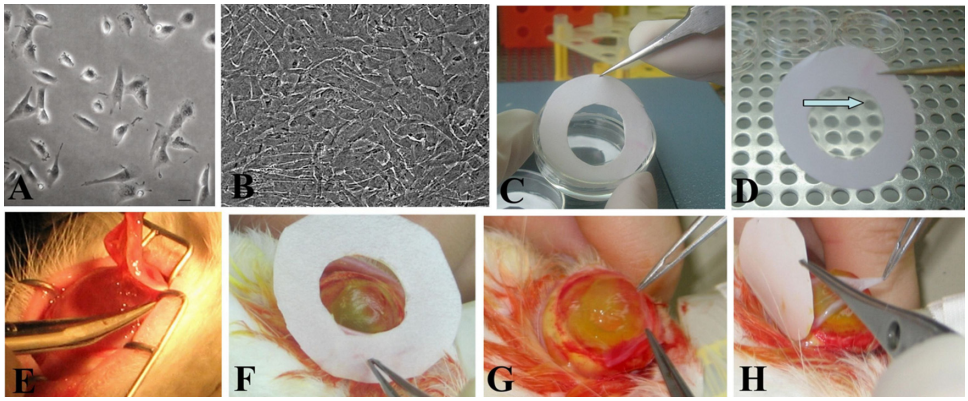

FIGURE 1. Transplantation of tissue-engineered cell sheet made from undifferentiated hIDPSC. (**A**) Morphology of hIDPSC (phase contrast [PC]). (**B**) Three days after reaching confluence, cells form a cell sheet (PC). (**C**) The viable cell sheet is harvested by reducing the temperature to 20°C for 30 minutes. (**D**) Sheet of tissue-engineered hIDPSC (*arrow*) is harvested with the use of a doughnut-shaped supporter (*white*). (**E**) Conjunctival tissue over the cornea is surgically removed to expose transparent corneal stroma. (**F**) Sheet of tissue-engineered hIDPSC is placed on the stromal bed. (**G**) The sheet adheres to the corneal stroma and the supporter is removed. (**H**) Amniotic membrane is placed over cell sheet and sutured. Scale bar (**A**, **B**) = 5 μm.

PBS, microscope slides were mounted in antifade solution (Vectashield mounting medium; Vector Laboratories, Hercules, CA) with 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) and analyzed by fluorescent microscopy (Axio Imager A1; Carl Zeiss) or confocal microscopy (LSM 510 META; Carl Zeiss). An argon ion laser set at 488 nm for FITC (Chemicon) and at 536 nm for Cy3 (Chemicon) excitation was used. The emitted light was filtered with a 505 nm (FITC) and 617 nm (Cy3) long pass filter in a laser scanning microscope.

Transmission Electron Microscopy

The corneal tissue samples obtained from normal control and experimental animals that received transplants of hIDPSC were washed twice in PBS solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The samples were washed three times in PBS and post-fixed for 15 minutes in 1% osmium tetroxide in PBS. They were then dehydrated in ethanol and embedded in Araldite resin. Ultrathin sections were made (Leica Ultracut; Leica, Wetzlar, Germany), stained with lead and uranyl acetate, and observed with a transmission electron microscope.

RESULTS

Clinical Results of IDPSC Transplantation in Animal Models of LSCD

After chemical injury, animals' eyes from the MCB group (n = 8) developed LSCD with corneal neovascularization and opacification (Figs. 2A1–E1), which was less intense when compared with those from the SCB group (n = 7; Figs. 2F1–I1). After 1 month, five animals from group MCB and four from group SCB were randomized to be transplanted with hIDPSC and deepithelialized AM. Three animals from each group were randomized to receive only AM and constituted the control groups for both MCB and SCB (Figs. 2J1–K1). Three months after transplantation, corneal transparency of eyes that underwent hIDPSC transplantation was clinically improved throughout the follow-up. Rabbits from the MCB group showed much clearer corneas with less neovascularization (Figs. 2A2–E2) than those from the SCB group (Figs. 2F2–I2). The control animals with MCB and SCB, which received only AM, developed total conjunctivalization and opacification (Figs. 2J1, 2K1), which worsened after 3 months (Figs. 2J2, 2K2). The results were consistent for all animals in each group.

Histologic Analysis of the Corneal Surface

Histologic analysis of the enucleated eyes of MCB animals performed 3 months after hIDPSC transplantation showed

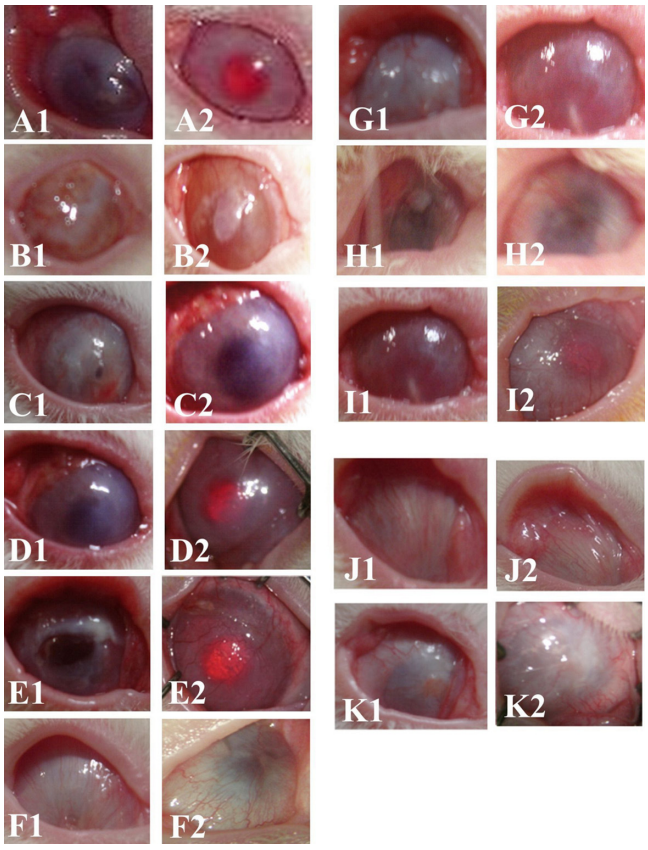

FIGURE 2. Representative figures of rabbit eyes one month after ocular surface damage and three months after transplantation of a tissue-engineered hIDPSC sheet. (**A1–E1**) and (**A2–E2**) show the eyes of MCB rabbits after chemical burn and cell sheet transplantation. (**F1–I1**) and (**F2–I2**) show the eyes of SCB rabbits after chemical burn and cell sheet transplantation. (**J1–K1**) and (**J2–K2**) show the eyes of rabbits after chemical burn and AM transplantation.

