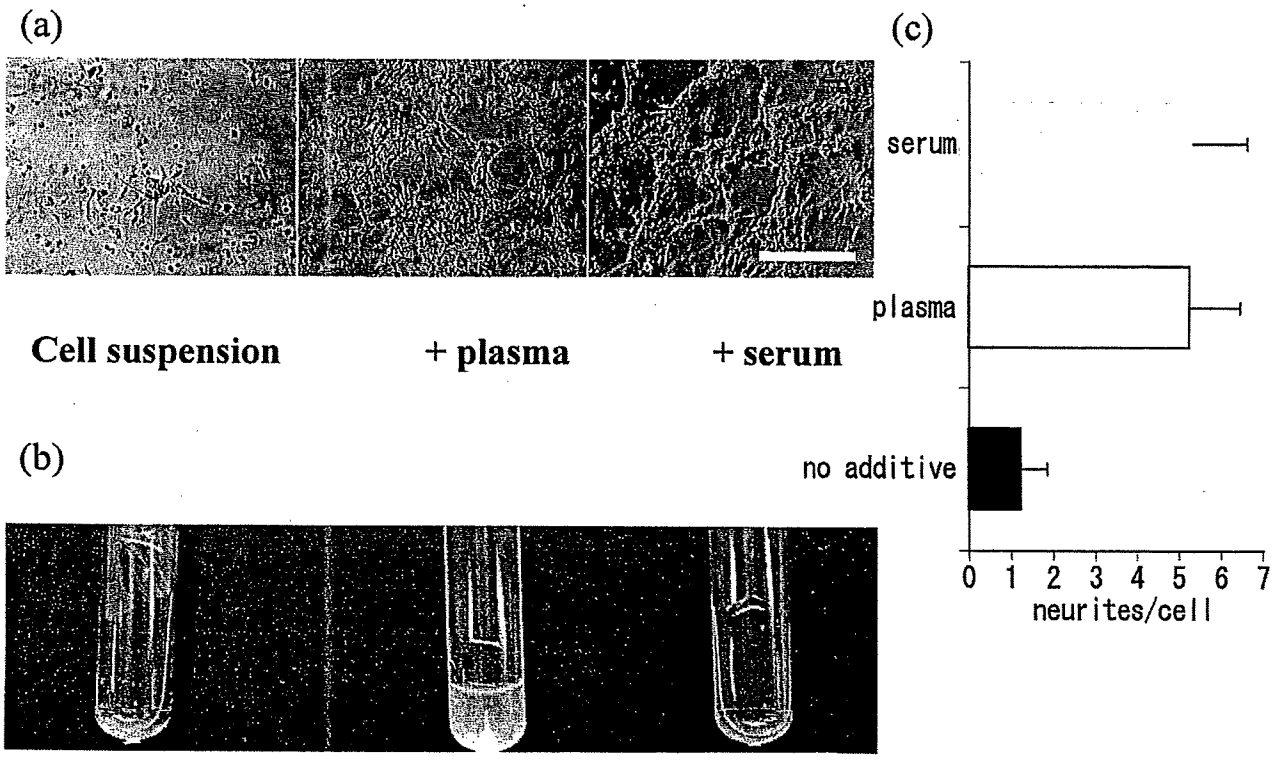

Figure 4. Neurite outgrowth and gel formation. (a) Plasma and serum promoted extensively neurite outgrowth. Neural precursor cells were incubated in the presence of plasma or serum (10%) for 24 h and then photographed. Scale bar: 100 μm. (b) A cell suspension with plasma (10%) forms a gel, while a suspension with serum (10%) does not. (c) Neural precursor cells were incubated with plasma or serum for 24 h and the number of neurites more than 25 μm long was counted from photographs taken under a phase-contrast microscope as shown in (a). Five separate experiments were carried out per group, and the number of neurites per cell was expressed as the mean ± SD.

Table 1. Effect of Plasma and Serum on the Thymidine Uptake Assay and MTT Assay

No. of Seeded Cells/ Treatment	Thymidine Uptake Assay (dpm)		MTT Assay (ABS)	
	24-h Incubation	48-h Incubation	24-h Incubation	48-h Incubation
1×10^5				
No additive	622.6 ± 46.4	237.8 ± 120.6	0.5872 ± 0.0162	0.5036 ± 0.0074
Plasma	176.3 ± 35.87*†	102.3 ± 58.06*‡	0.6343 ± 0.0162*	0.7177 ± 0.0527*
Serum	236.8 ± 37.0*	223.7 ± 60.6	0.6063 ± 0.0417	0.7166 ± 0.054*
2×10^5				
No additive	1134.3 ± 158.1	608.2 ± 319.1	0.9480 ± 0.0276	0.9671 ± 0.0231
Plasma	302.8 ± 104.8*	803.8 ± 199.3‡	1.0404 ± 0.1101§	0.9962 ± 0.0884
Serum	307.3 ± 89.4*	1290.7 ± 179.03*	0.8916 ± 0.1003§	0.9683 ± 0.1713

Cells were cultured in the presence or absence of serum or plasma (10%). Values are mean ± SD ($n = 3$).
*$p < 0.01$ versus RA alone—pulsed control cells (no additive).
†$p < 0.05$ versus serum-treated cells.
‡$p < 0.01$ versus serum-treated cells.
§$p < 0.05$ versus RA alone—pulsed control cells (no additive).

Table 2. Effect of Plasma and Serum on the Total RNA Content

Incubation Time/Treatment	RNA Content (mg/ml)
24 h	
No additive	0.332 ± 0.005
Plasma	0.382 ± 0.009*
Serum	0.412 ± 0.0045†
48 h	
No additive	0.381 ± 0.007
Plasma	0.575 ± 0.021*
Serum	0.656 ± 0.05*

Values are mean ± SD ($n = 3$). Cells (2×10^6/ml/well) were cultured in the presence or absence of serum or plasma. Total RNA was extracted and suspended in the same volume of DPEC-DW.
*$p < 0.05$ versus control cells (no additive).
†$p < 0.01$ versus control cells (no additive).

tion was significantly reduced by culture with plasma, it promoted cell growth and an increase of total RNA. Taken together, these results suggest that plasma increased cell growth slightly, but also promoted differentiation.

Unlike serum, plasma contains fibrinogen and forms a fibrin gel on exposure to air. Because culture was done under aerobic conditions, plasma formed a gel as shown in Figure 4. Although little air would be injected during cell transplantation with plasma under our experimental conditions, there is a possibility that fibrinogen would form a scaffold for the cells even if a gel did not develop. The superiority of plasma as a scaffold needs to be confirmed by more detailed comparison with serum, but plasma seems to be a good candidate scaffold for neural cell transplantation.

Scaffolds without cells have been reported to induce axonal regeneration, including substances such as algi-

