Transplantation of Transduced Retinal Pigment Epithelium in Rats

Yoko Saigo, Toshiaki Abe, Masayoshi Hojo, Hiroshi Tomita, Eriko Sugano, and Makoto Tamai

Purpose. To examine the effects of transplanting retinal pigment epithelial (RPE) cells transduced with neurotrophic factor genes into the subretinal space of rats.

Methods. RPE cells were transduced with plasmids carrying the cDNAs of Axokine (ciliary neurotrophic factor [CNTF]; Sumitomo Pharmaceuticals Co., Ltd., Tokyo, Japan), brain derived-neurotrophic factor (BDNF), and basic fibroblast growth factor (bFGF) genes. These RPE cells were transplanted into the subretinal space of rats, and the localization was examined. The expression of enhanced green fluorescent protein (eGFP)-BDNF–transduced RPE in the subretinal space was examined by real-time polymerase chain reaction (PCR) after the transplanted cells were collected by cell sorting. The expression of major histocompatibility complex (MHC) class I and -II after gene transduction was examined by real-time PCR. The ratio of $CD4^+$ and $CD8^+$ T cells and antibody production against transplanted cells were analyzed by flow cytometry.

Results. The transplant sites were not significantly different among the neurotrophic factors tested. The RPE cells expressed the BDNF gene in the subretinal region at approximately the same level as that in vitro. RPE cells transduced with Axokine stimulated MHC-I expression, and the cell transplantation changed the ratio of $CD4^+$ and $CD8^+$ T cells. A significant production of antibody against the Axokine-transduced RPE cells was also observed after Axokine-transduced RPE transplantation.

Conclusions. RPE cells transduced with neurotrophic factors express the factors after transplantation into the subretinal space. RPE transduced with Axokine or bFGF, in contrast to RPE transduced with BDNF, stimulate an immunologic reaction of the host. (*Invest Ophthalmol Vis Sci.* 2004;45:1996–2004) DOI:10.1167/iovs.03-0777

Transplantation of appropriate cells into animals with experimental or inherited retinal diseases can lead to significant changes in the progress of the disease process.[1–3] Patients with retinitis pigmentosa[4–7] and age-related macular degeneration (AMD)[8–13] have been treated by the transplantation of different types of cells, and some of the cells have been shown not to be rejected from the subretinal space, although their effect on visual function is not always satisfactory. The cells transplanted were fetal retinal pigment epithelial (RPE) cells,[9] autologous RPE cells,[13] and autologous iris pigment epithelial (IPE) cells.[12,14]

Intravitreal injections of neurotrophic factors (e.g., growth factors and cytokines) have also been shown to rescue degenerating photoreceptor cells in animals.[15–18] However, several problems limit their clinical usefulness (e.g., how to deliver the neurotrophic factor to the appropriate site and the short half-life of neurotrophic factors).

We have hypothesized that RPE cells can be transduced with the genes of neurotrophic factors and that these RPE cells, when injected into the subretinal space, would remain at the injection site and express the neurotrophic factors. To test this hypothesis, we have inserted the genes of different neurotrophic factors into a plasmid vector with or without a reporter gene, transduced these vectors into RPE cells, and transplanted the RPE cells into the subretinal space of rats.

Materials and Methods

Preparation of Cultured Rat RPE Cells

RPE cells from Long-Evans rats were grown in a standard medium,[19] and cells that had just attained confluence were used for all experiments. The cells were used between passages 1 and 4. The cells were first examined with pan anti-cytokeratin antibody (monoclonal antibody mixture; Sigma-Aldrich, St. Louis, MO) to confirm that they were epithelial in origin, as we have reported.[19]

Insertion of cDNA of Axokine, BDNF, and bFGF into Plasmids

The cDNA of rat brain-derived neurotrophic factor (BDNF) was generously supplied by Atsushi Takeda (Department of Neurology, Tohoku University, Miyagi, Japan).[20] The cDNA of basic fibroblast growth factor (bFGF) was supplied by Takeda Pharmaceuticals Co., Ltd. (Osaka, Japan) and the cDNA of Axokine was obtained from Sumitomo Pharmaceuticals Co., Ltd. (Tokyo, Japan).

These cDNAs were inserted into the plasmid vector pIRES2EGFP (eGFP-vector; BD Biosciences-Clontech, Palo Alto, CA) using multiple cloning sites.[21] This vector carried the G418 gene for anti-geneticin activity, the enhanced green fluorescence protein (eGFP) gene as a reporter gene, and an internal ribosomal entry site (IRES). The cells successfully transduced by the vector were selected by geneticin (100 μg/mL), and the expression of the different cDNAs was detected by the presence of green fluorescence. The cDNAs were also inserted into the plasmid Topo TA expression vector without eGFP (Invitrogen, San Diego, CA) but with an anti-bleomycin/phleomycin family gene for Zeocin (Invitrogen). The RPE cells successfully transduced were selected by growing the RPE cells in medium containing the antibiotic (25 μg/mL).