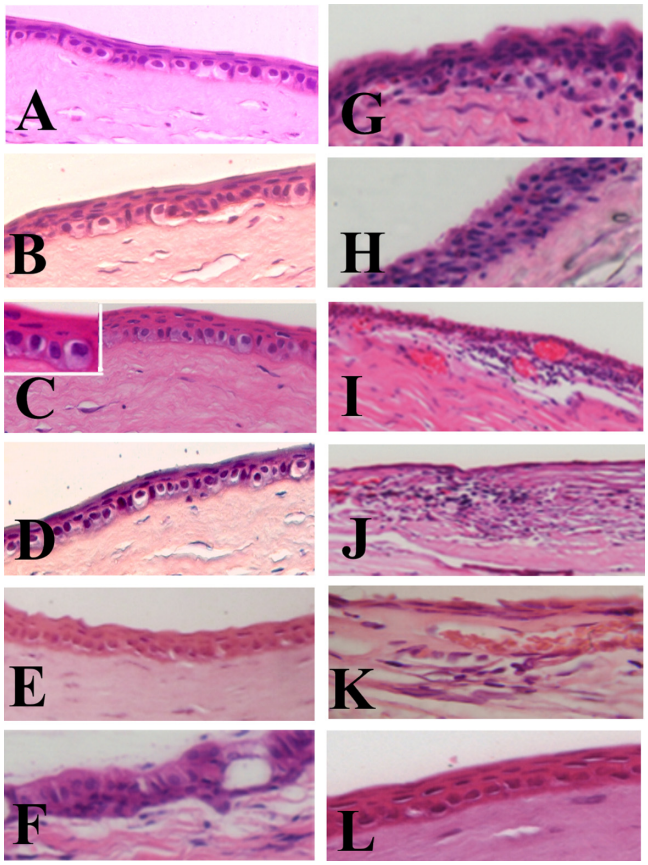

FIGURE 3. Histologic analysis of corneal epithelium obtained after transplantation of a tissue-engineered hIDPSC sheet (representative figures). Rabbit eyes from the MCB (**A–E**) and SCB (**F–I**) groups. *Inset* in (**C**) shows cuboidal epithelial cells at higher magnification. (**J**, **K**) Rabbits that received only amniotic membrane. (**L**) Corneal epithelium of a normal rabbit. The specimens were stained with hematoxylin and eosin. Light microscopy. (**A–H**, **K**, **L**) 40× magnification; (**I**, **J**) 20× magnification.

well-organized corneal epithelium with three to five cell layers consisting of small cuboidal basal cells, intermediate flattened cells, and polygonal and more flattened surface cells, with the corneal stroma structure preserved (Figs. 3A–E). In the SCB group, three eyes showed a less organized and loose corneal epithelium with inflammatory cells within the subepithelial and stromal layers (Figs. 3F–H), and one animal showed a very thin corneal epithelium and superficial neovascularization (Fig. 3I). All control eyes, which received AM only, demonstrated a disorganized conjunctivalized corneal epithelium, presence of inflammatory cells within the stroma, and neovascularization (Figs. 3J, 3K). Corneal epithelium and stromal layers from normal non-injured rabbit eyes showed morphology similar to that observed in the MCB group of animals (Figs. 3LA–E).

Ultrastructural Features of the Corneal Surface

Undifferentiated hIDPSC (Fig. 4A), the corneal epithelium from normal, SCB, and MCB eyes of rabbits (Figs. 4B–H), and the stromal layer from MCB eyes (Figs. 4I, 4J), were analyzed by transmission electron microscopy (TEM). Undifferentiated hIDPSC presented large nucleus and cytoplasm poor in organelles (Fig. 4A). The eyes of the SCB group, after cell transplantation, formed a multilayer epithelium, mainly composed of flattened cells and a loose stroma with fibroblast-like cells (Fig. 4C). The eyes of the MCB group displayed an architecture of healthy, multilayered and well-stratified epithelium overlying a compact stroma (Figs. 4D, 4E). We also observed in the corneas that received the hIDPSC sheets the presence of microstructures of native epithelial cells, such as microvilli, hemidesmosomes, and desmosomes (Figs. 4F–H). Stromal layer was well-organized in the eyes of MCB animals (Figs. 4I, 4J).

Reconstructed Rabbit Corneas Were of Human Origin

Three months after transplantation of cell sheets, the presence of hIDPSC was confirmed by positive staining with anti-hIDPSC antibody in both MCB and SCB rabbit eyes (Fig. 5A). MCB eyes showed positive immunostaining with anti-K3 antibody, which was stronger in suprabasal corneal epithelium (Fig. 5B). Other human anti-epithelial stem cell antibodies, such as anti-ß$_1$-integrin, anti-p63, and anti-ABCG2, demonstrated positive immunostaining for the corneal epithelial basal cells (Figs. 5C, 5D–F, 5G). As expected, epithelial cells were also positive for anti-K12 antibody (Fig. 5H). In contrast to MCB eyes, SCB reconstructed eyes showed positive immunostaining with anti-ß$_1$-integrin antibody in suprabasal corneal-like epithelium (Fig. 5I). SCB eyes also demonstrated positive reactivity with anti-CK18 antibody in both suprabasal and basal layers of corneal-like epithelium and in several stromal cells (Fig. 5J), but were negative to anti-K3, anti-ABCG2, and anti-p63 antibodies (data not shown) Control animals presented a thin layer of epithelial cells on the corneal surface, which had a similar pattern of immunolabeling with SCB eyes of rabbits (data not shown). Corneas of eyes of normal rabbits did not show any reactivity with anti-human ß$_1$-integrin, anti-CK18, and anti-p63 antibodies (data not shown). As expected, they were positive with anti-K3 antibody (Fig. 5K).