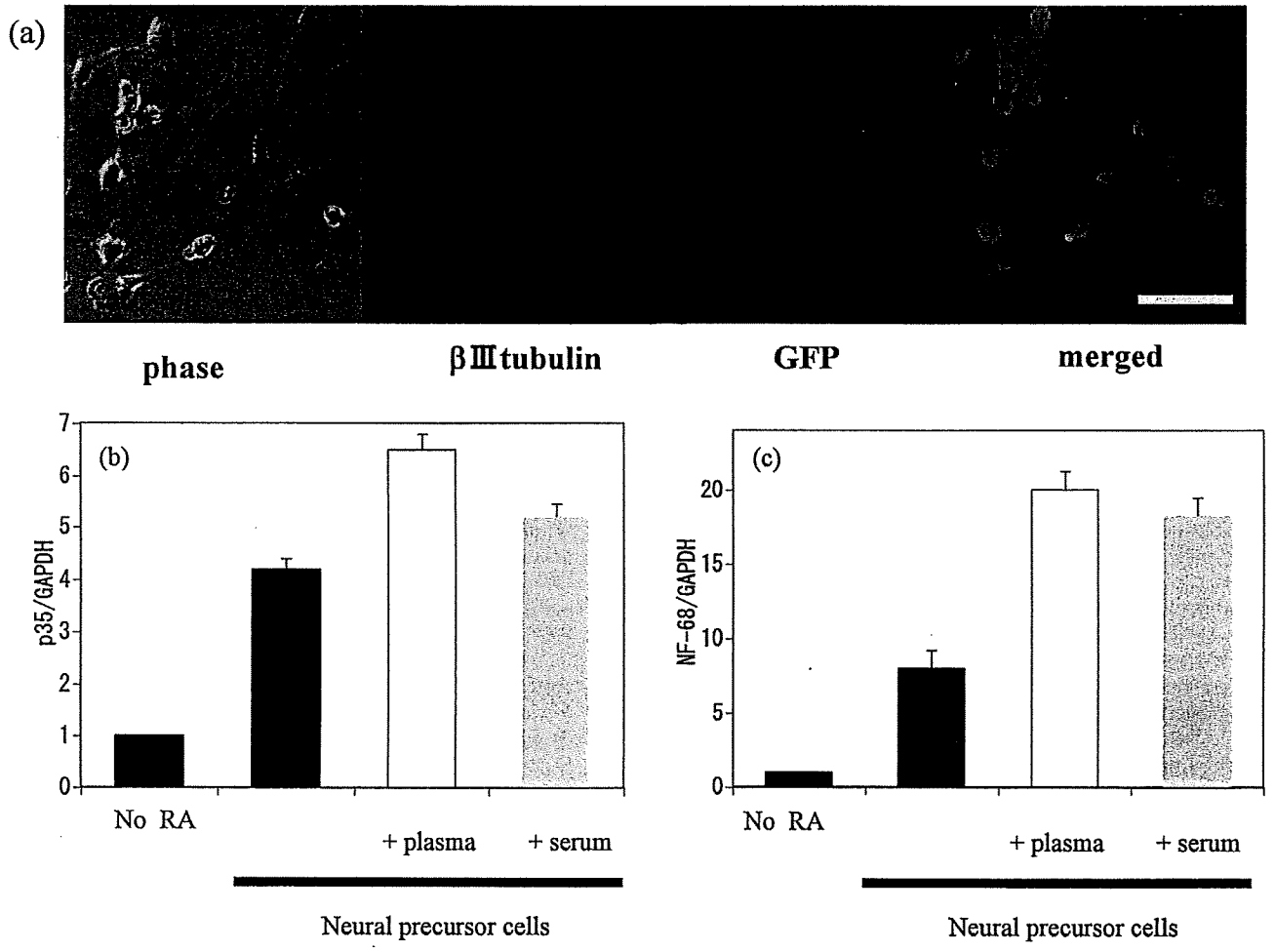

Figure 5. Promotion of neural marker and gene expression. Neural precursor cells derived from GFP-expressing ES were incubated with plasma or serum (10%) for 24 h. (a) Immunohistochemical staining. Scale bar: 50 μm. (b) Measurement of p35 and (c) 68kDa neurofilament (NF68) gene expression by RT-PCR. Values are shown as the mean ± SD ($n = 3$).

nate-based anisotropic hydrogel (21), freeze-dried agarose (22), and freeze-dried alginate cross-linked sponge (14). Bakshi et al. (3) studied a nonbiodegradable hydrogel, poly(2-hydroxyethylmethacrylate) (PHEMA), and they demonstrated that PHEMA combined with BDNF achieved sustained drug delivery to promote axonal growth and functional recovery after SCI. Jain et al. (13) used agarose gel as both a scaffold and a carrier of BDNF-releasing microtubules, and demonstrated that BDNF encouraged neurite outgrowth into the scaffold. Therefore, it might be important to combine cell therapy with the controlled delivery of neurotrophic factors by using a suitable scaffold.

Plasma is not only a scaffold but also provides the controlled release of various growth factors. In the future, the combination of plasma and other neurotrophic factors might lead to successful in vivo neural regeneration.

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Experimental Transplantation of Corneal Epithelium-like Cells Induced by *Pax6* Gene Transfection of Mouse Embryonic Stem Cells

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Purpose: Corneal epithelial stem cells are deficient in cases of limbal disorders, leading to conjunctival epithelial ingrowth, vascularization, and eventually visual disturbance. We introduced the eye development–associated transcription factor pax6 to embryonic stem (ES) cells and tested whether pax6-transfected cells resembling purified corneal epithelial cells were applicable as a cell source for corneal transplantation.

Methods: pax6 cDNA with green fluorescence protein was electrotransfected to ES cells and the cells were cultured with G418 for 14 days. They were characterized by reverse transcription–polymerase chain reaction and immunohistochemistry. The cells were transplanted onto experimentally damaged mouse corneas. Histologic reconstitution of the corneal epithelium was assessed.

Results: pax6-transfected cells formed a monolayer of epithelium-like cells in vitro. They expressed cytokeratin12, a specific keratin of corneal epithelial cells, E-cadherin, and CD44, which are important adhesion molecules of corneal epithelial cells on the cell membrane. They accumulated to make a colony that gave a staining pattern of reticular configuration for cytokeratin 12, E-cadherin, and CD44. When the cells were transplanted onto damaged cornea, they have been kept alive on the cornea.

Conclusions: The purified corneal epithelium-like cells derived from ES cells transfected with *pax6* gene adapted to the injured cornea and were kept alive on it. These results suggested application of ES cell–derived corneal epithelial cells for treating corneal injuries.

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Key Words: embryonic stem cells, pax6, corneal epithelial cells, transplantation

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Corneal transplantation is widely performed for treating patients with ocular trauma and diseases such as Stevens–Johnson syndrome and ocular cicatricial Pemphigoid.[1,2] Allogeneic corneal transplantation requires allogeneic donors and carries risks of immunologic rejection and infection. Autologous limbal transplantation was widely performed in patients with unilateral limbal stem cell deficiency.[3,4] Lately, limbal cells were cultured on the cell sheets or on the amniotic membrane to induce differentiation into corneal epithelial cells, and this technique has been introduced into clinical practice.[5–9]

Corneal epithelial stem cells reside in the palisades of Vogt.[10,11] The limbal stem cells produce undifferentiated progeny with limited proliferative potential that migrate centripetally from the periphery of the corneal epithelium to replace cells desquamating during healthy life.[12–16] Limbal stem cell deficiency causes conjunctival epithelial ingrowth, vascularization, chronic inflammation, recurrent erosions and persistent ulcer, destruction of the basement membrane, and fibrous tissue ingrowth leading to severe functional impairment.[1,2]

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocysts. They can differentiate into multiple cell types of all 3 germ layers, including epithelial progenitors. ES cells are capable of offering an unlimited number of cells for the transplantation therapy for various diseases. In mouse ES cells, controlled expression of specific genes promotes the differentiation into defined cell types, as seen in the generation of dopaminergic neurons and insulin-producing cells.[17–19] We have reported that *MASH1* gene transfection of ES cells led to preferential differentiation of motoneurons.[20,21]

Pax6 is a homeobox transcription factor acknowledged to have a critical and evolutionarily conserved role in eye development.[22–24] Pax6 is essential for the development and function of the cornea, lens, anterior eye segment, and neuroretina.[25]

Previously, we have reported on a system to induce differentiation into epithelial progenitor cells by culturing ES

cells on type IV collagen for transplantation.[26] Here, we report a modified strategy of generating corneal epithelial-like cells by *pax6* gene transfection into ES cells for yielding highly purified corneal epithelial cells.

MATERIALS AND METHODS

Culture of Mouse ES Cells

Undifferentiated ES cells (R-CMTI-1A, passage number 12–18) were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). They originated from a 129SV/EVJ mouse (normal karyotype) and had characteristics of prevalent undifferentiated ES cells, including the expression of alkaline phosphatase and OCT-4, a transcription factor essential for pluripotency. The cells were cultured on gelatin-coated dishes with a feeder layer consisting of mitomycin C–treated mouse fetal fibroblasts.[26] The culture medium consisted of Dulbecco modified Eagle medium, supplemented with 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1× nonessential amino acids, 1× pyruvate, 15% fetal calf serum, and 1000 U/mL recombinant mouse leukemia inhibitory factor (LIF; Invitrogen-Gibco, Grand Island, NY) to maintain the undifferentiated state of the ES cells.

Vector Constructions and Electroporation of Mouse ES Cells

Pax6 cDNA was kindly provided by Dr. Norman (National Eye Institute). We made a pcDNA3.1 vector (Invitrogen, Carlsbad, CA) containing pax6 cDNA with green fluorescence protein (GFP). As a control vector, we used a pcDNA3.1 vector designed to contain the GFP. All subsequent experiments were conducted as reported previously.[20,21] In brief, ES cells were electrotransfected with the pax6 vector. The cells were cultured in a medium containing 100 μg/mL G418 (Invitrogen-Gibco, Grand Island, NY).

Fourteen days later, stable G418-resistant cells were recovered. Although the pax6-transfected cells differentiated into neural cells as well, half of them differentiated into corneal epithelium-like cells and made a cell cluster (Figs. 1C, 4). The cell cluster of epithelial-like cells was easily distinguished from the neural cells and neural cell cluster with axonlike processes. The cells that adhered firmly to the dishes and made a flat cell aggregate were recovered because they represented mostly epithelium-like cells, and other cells including floating cell aggregates and those adhering weakly to the dishes (including neural cells) were removed by extensive washing and subsequent aspiration. The resultant cell population was used for analysis and transplantation. Transfection with empty vector did not cause morphologic changes suggestive of corneal epithelial cell differentiation, so further characterization was not carried out.