For control, the survival of nontransduced RPE cells incubated in various concentrations of Zeocin and geneticin were examined in a

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TABLE 1. Conditions for Real-Time PCR

	bFGF	BDNF	MHC-I	MHC-II	β-actin
Denaturation temp. (30 sec; °C)	94	94	94	94	94
Cycle	1	1	1	1	1
$MgCl_2$ (mM)	3.5	4.5	4	4	4.5
denaturation temp. (15 sec; °C)	94	94	94	94	94
annealing temp. (30 sec; °C)	59	59	60	57	60
extension temp. (30 sec; °C)	72	72	72	72	72
melting curve temp. (10 sec; °C)	82.5	87	86	85	86
Cycle	35	35	35	35	35

one-solution cell-proliferation assay (Cell titer96 AQueous; Promega Co., Madison, WI).[21]

Expression of Neurotrophic Factors by RPE-Transduced Cells

Reverse transcription–polymerase chain reaction (RT-PCR) and real-time PCR were performed to determine whether the neurotrophic factors were expressed by the transduced RPE cells. mRNA and cDNA were extracted from transduced and nontransduced RPE cells according to the manufacturer's protocol (Pharmacia Biotech Inc., Uppsala, Sweden). PCR was performed[22] with an annealing temperature dependent on the primer used: MHC-I, 58°C; MHC-II, 60°C; Axokine, 58°C; and β-actin, 57°C.

Real-time PCR was also performed to determine whether bFGF, BDNF, rat MHC-I, MHC-II, and β-actin genes were expressed. The PCR products were quantified by green fluorescent dye (SYBR Green I; BMA, Rockland, ME)[23] and a fluorescence-detecting thermal cycler (Smart Cycler; TaKaRa, Kyoto, Japan). The optimal conditions for amplification of each gene are listed in Table 1. After amplification, the PCR products were confirmed by melting-curve analysis[24] and gel electrophoresis. The concentrations were expressed as a ratio to that of β-actin.

For positive control of MHC-I and II, cDNAs from the spleen of Long-Evans rat were isolated and processed as with the growth factors.[25]

Primers

The primers for Axokine,[26] rat bFGF,[27] rat BDNF,[20] rat MHC-I[28] (GenBank accession number, M11071; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), rat MHC-II[29] (GenBank accession number, U65218), and β-actin[30] are listed in Table 2.

Quantification of Axokine, bFGF, and BDNF Expression

The amount of Axokine (DNT00; R&D Systems Inc., Minneapolis, MN), bFGF (DFB50; R&D Systems), and BDNF (Promega Co.) proteins expressed by the transduced RPE cells was quantified. Axokine is an analogue of human ciliary neurotrophic factor (CNTF)[31] and its concentration was determined as if it was human CNTF.

The color of the reaction products was measured with a microplate reader (MAXline; Molecular Devices Corp., Sunnyvale, CA). The total protein concentration was determined by a protein assay kit (Pierce, Rockford, IL). The measurements were made in duplicate.

TABLE 2. Sequences of Each Primer Set

Gene	Sequences	Amplification Size (bp)
Axokine	5′-ATGGCTTTCACAGAGCATTC-3′ 5′-ATTCCTATGGGATCCCAGTC-3′	572
BDNF	5′-GGAATTCCAGAGTGATGACC-3′ 5′-GGAATTCCTCCACTATCTTC-3′	130
bFGF	5′-GAGGAGTTGTGTCCATCAAGG-3′ 5′-TAGTTATTGGACTCCAGGCG-3′	359
MHC-I	5′-TTACACATGCCTTGTGGAGC-3′ 5′-TGGATGTCACAGGAGAGACC-3′	337
MHC-II	5′-GTCTGCAGACACAACTACGAGG-3′ 5′-TAAGCTGTGTGGACACGACC-3′	214
β-actin	5′-CTACAATGAGCTGCGTGTGG-3′ 5′-CGGTGAGGATCTTCATGAGG-3′	313

Animals, Transplantation, and Histologic Procedures

All procedures were preformed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Gene-transduced or nontransduced RPE cells were injected into the superior subretinal space of the right eye of anesthetized Sprague-Dawley rats with a 30-gauge needle (Hamilton, Reno, NV) .[22] The weight of the rats was approximately 250 g, and the age at the time of transplantation was approximately 3 months. Seventy-eight rats were used. Four rats were discarded because of subretinal or vitreous hemorrhage, and three rats died during the course of the experiments. Rats were anesthetized with an injection of pentobarbital sodium (60 mg/kg) followed by an injection of atropine sulfate (0.4 mg/kg). The needle passed through the sclera, and $4 \times 10^4/2$ μL cells were injected. For a control, the same volume of Hank's balanced salt solution (HBSS) was injected. Rats without injection were also examined as a control of the injection procedures.

The retina was examined by indirect ophthalmoscopy after the injection, and eyes showing massive subretinal hemorrhage, vitreous hemorrhage, or large retinal detachments were discarded.

Seven days after transplantation, the eyes were enucleated and fixed in 2% paraformaldehyde ($n = 4$ for each type of gene), and wholemounts of the RPE-choroid-scleral were examined. To determine the extent of the transplant site, the flatmounts were examined and photographed by fluorescence microscope (model DMIRE2; Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). The area of the fluorescence was analyzed by computer (IPLab; Scanalytics, Inc., Fairfax, VA).