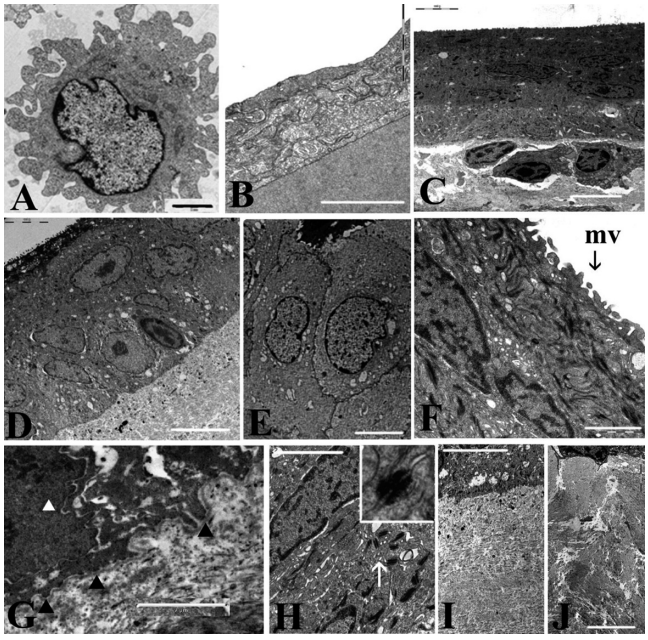

FIGURE 4. Representative figure of the corneal surface three months after hIDPSC transplantation (TEM). (**A**) Undifferentiated hIDPSC. (**B**) Cornea of a normal rabbit. Corneas of rabbits from the SCB (**C**) and MCB (**D–F**) groups. In (**C**), multilayer epithelium and fibroblast-like cells can be observed in the corneal stroma. Formation of corneal epithelium in a rabbit from MCB group with cuboidal cells (**D**, **E**), microvilli (mv; **F**), hemidesmosomes (*black arrowhead*; **G**), and desmosomes (**H**, *white arrows*; *Inset*: higher magnification), as well as the presence of a well-organized stromal layer (**I**), is shown. (**J**) Stromal layer of cornea from control animal, which received AM only. Scale bars: (**A**, **B**, **E**, **F**) 2 μm; (**C**, **D**, **I**, **J**) 5 μm; (**G**, **H**) 1 μm.