Transplantation to Recipient Mice

Female C57BL/6 mice 6 weeks of age (Charles River, Kanagawa, Japan) were used for transplantation recipients. The mice were categorized as normal (N), injury (I), and transplantation (T). Each group consisted of 5 mice. All subsequent experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by 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

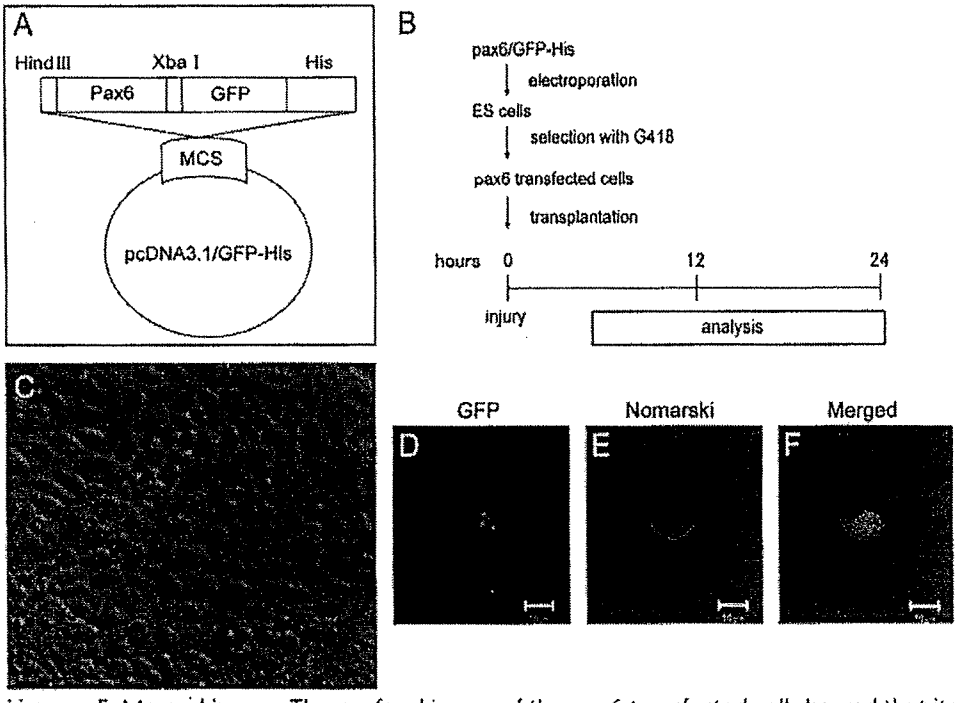

FIGURE 1. Characteristics of pax6-transfected cells. A, A map of Pax6/GFP-His expression vector. Pax6-encoding gene was inserted between *Hind*III site and *Xba*I site and GFP-encoding gene between *Xba*I and *Age*I of pcDNA3.1/myc-His. B, Experimental protocol. Pax6 cDNA was subcloned into GFP + His-tag expression vector (pPax6/GFP-His) and thereafter ES cells were electrotransfected with the vector. The transfected cells were cultivated with G418-containing media for 14 days. We confirmed that ES cells without transfection (control cells) have died in the G418-containing media no later than 14 days. C, Corneal epithelial progenitor cells derived from ES cells transfected with *pax6* gene in the culture. The cells showed a flagstonelike appearance that was similar to that of corneal epithelial cells. Magnification, ×200. D–F, Confocal images of the pax6-transfected cells in vitro. D, GFP. E, Nomarski image. F, Merged image. The confocal image of the pax6-transfected cell showed that its nucleus was GFP+, suggesting that the pax6 and GFP was expressed in the cell nucleus.

with a glass tube (Iwaki, Tokyo, Japan) to keep the eye open. After emplacement of the glass tube, a 2-mm diameter round paper of Kimwipe S-200 (Crecia, Tokyo, Japan), soaked in 2 μL of n-heptanol, was placed on the cornea for 1 minute after anesthesia to injure the corneal epithelium entirely, including limbal epithelium.[26] We used fluorescent staining to confirm complete removal of the mouse corneal epithelium, and mice with incomplete removal were excluded from the study. The graft cells (cell suspension, 2×10^6 cells/tube) were put into the tube and allowed to adhere to the damaged cornea for 1 hour. Both eyes of all mice were injured except the normal group. The graft cells were transplanted to both eyes of all the mice in the transplanted group, which were compared with the control eyes without transplantation (injury group).

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted and was reverse transcribed, and cDNA was synthesized.[26] The sequences and annealing temperature of the primers used in this study are summarized in Table 1.

Histologic Analyses

Cryostat sections (5 μm in thickness) 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.

Immunofluorescence Staining

Cells cultured on chamber slides and cryostat sections of samples were fixed in a mixture of methanol and acetone for 10 minutes at room temperature. The samples were blocked in PBSTG (0.2% Tween 20 and 0.2% gelatin in phosphate-buffered saline [PBS]) and were incubated overnight with the appropriate primary antibody. They were then treated with biotinylated second antibodies (Dako Cytomation, Kyoto, Japan) and finally with Cy3-conjugated streptavidin (Jackson Immuno Research, West Grove, PA). Immunofluorescence was observed by using confocal laser microscopy (LSM-510; Carl Zeiss, Jena, Germany). Sources and dilutions of the primary antibodies were summarized in Table 2. The same concentration of corresponding normal mouse immunoglobulin G1 (IgG1), normal rat IgG1, and normal rabbit IgG provided negative controls (Dako), and native mouse cornea served as positive controls. For all immunofluorescence procedures, adjacent wells in the chamber slides served as negative controls and were processed by using identical procedures, except for incubation without the primary antibody in each case.

RESULTS

Induction of Corneal Epithelium-like Cells by *pax6* Gene Transfection of Mouse ES Cells

We previously induced corneal epithelial cells from mouse ES cells by culturing them on type IV collagen. The purity of the differentiated epithelial cells from ES cells was not satisfactory at that time. Therefore, we tried to induce highly purified epithelial cells from ES cells by *pax6* gene transfection.

First, the pax6 expression plasmid (Fig. 1A) was electrotransfected to the undifferentiated mouse ES cells and the cells were cultured with G418 for 14 days (Fig. 1B). Although the pax6-transfected cells at day 14 differentiated into neural cells as well, half of them differentiated into corneal epithelium-like cells that made cell clusters (Fig. 1C). The cells that adhered firmly to the dishes and made a flat cell aggregate were recovered because they represented mostly epithelium-like cells (Fig. 4A, B). The other cells including floating cell aggregates and those adhering weakly to the dishes (including neural cells) were removed by extensive washing and subsequent aspiration (data not shown). The resultant cell population was used for analysis and transplantation. The pax6-transfected cells become 1 cell after their passage with trypsin/EDTA treatment. They gradually made cellular aggregates in vitro, and at day 5, they attached to each other and formed a cell sheet. Indeed, the pax6-transfected cells showed a flagstonelike appearance, resembling normal corneal epithelial cells (Fig. 1C). Confocal image of the pax6-transfected cells showed that their nuclei were GFP positive, indicating that the pax6/GFP was expressed in the cell nuclei (Figs. 1D–F).

We next examined mRNA expressions of the pax6-transfected cells resembling corneal epithelial cells by reverse transcription–polymerase chain reaction (RT-PCR). The pax6-transfected cells expressed mRNA of pax6, cytokeratin12 (CK12), which is a specific marker of corneal epithelial cells

TABLE 1. Primer Sequence Used for RT-PCR Analysis

Primer Name	Forward	Reverse	Annealing Temp (°C)	PCR Products (bp)
pax6	aacaacctgcctatgcaacc	cttggacgggaactgacact	54	206
CK12	cgagagtggtatgaaaca	tgggctctcatttcattg	48	437
CK14	ggtcgatttgatggatttgg	gttcagtgttggcctcttcc	48	199
E-cadherin	gagaacggtggtcaaagagc	catctcccatggtgccacac	58	489
CD44	accccagaaggctacattttgc	ctcataggaccagaagttgtgg	55	219
Integrin α4	tccaaaaatcccctatcctctc	aagccatcctgctgcaaac	54	438
Integrin β7	cagcaatggtgtctacacgaac	gcttgaagagtgacccagaaa	60	393
β-Catenin	gtggaccccaagccttaggta	atggtgggtgcaggagttta	60	547
p63	agtacctccctcagcacacg	atgggtacacggagtggttt	50	189
PCNA	gatgtggagcaacttggaat	agctctccacttgcagaaaa	56	160
β-Actin	gatgacgatatcgctgcgctg	gtacgaccagaggcatacagg	55	440

TABLE 2. Summary of Immunolabelling Reagents and Procedures

Primary Ig	Source of 1E Ig	Dilution	Secondary Ig	Source of 2E Ig
pax6	Covance	1:300	Biotinylated second antibody	DAKO
CK12	Fitzgerald	1:100	Biotinylated second antibody	DAKO
E-cadherin	TaKaRa Bio	1:200	Biotinylated second antibody	DAKO
CD44	Phamingen	1:20	Biotmylated second antibody	DAKO
p63	DAKO	1:50	M.O.M Kit	Vector
PCNA	Santa Cruz	1:500	M.O.M Kit	Vector

(Fig. 2). Cytokeratin14 (CK14) is known to be expressed in the squamous epithelial basal layer. However, the pax6-transfected cells did not express CK14. The pax6-transfected cells expressed E-cadherin mRNA, a member of the family of calcium-dependent cell adhesion molecules. β-Catenin is a central component of the cadherin cell adhesion complex and a key regulator of epithelial differentiation and proliferation.[27,28] The cells expressed mRNA of β-catenin and CD44, which was the hyaluronic acid receptor and acted as an adhesion molecule. E-cadherin, CD44, and integrin α4 have important roles in wound healing after corneal epithelial ablation.[29,30] pax6 directly regulates the expression of the integrin α4 during corneal wound healing, and the pax6-transfected cells expressed mRNA of integrin α4.