Frozen sections (10 μm) were made, with the sections passing through the optic nerve head and the region of transplantation to the ora serrata. The sections were placed on poly-L-lysine–coated slides and photographed.

Papain Dissociation and Cell Sorting

One week after transplantation, rats were killed, and the eyes were enucleated. After removal of the anterior segments, the eyecups were treated with papain/DNase solution (20 U/mL papain, 0.5 mM EDTA, 1 mM L-cysteine, 1000 U/mL DNase) for 30 minutes at 37°C followed by trituration. The cells were recovered by centrifugation at 300*g* for 5 minutes at room temperature (Worthington, Freehold, NJ). The cells showing green fluorescence (eGFP-BDNF) were collected by a cell-sorting system (FACS Vantage; Japan BD Biosciences, Ltd., Tokyo, Japan).

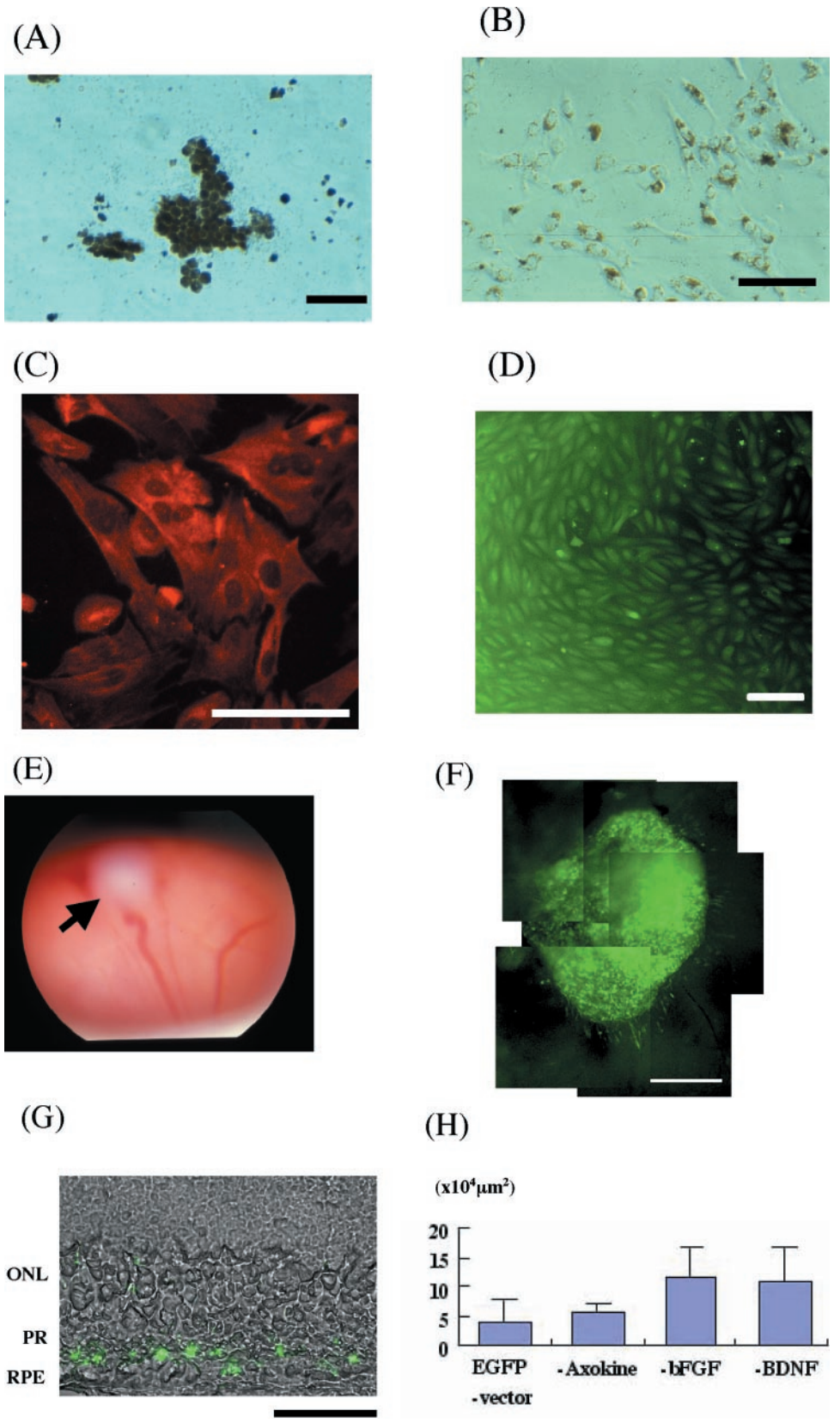

FIGURE 1. (**A**, **B**) Primary and first-passaged RPE cells. (**C**) Anti-cytokeratin staining of the cultured RPE. (**D**) Fluorescence microscopy showing eGFP expression spreading throughout the cells. (**E**) Fundus photograph 1 day after transplantation. Mild elevation of the retina was observed at the transplant site (*arrow*). (**F**) The area of transplanted cells with eGFP. (**G**) