Induction of Epithelial Progenitors In Vitro from Mouse Embryonic Stem Cells and Application for Reconstruction of Damaged Cornea in Mice

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Purpose. Severe ocular surface diseases and injuries cause loss of the corneal limbal epithelium, leading to re-epithelialization by bulbar conjunctival cells, resulting in vascularization of the cornea, conjunctival scarring, and loss of visual acuity. In this study, the optimal culture condition for induction of differentiation of epithelial progenitor cells from embryonic stem (ES) cells was determined for use in transplantation to damaged cornea in mice.

Methods. Mouse ES cells were cultured on Petri dishes coated with several extracellular matrix proteins, and the markers for epithelial cells were analyzed with RT-PCR and Western blot analysis. The optimal condition for induction of epithelial progenitor cells was determined, and the progenitors were transplanted onto mouse eyes with corneal epithelia that had been damaged by exposure to *n*-heptanol.

Results. Epithelial progenitors were successfully induced by culturing mouse ES cells on type IV collagen for 8 days. These progenitors expressed keratin (K)12, which is specific to corneal epithelial cells, and cell surface CD44 and E-cadherin, both of which are essential in corneal epithelial wound healing. Complete re-epithelialization of the corneal surface occurred within 24 hours after transplantation. The resultant corneal epithelial cells expressed markers of the grafted cells, and no teratomata were observed during the follow-up period.

Conclusions. Epithelial progenitors were successfully induced in vitro from ES cells and were applicable as grafts for treating corneal epithelial injury. ES cells may become an unlimited donor source of corneal epithelial cells for corneal transplantation and may restore useful vision in patients with a deficiency of limbal epithelial cells. This is an important first trial toward assessing the use of ES cells to reconstruct corneal epithelial cells. (*Invest Ophthalmol Vis Sci.* 2004;45: 4320–4326) DOI:10.1167/iovs.04-0044

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The corneal surface is covered by corneal epithelial cells that form the anterior ocular surface, together with limbal and conjunctival epithelial cells and tear film. Corneal epithelial cells are maintained by the centripetal migration of corneal epithelial stem cells, one of the region-specific epithelial progenitor cells that are also called limbal cells. The limbal cells play a role in the palisades of Vogt, located in the limbus—the narrow transitional zone of the ocular surface between the cornea and the bulbar conjunctiva.[1,2] In development, corneal epithelial stem cells originate from the surface ectoderm that supplies various kinds of region-specific epithelial progenitor cells. Embryonic stem (ES) cells are derived from pluripotent cells within preimplantation embryos and have the pluripotentiality to differentiate into ectoderm, mesoderm, or endoderm cells, assuming the form of any cell lineage, including epithelial progenitors.

Severe and widespread damage of the cornea in ocular surface diseases and injuries, such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid, severe microbial infection, and chemical or thermal burn, lead to loss of corneal and limbal epithelial cells. Limbal cell deficiency is manifested by chronic inflammation and vascularization, resulting in conjunctival scarring in the cornea and loss of visual acuity. The therapeutic strategies for these diseases involve two major approaches: one is the transplantation of the limbal graft taken from the healthy contralateral eye (limbal allograft),[3–5] and the other is regeneration of sheets of corneal epithelium in vitro and their transplantation in vivo.[6–17] Recently, limbal cells obtained by biopsy were cultured in the appropriate condition, or on the amniotic membrane to induce differentiation into corneal epithelial cells for transplantation to the damaged cornea.[7–17] More recently, successful culture and autologous transplantation of oral mucosal epithelial cells on the amniotic membrane has been reported in rabbit.[18] It should be noted that transplantation of corneal epithelial cells requires allogeneic donors and carries a risk of immunologic rejection. Although the successful reconstruction of the cornea by transplantation of autologous limbal epithelial cells has been reported,[3,8,11] such a procedure is impossible in severe ocular surface diseases such as Stevens-Johnson syndrome, because the effects of the diseases are usually bilateral, and often the oral mucosa is damaged.

In this study, we report a new strategy for generating corneal epithelial cells from mouse ES cells in vitro and successful reconstruction of damaged corneas by transplantation of the ES-cell–derived epithelial progenitor cells. This is one of the first steps toward using ES cells to reconstruct the corneal epithelium.

Materials and Methods

Cell Culture and Induction of ES Cell Differentiation

Undifferentiated ES cells (R-CMTI-1A; passages 12–18) were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). They originated

from a 129SV/EVJ mouse and had a normal karyotype and characteristics of prevalent undifferentiated ES cells, including the expression of alkaline phosphatase and OCT-4, a transcription factor essential for pluripotency. The cells were maintained in the presence of mitomycin-C–treated mouse fetal fibroblasts on gelatin-coated dishes.[19,20] The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1× nonessential amino acids, 1× pyruvate, 15% fetal calf serum (FCS), and 1000 U/mL recombinant mouse leukemia inhibitory factor (LIF; Invitrogen-Gibco, Grand Island, NY), to maintain the undifferentiated state of the ES cells. For induction of epithelial cell differentiation, ES cells were first cultured on bacterial-grade Petri dishes in Iscove's modified Dulbecco's medium (Invitrogen-Gibco) containing 10% FCS. After the 4-day culture, floating cell aggregates, called embryoid bodies (EBs), were transferred to plates coated with type IV or VI collagen, poly-L-lysine, or fibronectin (Iwaki, Tokyo, Japan) and cultured in the same medium for 8 days. The cells adhering firmly to the dishes were recovered, and other cell types, including floating cell aggregates and those adhering weakly, were removed by aspiration. The resultant cell population was used for analysis and transplantation.

In some experiments, to trace the ES-cell–derived epithelial progenitor cells, the cells were labeled with $Fe3^+$. The cells were incubated in culture medium supplemented with 20 μg/mL iron oxide (Tanabe, Osaka, Japan) and 20 μg/mL transfection reagent (Roche, Basel, Switzerland).[21–23] We used 2×10^6 cells labeled with $Fe3^+$ as grafts for each damaged cornea.

Transplantation to Recipient Mice

C57BL/6 female mice aged 6 weeks (Charles River, Kanagawa, Japan) served as recipients. All subsequent experiments were conducted in accord with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and received the approval of the Animal Care Facility of St. Marianna University School of Medicine. Mice were anesthetized and placed in a stereotaxic frame (Narishige, Tokyo, Japan). Each mouse cornea was enclosed with a glass tube (Iwaki) to keep the eye open after emplacement, and paper wiper (Kimwipe S-200; Crecia, Tokyo, Japan) with a 2-mm diameter, soaked in 2 μL of *n*-heptanol, was placed on the center of the cornea for 1 minute after anesthesia to injure the corneal epithelium.[24] The graft cells (2×10^6 cells/tube) were put into the tube and allowed to adhere to the damaged cornea for 1 hour. Each group of mice with or without transplantation included 10 mice, and both eyes of all mice were injured. The graft cells were transplanted to both eyes of all the mice in the transplant-recipient group, and the results were compared to the control eye corneas without transplantation.

Flow Cytometric Analysis

Cell surface antigen expression was studied by a flow cytometer (EPICS XL; Beckman Coulter, Tokyo, Japan) using anti-mouse CD44 (BD-PharMingen, Lexington, KY) and anti-mouse E-cadherin (Takara, Ohtsu, Japan). The single cell suspension was incubated with each primary antibody or isotype control IgG, followed by incubation with FITC-conjugated secondary antibody and analysis by flow cytometry.[19]

Reverse Transcription–Polymerase Chain Reaction

Total RNA extraction and cDNA synthesis were conducted as reported previously.[19] Briefly, total RNA was extracted, and then 1 μg of total RNA was reverse transcribed, and complementary DNA (cDNA) was synthesized. β-Actin was used for detection of housekeeping gene expression in all RT-PCRs. The primers used and the expected size of amplified polymerase chain reaction (PCR) products were as follows: β-actin (440 bp), sense gatgacgatatcgctgcgctg and antisense gtacgaccagaggcatacagg; pax-6 (206 bp), sense aacaacctgcctatgcaacc and antisense cttggacgggaactgacact; K12 (437 bp), sense cgagagtggtatgaaaca and antisense tgggctctcatttcattg; and K14 (199 bp), sense ggtcgatttgatggatttgg and antisense gttcagtgttggcctcttcc. Cycling parameters were as follows: a hot start at 94°C for 2 minutes, denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 60 seconds. The reaction was repeated for 30 cycles and followed by elongation at 72°C for 10 minutes.

Immunoblot Analysis

Immunoblot analysis was conducted as described previously.[19] Briefly, cells were lysed in 2× SDS sample buffer, and the lysates were electrophoresed on 5% SDS-polyacrylamide gels. Proteins were electrically transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Tokyo, Japan). The membrane was incubated with anti-Pax-6 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-keratin 12 (Santa Cruz Biotechnology), and anti-E-cadherin (Chemicon International, Temecula, CA) antibodies, followed by incubation with a biotin-conjugated secondary antibody (Dako, Kyoto, Japan) and streptavidin-conjugated horseradish peroxidase (HRP). Detection was performed by chemiluminescence (Amersham Pharmacia Biotech).

Histologic Analyses

Cryostat sections (5 μm) of the mouse eyes were mounted on slides. For hematoxylin and eosin (H&E) staining, the sections were fixed with 20% formaldehyde in methanol, washed extensively, and stained with H&E. For the detection of intracellular $Fe3^+$ of colloidal iron-transfected donor cells, we performed Berlin blue staining. Briefly, frozen sections were fixed with 10% formaldehyde solution, extensively washed, and stained with Berlin blue solution, followed by counterstaining of the nucleus with fast red solution. For immunostaining, the samples were incubated with primary antibody followed by a peroxidase-conjugated secondary antibody. Reactivity was visualized with 3-amino-9-ethylcarbazole.

Sry Gene PCR

PCR was used to detect the male-specific sequence (*Sry* gene of ES cells) in genomic DNA extracted from the cornea of recipient female mice by a conventional method.[25,26] The reaction was performed with primers specific for the *Sry* gene on chromosome Y (sense, gttttggactggtgacaattg; antisense, gtcttgcctgtatgtgg). We know that the 402-bp band (*Sry)* identifies the male genotype, since we confirmed the DNA sequence of the amplified product by TA cloning and subsequent DNA sequencing (data not shown). β-Actin–specific primers were used to monitor the correct amplification of the template DNA (data not shown). This method allowed the identification of the ES-cell–derived epithelial cells in the recipient female cornea.

Results

Establishment of Optimal Culture Condition for ES Cells to Differentiate into Epithelial Progenitor Cells

Based on the evidence that (1) type IV collagen is the basic structural component of all basement membranes (BMs)[27]; (2) the cornea is initially covered with an epithelium that attaches to normal BM in the development of eyes[27]; (3) the conjunctival, limbal, and corneal BMs each include diverse collagen components[28,29]; and (4) the limbal epithelial cells can be successfully cultured on amniotic membrane[17] that consists of collagen and BM, we attempted to culture ES cells on several extracellular matrices, including type-IV and -VI collagen, poly-L-lysine, and fibronectin, to induce differentiation into the epithelial lineage. Undifferentiated ES cells were recovered from the maintenance culture consisting of LIF and mouse fetal fibroblasts. The ES cells were cultured in noncoated dishes for 4 days to form EBs. Then the EBs were transferred to a plate coated with each extracellular matrix. The cells cultured in the presence of type IV collagen began to grow and form an epithelium-like monolayer at day 8 of culture (Fig. 1), suggest-

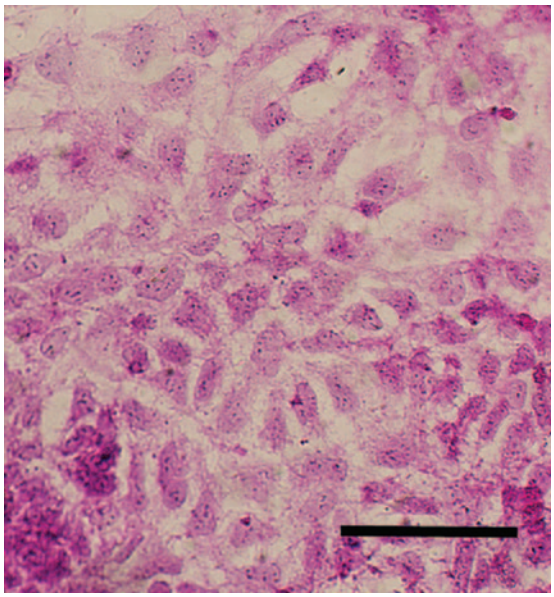

FIGURE 1. Induction of differentiation of epithelial progenitor cells from ES cells in vitro. ES cells were cultured in noncoated dishes for 4 days to form EBs, and the EBs were cultured on type-IV collagen for 8 days. The adhering cells had an epithelial-cell–like appearance. Staining, H&E. Scale bar, 50 μm.

ing that the ES cells had differentiated into cells committed to an epithelial lineage. To confirm and characterize the differentiation of ES cells into corneal epithelial cells, we investigated the expression pattern of cytokeratins in addition to the eye development marker *pax-6*. As shown in Figure 2A, RT-PCR demonstrated that ES-cell–derived epithelial cells cultured on type-IV or -VI collagen expressed K12, a specific marker of corneal epithelial cells. They did not express K14, which was expressed in the squamous epithelial basal layer. Moreover, these cells expressed *pax-6*, which is necessary for early development of eyes (Fig. 2A). As shown in Figure 2B, immunoblot analysis confirmed the expression of K12 in ES-cell–derived epithelial cells cultured with type IV collagen. Pax-6 appeared in the early stage of differentiation and was hardly detected at day 8 of culture (Fig. 2B). These results indicated that ES-cell–derived cells cultured on type-IV-collagen–coated plates had the characteristics of epithelial cells necessary for eye development.

Cell Surface Expression of E-Cadherin and CD44 on ES-Cell–Derived Epithelial Progenitor Cells

We established epithelial progenitor cells from mouse ES cells cultured on plates coated with type-IV or -VI collagen. We next evaluated whether the cells were appropriate for transplantation to the damaged cornea. We focused on studying the expression of cell adhesion molecules. E-cadherin has been deemed to have an important role in wound healing after corneal epithelial ablation.[30] Similarly, an earlier increase of CD44 transcription is observed during corneal epithelial wound healing.[31] We investigated the expression of E-cadherin and CD44 on ES-cell–derived epithelial progenitor cells. The cells cultured on type IV collagen expressed E-cadherin, and its expression level gradually increased along with epithelial differentiation (Fig. 3A). We next analyzed the cells by flow cytometry. We detected cell surface expression of CD44 and E-cadherin on the ES-cell–derived epithelial progenitor cells. The percentage of CD44 and E-cadherin–positive cells was 33.6% and 24.3%, respectively (Fig. 3B). The adhesion molecules expressed on the ES-cell–derived progenitor cells may facilitate their adhesion to the injured corneal surfaces. These findings suggested that these cells could be used for transplantation and corneal wound healing.

Successful Transplantation of ES-Cell–Derived Epithelial Progenitor Cells to Damaged Cornea

After corneal surface injury with *n*-heptanol, the ES-cell–derived epithelial progenitor cells cultured on type IV collagen for 8 days were transplanted to the injured cornea. At 1, 12, and 24 hours after transplantation, histologic examination of the eyes disclosed that the epithelial progenitor cells adhered well to the recipient corneal stroma and completely covered the damaged corneal surface (Figs. 4C–F, 4J, 5A). After injury, almost all epithelial cells were gone from the corneal surface, the stroma shrank, and inflammatory cells infiltrated the stroma and anterior chamber (Fig. 4B), compared with normal cornea (Fig. 4A). The stromal shrinkage was inhibited by the transplantation of ES-cell–derived epithelial progenitor cells, and subepithelial infiltration of inflammatory cells was greatly reduced after transplantation (Figs. 4C–F). At a higher magnification, normal corneal epithelium showed complex layering consisting of basal cells, wing cells, and superficial cells (Fig. 4G). In contrast, transplanted cells formed a monolayer (in places, two layers) on the stroma; however, they had nuclei, and some of them showed a basal or wing-cell–like appearance (Fig. 4H, arrows), indicating that transplanted cells had characteristics of nonkeratinized corneal epithelial cells. Cells were not observed on the stroma, however, either in the center (Fig. 4I) or in the limbus (Fig. 4J) of injured cornea without transplantation 24 hours after the injury, suggesting that the epithe-

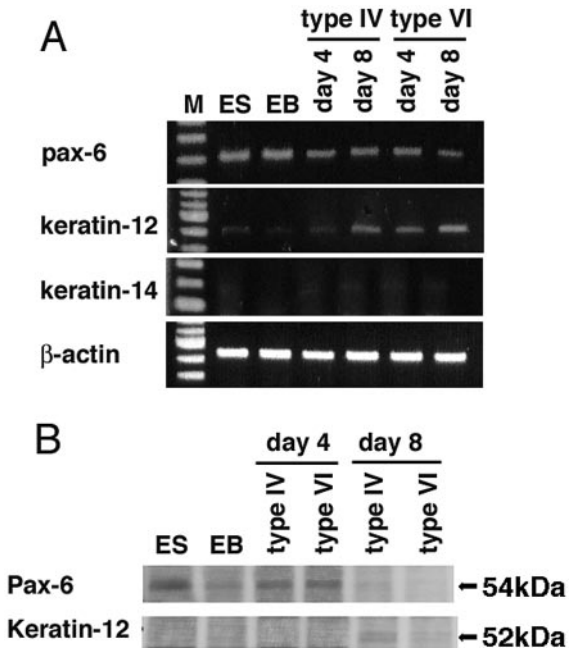

FIGURE 2. Expressions of keratins and *Pax-6* in ES-cell–derived cells. (**A**) ES cells were induced to differentiate into epithelial progenitor cells. Total RNA was extracted at each differential stage, reverse transcribed, and PCR amplified. The product was electrophoresed on 1.5% agarose gel and visualized with ethidium bromide. Data shown are representative of results in three independent experiments. (**B**) At each stage of differentiation of ES-cell–derived cells cultured on plates coated with type-IV or -VI collagen, cell lysates were extracted. Then immunoblot analyses with Abs to K12 and Pax-6 were performed. *Arrows*: bands corresponding to the size of Pax-6 and K12. Data are representative of results in three independent experiments.

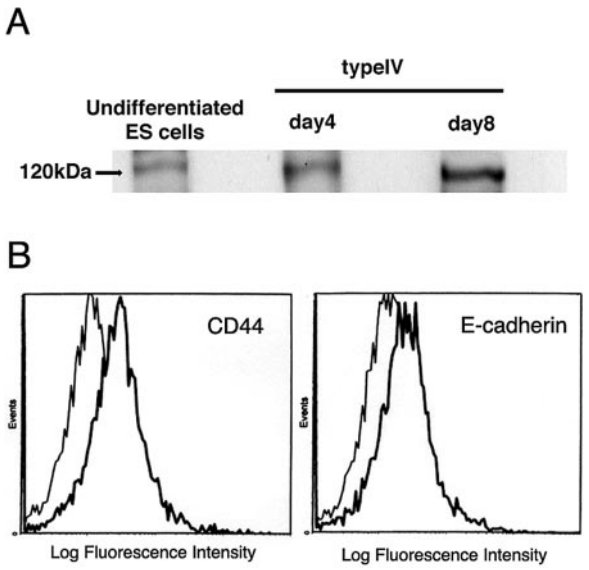

FIGURE 3. Expressions of E-cadherin in ES-cell–derived epithelial progenitor cells. (**A**) ES cells were induced to differentiate into epithelial progenitor cells by culture on type-IV collagen–coated plates. At each stage of differentiation, cell lysates were prepared, and expression of E-cadherin was analyzed by immunoblot analysis. *Arrow*: a band corresponding to the size of E-cadherin. (**B**) Cell surface expressions of CD44 and E-cadherin on the epithelial progenitor cells induced from ES cells were examined by flow cytometry. The results using control IgG (*thin line*) and each Ab (*thick line*) are shown. A representative of three independent experiments is shown in each panel.

lial cells covering the corneal surface after transplantation originated from grafted cells, not host-derived epithelial progenitor cells. Moreover, immunochemical staining for E-cadherin, one of the epithelial markers, demonstrated that the cells covering the corneal surface were of E-cadherin–positive epithelial lineages but not other lineages, such as myofibroblasts.

Origin of Corneal Epithelial Cells Covering the Damaged Corneal Surface

To examine further whether the epithelial cells covering the damaged cornea after transplantation originates from ES-cell–derived epithelial progenitor cells, we used two different approaches. First, we introduced colloidal iron ($Fe3^{+}$) into the grafted cells by using a transfection reagent.[21–23] We detected the cells labeled with colloidal iron by Berlin blue staining after corneal transplantation. Our labeling procedure with colloidal iron was simple and seemed essentially to make no change in the characteristics and viability of epithelial progenitor cells, as reported in earlier studies.[21–23] The epithelial cells covering the damaged corneal surface were stained well with Berlin blue (Fig. 5A, 5B; blue spots) at 24 hours after transplantation, suggesting that these cells originated from the ES-cell–derived epithelial progenitor cells, not the host. To confirm this fact, we next examined the expression of the *Sry* gene, which is located on the Y chromosome of ES cells. Recipient female mice were negative for the gene. At 1 and 24 hours after transplantation, the epithelial cells on the cornea had *Sry* gene expression (Fig. 5C). The epithelial cells covering the damaged cornea may have originated from the ES cells.

DISCUSSION

Severe and wide ocular surface diseases and chemical or thermal burn are the most difficult problems for ophthalmologists. In recent years, surgical reconstruction after ocular surface damage, even in the acute phase of inflammation or injury, has been advanced. Application of a limbal graft taken from the healthy contralateral eye,[3–5] transplantation of amniotic membrane,[7,9,15,16] transplantation of cultured epithelial cells in vitro,[7–14,17] and autologous transplantation of oral mucosal epithelial cells on amniotic membrane[18] have been reported. However, the most important key to making the reconstruction possible is the provision of sufficient materials for transplantation. Thus, we focused on developing a method for corneal epithelial transplantation using epithelial progenitor cells derived from ES cells.

ES cells are derived from the pluripotent cells of early embryos and can maintain a normal karyotype infinitely on culture in vitro and can differentiate into any cell type under appropriate conditions. Recently, human ES cells have been established, and the production of any kind of cell and tissues derived from ES cells for transplantation has logically become a reality.[32–38] This experimental therapeutic approach has already been reported in the nervous system.[20,39–41] In addition, some differentiation of epithelial lineages, such as cutaneous epithelial cells,[42] lung alveolar epithelial cells,[43] epithelial islets of thymus,[44] and pigmented epithelial cells,[45] has been induced from ES cells in vitro. In this report, we established a system to induce differentiation into epithelial progenitor cells by culturing ES cells on plates coated with type IV collagen for transplantation and further differentiation into corneal epithelial cells in vivo. Previous reports have demonstrated that mouse ES cells cultured without LIF on plates coated with type IV collagen differentiate into Flk-1–positive cells and are approximately 40% of the total ES cells after culturing for 4 days.[46,47] In addition, all the Flk-1–positive cells further differentiate into either vascular endothelial cells or vascular smooth muscle cells in the presence of vascular endothelial growth factor (VEGF).[46,47] In this study, a considerable portion of the ES cells cultured without LIF on the plate coated with type-IV collagen differentiated into epithelial cells in vitro. They may have further differentiated into corneal epithelial cells in vivo. This means that the epithelial progenitor cells established in our study were not completely committed to becoming corneal epithelial cells and may have the plasticity to differentiate into other epithelial cells. Thus, further identification of our epithelial progenitor cells is warranted. However, the partial or incomplete differentiation of ES cells into some lineages has the advantage of avoiding the development of teratoma, which is an obstacle to the successful transplantation of ES-cell–derived cells. Moreover, we can directly observe the corneal surface, facilitating early detection of unwanted events on the cornea. Indeed, we have not noted development of teratoma to date.

Keratins are the intermediate filamentous proteins of epithelial cells. A large number of mammalian keratins have been identified and, based on their biochemical properties, have been divided into two groups. The type-I keratins are designated K1 to K8 and the type II, K9 to K20. The keratin proteins form heterodimers with one member from each group.[48] Specific members of type-I and -II keratins are characteristically associated with each other in different cell and tissue types. Simple epithelia, such as the gut, express predominantly K8 and K18. Stratified squamous epithelia express mainly K5 and K14 in their basal layers, whereas the suprabasal layers express K1 and K10 in skin and K4 and K13 in some other epithelia, such as the esophagus.[49]

In ocular epithelia, it is important to distinguish the corneal epithelia from conjunctival epithelia because they have inverse characteristics, and successful transplantation of pure corneal epithelium depends on the exclusion of conjunctival epithelium. Recent reports have suggested that K3 and K12 are

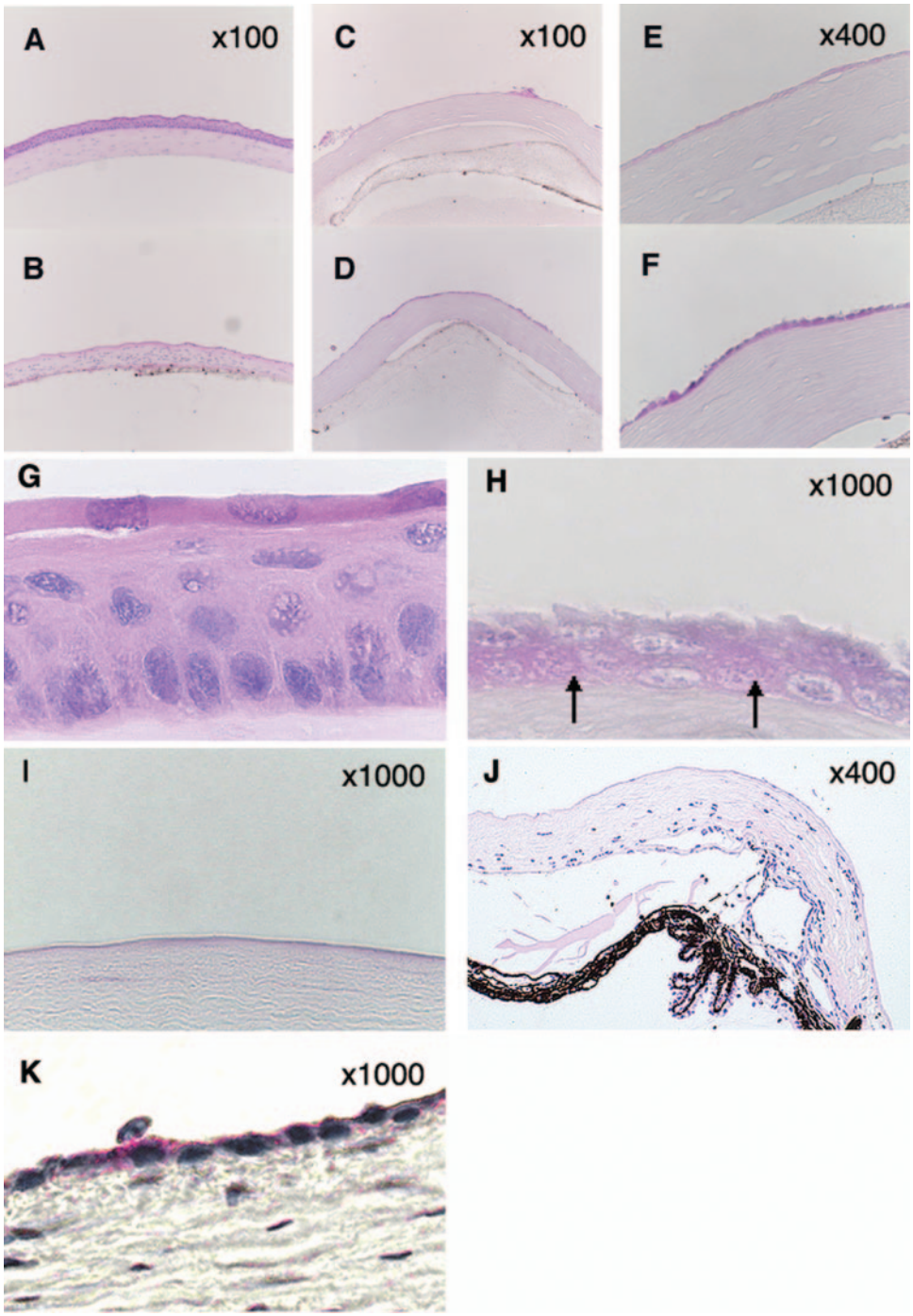

FIGURE 4. Histologic analysis of injured cornea, with or without transplantation of the ES-cell–derived epithelial progenitor cells. The ES-cell–derived epithelial progenitor cells (day 8 culture) were transplanted to *n*-heptanol–injured cornea of mice. (**A**) Normal mouse cornea. (**B**). *n*-Heptanol–injured cornea without transplantation. (**C**–**F**). Mouse eyes were injured with *n*-heptanol. At 1 hour (**C**, **E**) and 12 hours (**D**, **F**) after transplantation, the eyes were enucleated. Cryostat sections were fixed with 20% formaldehyde in methanol, stained with H&E, and compared with those of normal cornea. (**G**) Higher magnification of the normal corneal epithelium shown in (**A**). (**H**) Higher magnification of another preparation of the ES-cell–derived epithelial progenitor cells at 12 hours after transplantation. *Arrows*: the basal or wing-cell–like transplanted cells. (**I**) Higher magnification of *n*-heptanol–injured cornea without transplantation 12 hours after the injury. No corneal epithelial cells were observed. (**J**) Limbus of *n*-heptanol–injured cornea without transplantation 24 hours after the injury. Migration of the host-originated progenitor cells onto the corneal surface was not observed. (**K**) Immunostaining for E-cadherin of the corneal epithelial cells 12 hours after transplantation of ES-cell–derived graft cells. E-cadherin–positive epithelial cells are stained *red.* Data shown are representatives of results in 10 independent experiments.

specifically expressed in the corneal epithelium. Whereas K4 and K13 are expressed in the conjunctival epithelium, at a lesser level, K4, has also been observed in the corneal epithelium.[18,50] The ES-cell–derived epithelial progenitor cells express a low level of K12, which is a specific corneal epithelium cell marker, suggesting the possibility that ES-cell–derived epithelial progenitor cells can differentiate further into mature corneal epithelial cells in appropriate microenvironments. Pax-6 expression was evident in the ES-cell–derived progenitor cells. Pax-6 is very much involved in controlling eye development, including forming the cornea.[51] It is necessary to stimulate K12 promoter activity.[52,53] Western blot analysis demonstrated that the expression of Pax-6 preceded K12 expression in the epithelial progenitor cells cultured in the presence of type IV collagen. This suggests that Pax-6 and other signals generated by interaction with type IV collagen were necessary for K12 promoter activity.

We successfully transplanted ES-cell–derived epithelial progenitor cells in conventional mice. Detection of the *Sry* gene specific to the male genotype is one of the most useful methods for identification of the donor cells that originate from ES cells (male mouse origin) in the female recipient cornea.[25,26] The evidence that PCR detected a male-specific sequence (*Sry* gene on the Y chromosome of ES cells) in genomic DNA extracted from the cornea of female recipient mice definitely confirmed histologically the survival of transplanted cells and reconstruction of the corneal epithelium. The ES-cell–derived epithelial progenitor cells expressed both E-cadherin and

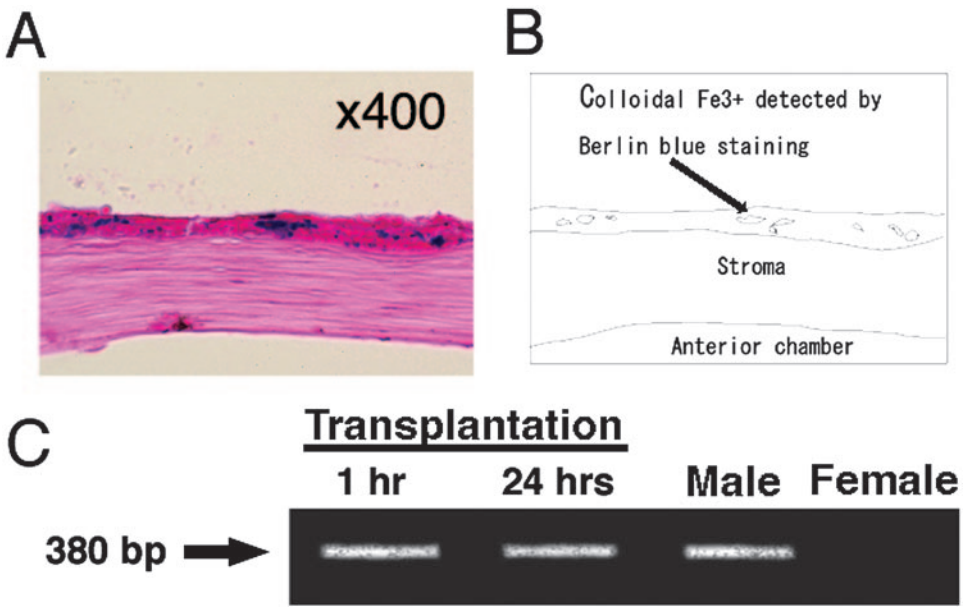

FIGURE 5. Expression of donor-cell–specific markers in epithelial cells covering the damaged cornea after transplantation of ES-cell–derived epithelial progenitor cells. (**A**) The ES-cell–derived epithelial progenitor cells were labeled with colloidal iron ($Fe3^{+}$) and transplanted to the *n*-heptanol–injured cornea of mice. At 24 hours after transplantation, cryostat sections were stained with Berlin blue solution, followed by counterstaining of nucleus with fast red solution. We detected the $Fe3^{+}$ positive ES-cell–derived cells as those with *blue* particles in their cytoplasm. (**B**) A schematic representation of (**A**). (**C**) At 1 hour and 24 hours after transplantation of the ES-cell–derived epithelial progenitor cells with a Y chromosome to *n*-heptanol–injured corneas of female recipient mice, genomic DNA was extracted from the corneal surface, and the *Sry* gene was detected by PCR. The *Sry* gene-specific PCR products from male and female mouse corneal DNA served as the positive and negative control, respectively.

CD44. The critical roles of E-cadherin during wound healing after corneal epithelial injury has been reported,[30] whereas CD44 has also been reported with its earlier increase of transcription during corneal epithelial wound healing.[31] Thus, the expression of adhesion molecules may contribute to successful transplantation by inducing tight cell-to-cell and/or cell-to-matrix interaction.

Taken together, both the location of cornea and the characteristics of ES-cell–derived progenitor cells may make it clinically possible to transplant ES-cell–derived cells to the cornea. This is one of the first successful trials toward using ES cells to reconstruct the corneal epithelium.

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