Control vector transfected cells did not express mRNA of E-cadherin and CD44 (Fig. 2). Thereafter, we examined protein expressions of the pax6-transfected cells. Pax6 protein was expressed in their nuclei (Figs. 3A–C).[31] CK12 (Figs. 3D–F), E-cadherin (Figs. 3G–I), and CD44 (Figs. 3J–L) proteins were expressed on the cell membrane and/or cytoplasm.[32,33] As mentioned, the pax6-transfected cells cultured on gelatin-coated dishes formed 1 layer of epithelium-like cell sheet in vitro (Figs. 4A, B). They grew in an adherent fashion. The cell cluster of the pax6-transfected cells with corneal epithelium-like appearance expressed CK12, E-cadherin, and CD44 proteins, giving a staining pattern of reticular configuration (Figs. 4C–E).

We next examined p63 and PCNA mRNA and protein expressions of the pax6-transfected cells to test whether the pax6-transfected cells had proliferative potential and shared, at least partly, the characteristics with the corneal limbal stem cells.[34] p63 plays a key role in morphogenesis of corneal epithelial cells by regulating epithelial development and differentiation. p63-knockout mice have a remarkable absence of stratified squamous epithelia and their derivatives. Therefore, p63 is related to maintenance of stem cells.[35–37] The cells expressed p63 and PCNA mRNA and proteins (Figs. 2 and 3M–R).

Transplantation of pax6-Transfected Cells to the Damaged Mouse Cornea

We first removed corneal epithelial cells including limbal stem cells from the stroma by n-heptanol treatment

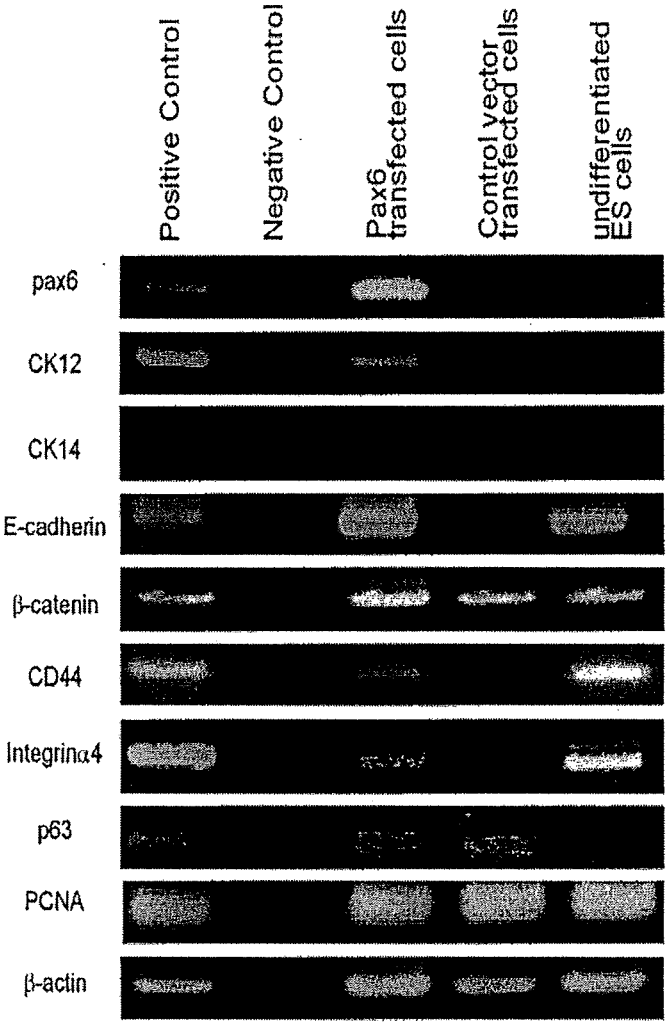

FIGURE 2. RT-PCR analysis of the pax6-transfected cells. Total RNA of the pax6-transfected cells was extracted, reverse transcribed, and PCR amplified. The products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide. The pax6-transfected cells expressed mRNAs of pax6, CK12, E-cadherin, β-catenin, CD44, and integrin α4. Similarly, p63 and PCNA mRNA was expressed in the pax6-transfected cells.

(Figs. 5A, normal, B; defect). We transplanted the pax6-transfected cells to the damaged cornea. Twelve hours after the transplantation, the grafted eyes were recovered for histologic analysis. Twelve hours after transplantation (Fig. 5C), the pax6-transfected cells formed 3 layers of epithelial cells on the damaged cornea. We conducted transfection experiments of the ES cells with the same vector without an insert. After the selection culture of the G418-resistant cells, we transplanted them the same way as with the pax6-transfected cells. The transfected cells with the same vector without an insert (designated as "control vector") hardly attached to the damaged cornea (Fig. 5D). We repeated the experiments several times to compare the histologic recovery between the pax6-transfected cells and the transfected cells with the same vector

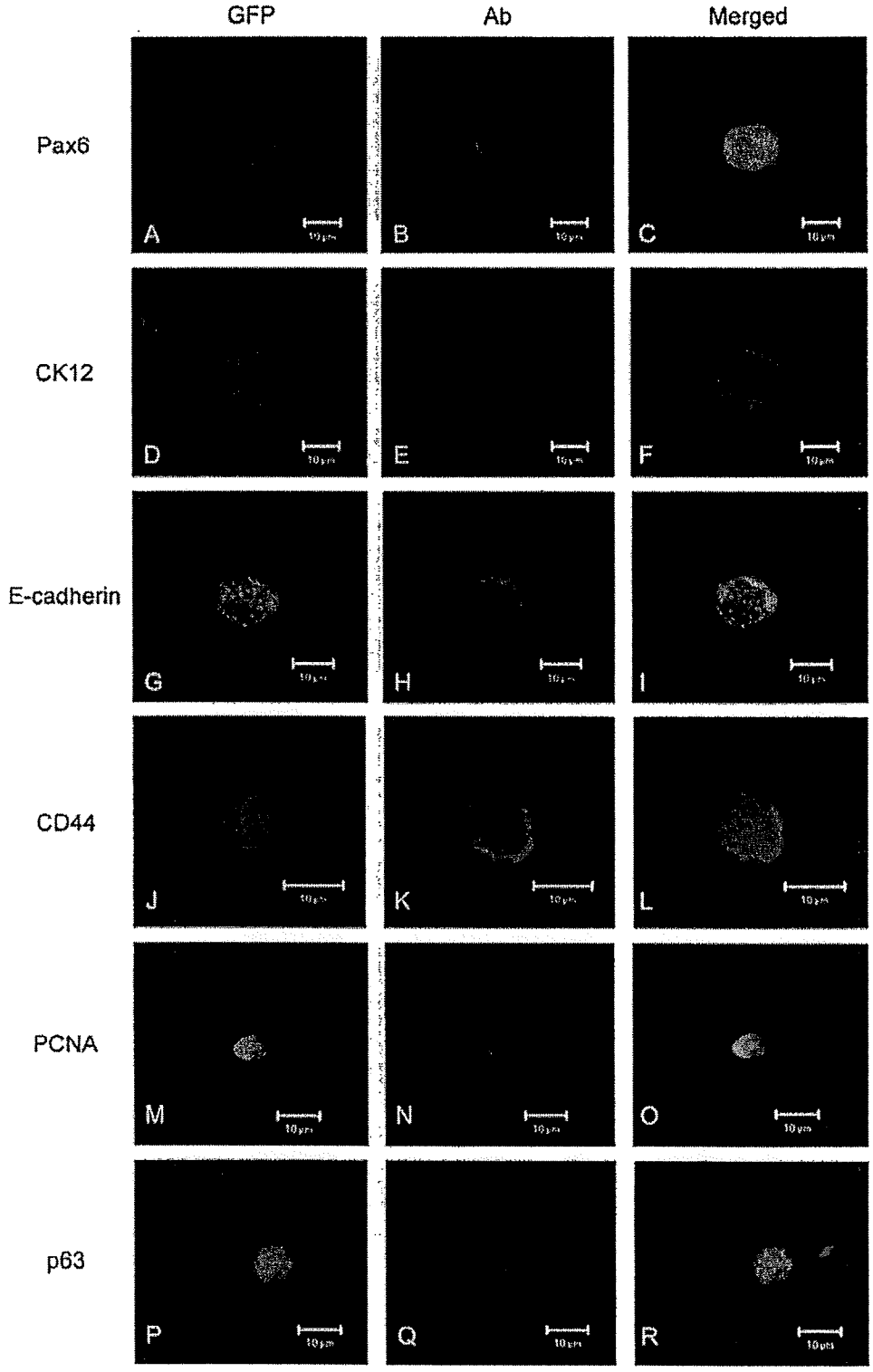

FIGURE 3. Confocal analysis of the pax6-transfected cells. The pax6-transfected cells were immunostained by specific antibodies including pax6 (A, GFP; B, anti-Pax6; C, merged image), CK12 (D, GFP; E, anti-CK12; F, Merged image), E-cadherin (G, GFP; H, anti–E-cadherin; I, merged image), CD44 (J, GFP; K, anti-CD44; L, merged image), PCNA (M, GFP; N, anti-PCNA; O, merged image) and p63 (P, GFP; Q, anti-p63; R, merged image). Pax6, p63, and PCNA proteins were expressed in the nuclei. CK12, E-cadherin, and CD44 proteins were expressed on the cell membrane and/or cytoplasm. Magnification, ×600.

without an insert. Thus, for efficient attachment to the damaged cornea, transplantation of the pax6-transfected cells seemed to be necessary.

This result showed that the pax6-transfected cells have been kept alive on the damaged cornea for 12 hours after the transplantation. The confocal image of the injured mouse cornea transplanted with the pax6-transfected cells (Fig. 5E, 24 hours after transplantation) showed that GFP-positive cells that had been transfected with pax6 gene attached to the damaged cornea 24 hours after the transplantation.

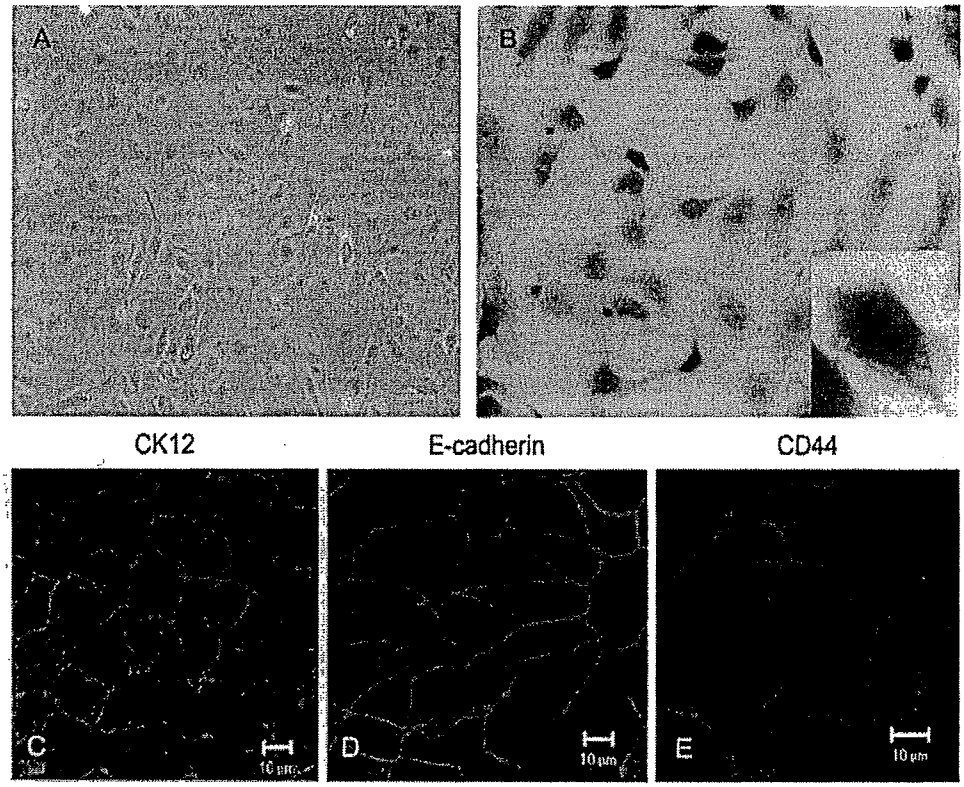

FIGURE 4. Characterization of the cell sheets of the pax6-transfected cells. Pax6-transfected cells were cultured on gelatin-coated dishes and formed 1 layer of their cell sheets. The cells grow in an adherent fashion. The pax6-transfected cells were observed by using inverted microscopy image (A, magnification, ×100; 5 days after the passage). These cells showed a flagstonelike appearance similar to that of normal corneal epithelial cells. The pax6-transfected cells were stained with H&E (B, magnification, ×100; inset, magnification, ×1000; 3 days after the passage). Protein expression was examined by immunofluorescence staining. The cell sheets of pax6-transfected cells with corneal epithelium-like appearance expressed CK12 (C), E-cadherin (D), and CD44 (E) proteins with reticular configuration. Magnification, ×100. C–E, 5 days after the passage.

DISCUSSION

Limbal disorders such as severe trauma and ocular surface diseases lead to corneal epithelial stem cell deficiency in the corneal limbs. Although allogeneic corneal transplantation has a useful strategy for these diseases, it may be complicated by high irregular astigmatism and corneal graft rejection.[38] It is desirable to establish an alternative method for supplying sufficient cells for reconstruction of the damaged cornea. Recently, autologous limbal stem cell transplantation and tissue-engineered epithelial cell sheets have been developed and have already entered into the clinical realm.[39,40]

It is difficult to perform an autologous limbal transplantation in the case with bilateral eye lesions because of bilateral limbal stem cell deficiency. Tissue-engineered epithelial cell sheets made from autologous oral mucosal epithelium were useful. However, patients suffering from ocular pemphigoid and Stevens–Johnson syndrome were often affected in their oral mucosal epithelium. In such cases, it will be difficult to use the patients' own oral mucosal epithelium for the reconstitution. ES cells can be applicable as donor cells of any cell type. Thus, we focused on developing a technique to differentiate ES cells into purified corneal epithelial cells applicable for corneal transplantation. pax6 is a transcription factor essential for the eye development and regulates corneal epithelial differentiation.[32,41] Therefore, we introduced the *pax6* gene into mouse ES cells. As expected, the resultant cells contained both neural cells and epithelial cells. The epithelial cells adhered firmly, forming relatively homogenous cell clusters and can be distinguished from the neural cells by extensive washing and subsequent aspiration. Half of this cell population at day 14 was epithelial cell positive for CK12. CK12 is a major intermediate filament-forming keratin of mouse corneal epithelial cells. CK12 is generally believed to be the specific marker for mature corneal epithelial cells.[42] We confirmed that the pax6-transfected cells expressed CK12 and corneal epithelial cell associated adhesion molecules, E-cadherin and CD44 proteins. The remaining half at day 14 included neuroepithelial cells and other neural cells and rarely contained cells of endodermal and mesodermal origin (M. Kayama and N. Suzuki, unpublished data). In our previous study where undifferentiated ES cells were cultured on type IV collagen to induce epithelial cell differentiation, we found that 20% of them differentiated into CK12-positive corneal epithelium-like cells and that they contained other cell types than ectodermal origin, such as endothelial cells.[26] The purity of the CK12-positive corneal epithelium-like cells in the pax6-transfected cells is much higher than that in the type IV collagen–induced corneal epithelial cells. It is possible to further enrich this population by cell sorting with anti-CK12 antibody.

Because we successfully transfected the *pax6* gene into mouse ES cells by using electroporation for yielding purified corneal epithelial cells, we used this cell type for the transplantation of the damaged cornea. The pax6-transfected cells expressed E-cadherin and CD44 in vitro. They may contribute to tissue regeneration by inducing cell-to-cell and/or cell-to-matrix adhesion of the grafted cells.

We wanted to know whether the pax6-transfected cells had characteristics of mature corneal epithelial cells or whether they had potent proliferative capability resembling limbal stem cells. We found p63 expression and PCNA

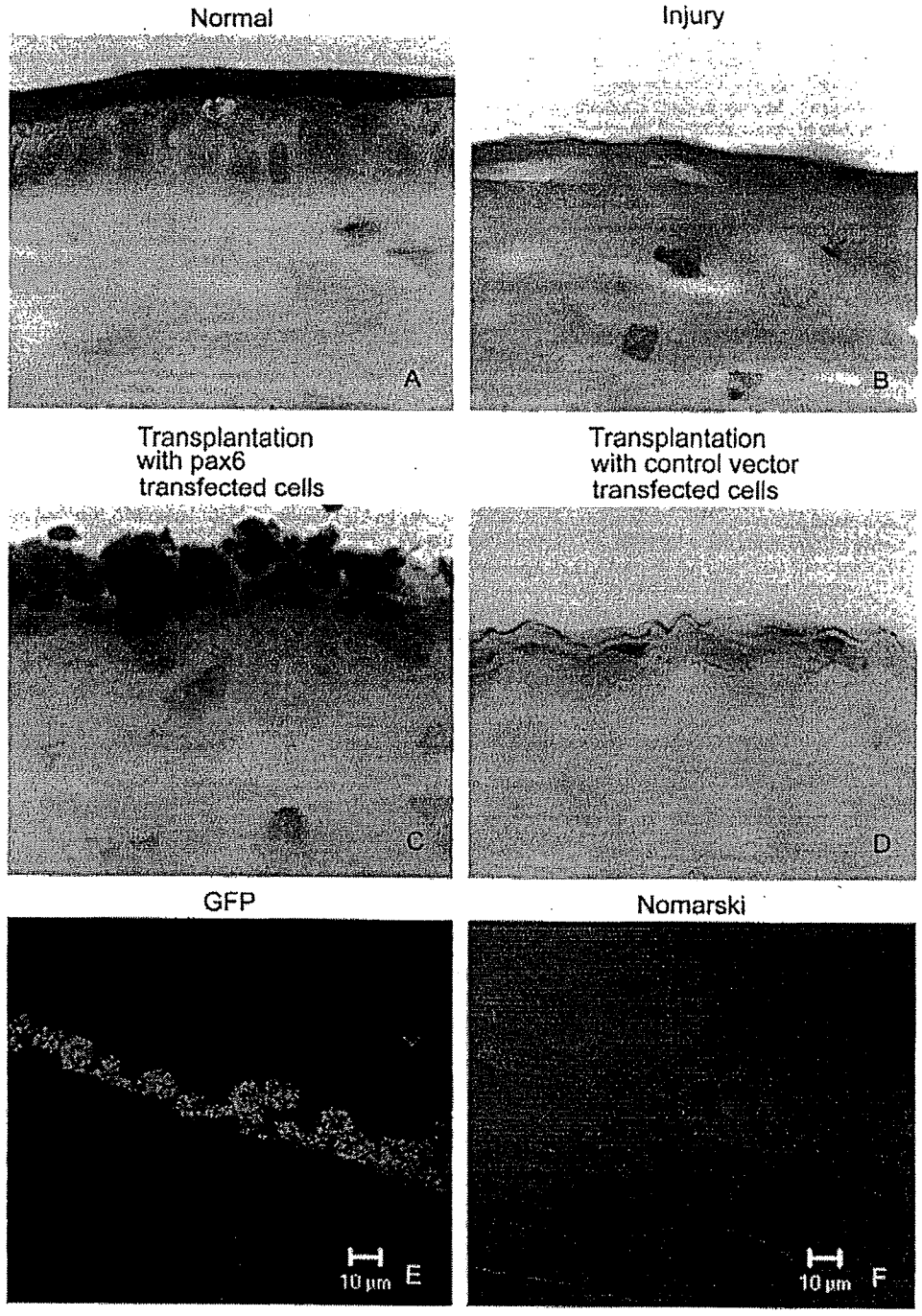

FIGURE 5. Experimental transplantation of the pax6-transfected cells to the damaged cornea. Cryostat sections of normal mouse cornea (A), the injured mouse cornea (B), the injured mouse cornea transplanted with the pax6-transfected cells (C, 12 hours after transplantation), and the injured mouse cornea transplanted with the control vector transfected cells (D, 12 hours after transplantation) were stained with H&E. Magnification, ×400. The confocal image of the injured mouse cornea transplanted with the pax6-transfected cells (E, 24 hours after transplantation) showed that GFP-positive cells that had been transfected with *pax6* gene attached to the damaged cornea 24 hours after the transplantation. F, Nomarski image of E.

expression of the pax6-transfected cells. The results suggested that the pax6-transfected cells shared at least part of the characteristics of limbal stem cells.

When the pax6-transfected cells were transplanted onto the damaged cornea, they formed bi- and trilayers of epithelial cells on the cornea at 12 hours. We need to conduct a histologic examination of the graft after a longer time span for efficacy and safety. We saw substantial detachment of the grafted cells at 24 hours after transplantation (data not shown). The mechanical forces caused by eye lid opening/closing induces detachment of the grafted corneal epithelial cells. Some device, such as contact lenses of mice, was awaited in this series of experiments for preventing the detachment. Nonetheless, the epithelial cells derived from ES cells were applicable as grafts for treating the corneal injury. ES cells may become an unlimited donor source of corneal epithelial cells for corneal transplantation.

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特　集

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マウス胚性幹細胞由来血管内皮細胞および壁細胞の分化誘導

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再生医療における血管誘導の必要性

脳梗塞，心筋梗塞，脊髄損傷，筋ジストロフィーをはじめとして，成体において再生能力の低い組織の損傷やこれらの変性疾患は，一旦罹患すると不可逆性であり，当該組織および機能の回復はほぼ不可能である。近年，再生医学の分野において，組織幹細胞または胚性幹（embryonic stem, ES）細胞から神経，筋など様々な生体の細胞を分化誘導する基礎的検討がなされ，一部は臨床の現場でも臨床試験または治療として行われつつある。

これらの分化誘導された移植組織細胞が宿主組織に確実に生着し，十分な機能発現をするためには，宿主組織において移植組織細胞に十分量の血流を供給する血管新生が不可欠である。実際には，ES細胞から目的の組織細胞への分化誘導を行う場合には同時に血管内皮細胞への分化を伴うことは良く認める。しかし分化誘導を高い効率で行う場合には十分な新生血管が誘導されず，宿主組織への生着率低下の原因となる。この危険性を回避するために，目的の組織細胞とは別にin vitroで血管を誘導し，目的の組織細胞と合わせて移植することで移植組織細胞が必要とする血流量を増加させることが可能になる。血管内皮細胞を分化誘導し移植するために，必要な移植細胞数を含め調整性に富み，再生させる血管の量や構造すら調節可能と考えられる。in vitroで血管内皮細胞を分化誘導する場合，その分子生物学的な機序を解明することが必要となるが，これは同時に分化誘導を妨げる因子についての理解も必要となる。このことは，角膜上皮細胞の分化誘導など逆に血管新生を阻まなければいけない場合に，そのメカニズムを理解し，臨床応用へと結びつける事も可能となる。

我々は，マウスおよびカニクイザルのES細胞から神経細胞，筋細胞，角膜細胞，血球細胞などを分化誘導し，疾患モデルマウスに移植することにより運動機能等失われた機能が回復することを報告してきた[1-7]。特に神経細胞（ニューロン）の誘導においては，数種類の方法で分化効率を検討しているが，例えばマウスES細胞をレチノイン酸処理することで分化誘導した神経細胞に比べ，神経分化に重要な転写因子Mash1遺伝子を導入することで分化誘導した神経細胞では，運動ニューロンの出現率は高くなるが逆に血管の新生は低くなることが分かっている[1,2]。

我々はこれらの神経細胞を片麻痺モデルマウスに移植し，比較対照として溶媒である生理食塩水のみを注入した片麻痺マウスとともに経時的に麻痺側の運動機能を検討した経験がある。神経細胞移植マウスでは対照マウスに比較して有意に運動機能が回復し，移植後1か月程で全く正常なマウスの運動機能の50%程度まで回復した。しかし完全に回復するにはいたらない[1-3]。この移植マウスの脳血流をfunctional MRIで観察した。障害側大脳半球では上記対照マウスより血流は増加しているものの，正常マウスよりは血流量が低下している[3]。このため，神経細胞と血管を構成する細胞とを別々に分化誘導し合わせて移植することにより，血管新生を促進し

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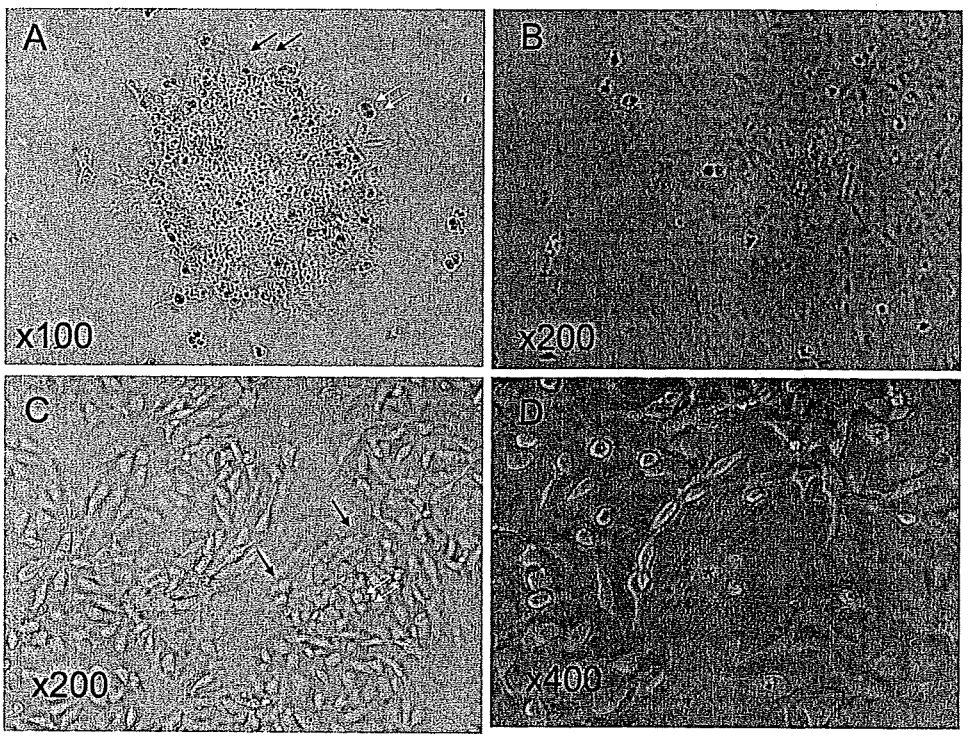

図1　マウス ES 細胞をメチルセルロース処理および OP9 細胞と共培養した細胞の形態
A, B メチルセルロース処理後 4 日目のコロニーを示す。培養皿に付着して比較的扁平な細胞が増生し(黒矢印)，培養液中に類円形の細胞が現れる(白矢印)。
C, D メチルセルロース処理 4 日後 OP9 細胞と共培養を開始し 24 時間後のコロニーの形態を示す。類円形の細胞(白矢印)を扁平な細胞が取り囲む(黒矢印)形のコロニーが多数見られる (**C**)。この扁平な細胞は別なコロニーの扁平な細胞と両端で接合し，管腔を形成する傾向を認めた (**D**)。*は OP9 細胞の核を示す。

損傷部位の血流を十分回復させ，運動機能も正常に近い状態まで回復できる可能性を考えている。

同様にマウス ES 細胞に成長因子である IGFII 遺伝子を導入して誘導した骨格筋細胞を，前脛骨筋を損傷して作製した筋損傷モデルマウスに移植すると，溶媒のみを注入した対照の動物より運動機能が有意に改善するが，やはり正常の動物ほどは回復しない[5]。この場合も血管構成細胞を同時に移植し血流を増強すれば，より機能を高める可能性がある。脊髄損傷モデルマウスに上記の Mash1 導入細胞を移植した場合も，組織の観察では血管新生が少なく，機能改善を妨げていることが伺える[4]。

加えれば，血管や血管内皮細胞そのものの異常を伴う疾患では，例えばバージャー病や狭心症・心筋梗塞，さらには緑内障などでは，ES 細胞から分化誘導した血管内皮細胞そのものが治療応用可能である。

ES 細胞からの血管構成細胞の誘導

血球細胞と血管構成細胞は共通の幹細胞である血液血管芽細胞 (hemangioblast) から分化する[8-10]。

血管の形成は脈管形成 (vasculogenesis)，血管新生 (angiogenesis)，そして動脈形成を含む血管の成熟とリモデリング (arteriogenesis) の 3 段階に分けられる[11, 12]。ヒトでは胎生 17 日目に卵黄嚢の臓側板中胚葉において，この血液血管芽細胞が集合した血島の出現がみられ，内部の細胞は血球芽細胞となり，外側の細胞が血管内皮細胞となる。この血管内皮細胞が癒合し原始血管叢を形成する過程が，最初の脈管形成の段階となる。その後血管新生の過程では発芽や崁入によりよりきめ細かな血管網が形成され，最後のリモデリングの過程では動脈・静脈等の成熟血管の形成が行われる。このように血管内皮細胞は血管発生の初期段階から主役をなす細胞であ

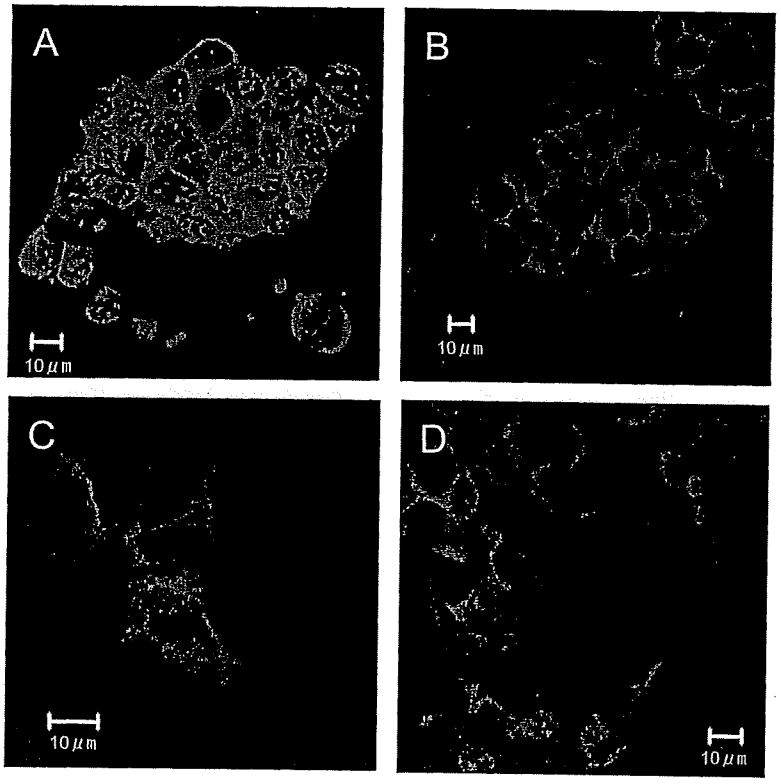

図2 血液血管芽細胞および血管内皮細胞特異的な分子マーカーの発現
A, B メチルセルロース処理後4日目 (**A**) およびその後 OP9 細胞と共培養 24 時間後 (**B**) のコロニーにおいて，血液血管芽細胞のマーカーである Flk-1 の発現を認めた。
C, D OP9 細胞と共培養 24 時間後のコロニーにおいて，血管内皮細胞のマーカーである CD31 (**C**) と VE-cadherin (**D**) の発現を認めた。VE-cadherin は特にコロニー周囲の扁平な細胞や管腔を形成した細胞に強く発現する傾向を認めた。

り，血管再生を成功させるためには，構造・機能共により自然発生に近い血管内皮細胞を試験管内で誘導することが鍵となると考えられる。既にマウスおよびサルの ES 細胞から Flk-1 陽性細胞が分化され，血管内皮細胞や壁細胞の誘導，そして試験管内・生体内での血管構築が報告されている[13-15]。またヒト ES 細胞からも試験管内で血管内皮細胞・壁細胞の誘導が報告されている[16-17]。しかし ES 細胞から誘導した他の細胞と共にモデル動物に移植し，血流改善を図る研究は殆ど行われていない。

ES 細胞からのメチルセルロース半固形化培養法を用いた血管内皮細胞および壁細胞の分化誘導

メチルセルロース (MC) を用いた半固形化培養法は，造血系の細胞の誘導に広く用いられている。我々はマウス ES 細胞をメチルセルロース中にて培養することにより，血液血管芽細胞および血管内皮細胞の誘導を試みた。未分化能を保持して継代維持培養したマウス ES 細胞を，0.8%メチルセルロースを含む培地で培養を開始すると，3日目頃より付着性の細胞が増生し，4日目には 1-2 層の細胞からなるシート状のコロニーを形成する(図 **1 A, B**)。同時に培地中またはこの付着細胞の上に乗った形で，類円形の細胞が分化し出現してくるのが認められ，これが血球系に分化した細胞と考えられる[7]。

これを op/op マウス由来の骨髄ストローマ細胞である OP9 細胞上で 24 時間共培養すると，各コロニーの辺縁の細胞が扁平化する傾向を認め，類円形の細胞を扁平化した細胞が囲む特徴的なコロニーの形態が認められた(図 **1C**)。ヒトでは胎生 17 日に

表1 血管構成細胞特異的な分子の mRNA の発現

	NC	ES	MC	OP9	MC+OP9
Flk-1	-	-	+	-	+
CD34	-	+	+	-	+
CD31	-	+	+	-	+
VE-cadherin	-	-	+	-	+
Ets-1	-	-	+	+	+
Vezf-1	-	-	+	-	+
Hex	-	-	+	-	+
EphrinB2	-	-	+	+	+
EphB4	-	-	+	+	+
nAChRα2	-	+	+	-	+
nAChRβ1	-	+	+	-	+
Tie2	-	+	+	-	+
PDGF-B	-	-	+	-	+
αSMA	-	+	+	+	+
βactin	-	+	+	+	+

NC, 陰性対照(DDW); ES, ES 細胞; MC, メチルセルロース処理細胞; OP9, OP9 細胞; MC+OP9, OP9 細胞と共培養したメチルセルロース処理細胞

卵黄嚢の臓側板中胚葉で血管系・血球系の起源となる血島が形成されるが，ここで増殖する血液血管芽細胞のコロニーでは，周辺部の細胞は扁平化して血管内皮細胞となり，内部に位置する細胞は血球芽細胞となる。

我々が誘導した細胞のコロニーは，この血島で形成されるコロニーに相似した形態をとることが分かった。またこの扁平化した細胞の一部は，互いに両端にて結合し管腔構造を形成した(図 1D)。発生過程において血管内皮細胞は互いに癒合して原始血管叢を形成するが，この過程に沿った誘導が試験管内で行われていると考えられた。

分化誘導した細胞に血管内皮または壁細胞に特異的な分子マーカーが発現しているか，RT-PCR 法および免疫染色にて検討した。血液血管芽細胞のマーカーと考えられる Flk-1 は[18]，マウス ES 細胞では発現しておらず，メチルセルロース処理細胞およびメチルセルロース処理後 OP9 と共培養後した細胞に発現を認めた(表 1)。即ち，ES 細胞はメチルセルロースと培養することで血液血管芽細胞に分化誘導されることが示唆された。

造血幹細胞と血管内皮細胞で発現される CD34 は ES 細胞とメチルセルロース処理後の細胞，OP9 と共培養後の細胞に発現を認めた。血管内皮のマーカーでは，VE-cadherin は Flk-1 と同様の発現を示したが，CD31 は ES 細胞から発現を認めた。

ニコチン性アセチルコリン受容体 (nicotinic acethylcholine receptor, nAChR) は α, α, β, γ または ε, δ のサブユニットからなる 5 量体であり，血管内皮細胞において a7 を含む nAChR の刺激により血管新生が促進されることが報告された[19]。nAChR の発現を α1–7, β1–4 のサブタイプについて検討したところ，α2 および β1 の発現をメチルセルロース処理細胞およびメチルセルロース処理後 OP9 細胞と共培養した細胞に認めた。血管新生を促進する Est-1[20]，Vezf-1[21]と血管新生を抑制する Hex[22]の発現も Flk-1 と同様に認めたが，Est-1 は OP9 細胞でも発現を認めた。

動脈・静脈を規定する分子機構として，受容体型チロシンキナーゼである erythropoietin producing human hepatocellular carcinoma(Eph) とそのリガンドの Ephrin (Eph-receptor-interacting protein) のシステムが重要である。EphrinB2 は動脈内皮細胞に発現しており[23]，一方静脈内皮細胞には EphB4

が特異的に発現している[24]。動脈系，静脈系のどちらかに欠損が生じても血管系のリモデリングに異常を来し，胎生期の成熟した血管系がうまく構成されない[25]。我々の誘導した細胞には EphrinB2, EphB 4 とも認められ，動脈系・静脈系いずれの内皮細胞も誘導されることが明らかになった。血管内皮細胞に発現され壁細胞の動員・解離に関わる Tie2 は，ES 細胞，メチルセルロース処理細胞，メチルセルロース処理後 OP9 細胞と共培養した細胞にて発現が認められた。内皮細胞等から分泌される PDGF は成熟した血管形成の為に壁細胞を増殖・動員する働きをもつが，この PDGF の B 鎖もメチルセルロース処理細胞，メチルセルロース処理後 OP9 細胞と共培養した細胞で発現が認められた。血管平滑筋細胞のマーカーとなる aSMA の発現は，OP9 細胞を含む全ての細胞において発現が認められた。メチルセルロース処理の段階にて既に壁細胞が誘導されてきていることが示唆される。蛋白レベルの発現を知る目的で免疫染色を行ったが，メチルセルロース処理細胞に Flk-1 の発現を認め，メチルセルロース処理後 OP9 細胞と共培養した細胞に血管内皮細胞のマーカーである VE-cadherin, CD31 の発現が認められた(図 2)。

これらの成績から，メチルセルロース処理および OP9 細胞との共培養により血液血管芽細胞および血管内皮細胞・壁細胞が誘導できることが示唆された。今後この細胞群について，さらに機能や分化に関する情報伝達機構等を解析すると共に，これらの細胞を直接，或いは 3 次元培養により血管を構築してから動物に移植し，体内での血管の構造や機能を検討していきたい。そして ES 細胞から誘導した神経細胞や骨格筋細胞と共にモデル動物に移植することにより，どの部位にどの血管(動・静脈，リンパ管)が形成され宿主の血管とどのようなメカニズムで交通するのか，正常の動物のレベルまで回復しなかった機能が血流の増加により十分機能が回復するかを検討する予定である。

おわりに

我々のこれまでのマウスを用いた検討から，ES 細胞から分化誘導した各種の細胞の移植がこれまで有効な治療法が無い疾患に大変有効であることが示された。ことに神経機能や骨格筋の機能回復には非常に有効であった。しかしながら，ES 細胞には奇形腫発生をはじめ，臨床応用を目指す際に，乗り越えて行かなければならない課題も多い。倫理的な課題も十分配慮する必要がある。

我々は将来の臨床応用を目指して，今後も必要な基礎的検討を積み上げていく予定である。

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Recent advances in corneal regeneration and possible application of embryonic stem cell-derived corneal epithelial cells

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Abstract: The depletion of limbal stem cells due to various diseases leads to corneal opacification and visual loss. The unequivocal identification and isolation of limbal stem cells may be a considerable advantage because long-term, functional recovery of corneal epithelium is linked to graft constructs that retain viable stem cell populations. As specific markers of limbal stem cells, the ATP-binding cassette, sub-family G, member2 (ABCG2), a member of the multiple drug-resistance (MDR) family of membrane transporters which leads to a side population phenotype, and transcription factor p63 were proposed recently. Conventional corneal transplantation is not applicable for patients with limbal stem cells deficiency, because the conventional allograft lacks limbal stem cells. The introduction of limbal epithelial cell transplantation was a major advance in the therapeutic techniques for reconstruction of the corneal surface. Limbal epithelial cell transplantation is clinically conducted when cultured allografts as well as autografts are available; however, allografts have a risk of immunologic rejection and autografts are hardly available for patients with bilateral ocular surface disorders. Embryonic stem (ES) cells are characterized by their capacity to proliferate indefinitely and to differentiate into any cell type. We induced corneal epithelial cells from ES cells by culturing them on type IV collagen or alternatively, by introduction of the pax6 gene into ES cells. Recent advances in our study supports the possibility of their clinical use as a cell source for reconstruction of the damaged corneal surface. This review summarizes the recent advances in corneal regeneration therapies and the possible application of ES cell-derived corneal epithelial cells.
Keywords: corneal epithelial cell, limbal stem cell, transplantation, embryonic stem cell

Introduction

Limbal stem cell deficiencies cause conjunctival epithelial ingrowth, neovascularization, chronic inflammation, recurrent epithelial erosions and defects, destruction of the basement membrane, and fibrous tissue ingrowth, leading to severe functional impairment (Tsai et al 1990; Tsubota et al 1995; Kruse and Reinhard 2001). The pathology includes Stevens-Johnson syndrome, ocular cicatricial pemphigoid, chemicals and thermal burns and radiation injury.

Proper visual function requires an intact ocular surface. The integrity of the corneal surface is maintained by two specialized epithelia, the conjunctival epithelium and the limbal corneal epithelium. Corneal epithelial stem cells reside in the palisades of Vogt, located in the basal layer of the limbus, coinciding with the transitional zone between the cornea and the bulbar conjunctiva (Schermer et al 1986; Cotsarelis et al 1989). Limbal stem cells produce undifferentiated progeny with limited proliferative potential that migrate centripetally from the periphery of the corneal epithelium to replace cells desquamating during normal cell turnover (Kinoshita et al 1981; Buck 1985; Kruse 1994; Beebe and Masters 1996; Lehrer et al 1998; Collinson et al 2003; Nagasaki and Zhao 2003).

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