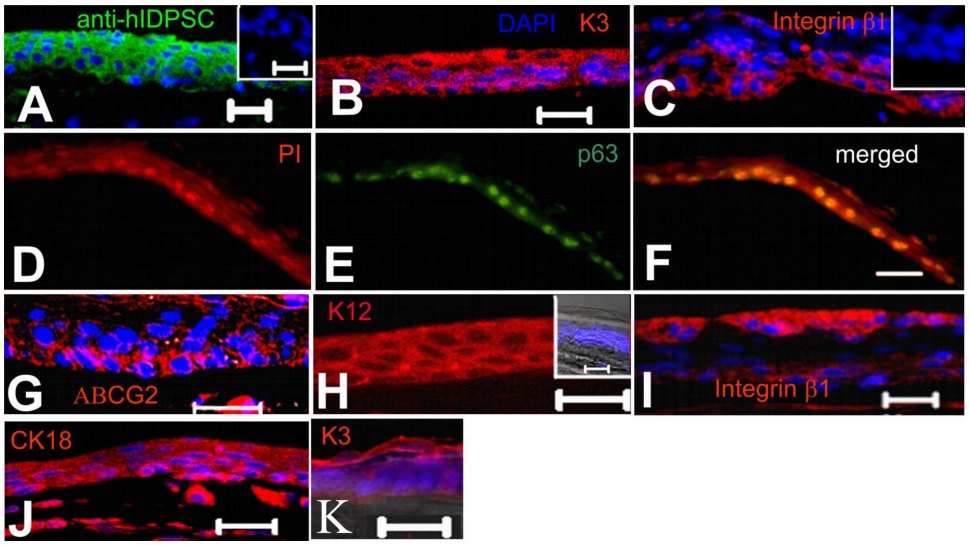

FIGURE 5. Representative figure of rabbit eyes with LSCD three months after hIDPSC transplantation (confocal microscopy). (**A–H**) MCB 3 months after the transplantation of hIDPSC sheets: (**A**) Positive anti-hIDPSC antibody immunostaining (*green*); *inset* shows negative control: rabbit cornea that does not react with this antibody. (**B**) Anti-K3 antibody showed positive immunostaining, which was stronger in the suprabasal layers and weaker in the basal layers of the corneal epithelium (*red*). (**C**) Positive immunostaining with human anti-ß$_1$-integrin antibody (*red*) observed in basal layer of corneas. *Inset* shows control for secondary antibody. (**D–F**) Positive immunostaining with human anti-p63 antibody: (**D**) nucleus stained with PI (*red*), (**E**) nuclear localization of anti-p63 antibody (*green*), (**F**) overlapping images of (**D**) and (**E**) (*yellow*). (**G**) Positive immunostaining with human anti-ABCG2 antibody observed in basal layer. (**H**) Positive immunostaining with anti-K12 antibody. *Inset* shows negative control. (**I–J**) SCB group 3 months after the transplantation of hIDPSC sheets: (**I**) Positive immunostaining with anti-ß$_1$-integrin antibody in the corneal epithelium: stronger in suprabasal layers and weaker in basal layers. (**J**) Positive immunostaining with anti-CK18 antibody in corneal epithelium and also in several stromal cells. (**K**) As expected, the corneas of the eyes of normal rabbits were positive with anti-K3 antibody. Scale bars: 20 μm.

DISCUSSION

During the last years, multipotent MSC isolated from different tissues of an adult organism have been shown to be a promising new source for use in the regeneration of damaged tissues and organs.[22–26] It has also been reported that they are immunoprivileged as well as immunosuppressive.[27–29] Hence, these cells are not rejected by an immunologically unmatched recipient and may represent the ideal candidates for transplantation. MSC isolated from sources such as human bone marrow have already been used to reconstruct the ocular surface of an animal model of LSCD induced by chemical burn. Although these cells were detected in the grafted area after transplantation, their differentiation into corneal epithelium was not confirmed by the authors.[11]

We reported on the isolation and characterization of another type of MSC, the hIDPSC from human dental pulp of deciduous teeth.[12] We showed that these cells were multipotent and capable of contributing to the reconstruction of large cranial defects produced in non-immunosuppressed rats after their transplantation onto collagen membrane, as well as the contribution of these cells to chimeric myofibers in golden retriever muscular dystrophy (GRMD) dogs without demonstrating any graft rejection.[13,14] Other authors suggest that benefits observed after transplantation of dental pulp stem cells (isolated from the teeth of adult patients) was due to secretion of paracrine factors by these cells.[30]

In addition, we showed that hIDPSC during in vitro culture express markers of LSC.[16] Based on these findings, we suggested that such cells could be a promising candidate to be used for corneal epithelial reconstruction to avoid the problems of graft rejection. Since these cells are isolated from deciduous teeth, they can be cryopreserved and used as an autologous source of cells in case of corneal damage during the patient's life or can be transplanted in related and unrelated patients.

In the present study, we demonstrated for the first time that tissue-engineered cell sheets of hIDPSC can effectively substitute for allografts of limbal tissues in the reconstruction of the corneal and limbal surfaces in an animal model of LSCD. The rabbits' eyes that were treated with this approach displayed improvement of corneal transparency in both experimental groups with MCB and SCB. During the follow-up period, the MCB group maintained well-transparent corneas, while the SCB group showed less evident improvement of corneal transparency. Also, we did not observe serious complications during the 3 months of postoperative follow-up. Histologic and TEM analyses confirmed these findings, demonstrating significant differences in the two types of chemical injury between transplanted eyes and eyes that received only AM. Only corneas of animals from the MCB group showed well-differentiated multilayered corneal epithelium with adequate hemidesmosome density and stromal architecture similar to that of native human corneas. On the other hand, the SCB group that received hIDPSC formed a multilayered, less organized, and flattened epithelium with the presence of inflammatory cells and neovascularization. Probably the severity of the chemical injury in the SCB group and the resultant change in the microenvironment of the ocular surface with more tissue destruction and inflammation interfered with hIDPSC survival and their capacity for regenerating the organized and transparent corneal epithelium.

Following the same reasoning, the corneal reconstructed surface of the MCB and SCB groups were composed of hIDPSC that showed a different pattern of expression of epithelial markers after transplantation. According to morphologic observations, reconstructed corneas of the MCB group stained strongly positive for cytokeratin K3, indicating the formation of functional corneal epithelium. In addition, they appropriately expressed ABCG2 and ß$_1$-integrin, which are considered epithelial stem cell and progenitor cell markers, and p63, a putative epithelial stem-cell marker, all localized in the basal layer in the multilayered corneal epithelium.[31,32] In contrast, the expression of cytokeratin K3 and p63 was not observed in the corneal epithelium of the SCB group. ß$_1$-integrin–positive cells were also found in the corneal epithelium of the SCB group; however, they were localized in the basal as well as in the suprabasal layers, suggesting the presence of undifferentiated cells. In both MCB and SCB groups, corneal epithelium was positive for cytokeratin CK18 which is considered a marker of epithelial cells.[33] According to Elder et al.,[33] differ-

entiated human corneal epithelium showed positive staining for K3 and negative for K18. However, other authors reported a different distribution of cytokeratins in definite areas of human corneal epithelium.[34] The expression of CK18 with the other epithelial cell markers in the MCB group suggests that the process of corneal epithelial reconstruction was not completed in 3 months and that additional time may be needed to form a more mature corneal epithelium.

We also showed that the method previously used in the preparation of tissue-engineered cell sheets composed of autologous oral mucosal epithelium can be successfully adopted for hIDPSC.[9] However, Nishida's multilayered cell sheets can be made only in the presence of mouse 3T3 cells, and, consequently, animal material is transferred to the patients. Our culture system avoids using 3T3 cells. The other advantage of our system consists of the use of undifferentiated cells, which may provide a better source for corneal epithelial regeneration for a longer period.

Deepithelialized human AM was used to fix the cell sheets in the present work. In the future, in experiments involving human beings, AM can be substituted with contact lenses.[9]

In the SCB group, the animals' eyes showed a less organized stromal layer with inflammatory cells. Interestingly, we detected the presence of hIDPSC in the corneal stroma. A similar finding of limbal epithelial progenitor cells invading the limbal stroma was described by Kawakita et al.,[35] when rabbit limbal explants were cultured on a collagen-coated substrate, and by Li et al.,[36] during human limbal explant culture on intact amniotic membrane. Double staining results of p63(+)/pancytokeratin(−) and p63(+)/vimentin(+) in the limbal stroma, which increases during serial passages, suggest that such invading cells not only lose their epithelial progenitor status but also undergo epithelium–mesenchymal transition into fibroblasts.[36]

Overall, we showed that the transplantation of tissue-engineered hIDPSC sheets was successful for the reconstruction of corneal epithelium in the MCB animal model of LSCD. It seems that, in the SCB model, additional procedures may be required to determine whether this method could be used in severely damaged eyes. Based on the results of our research, we believe that transplantation of tissue-engineered hIDPSC sheets represents a valid alternative for ocular surface reconstruction in cases of bilateral LSCD and provides a new perspective in the field with important clinical implications.

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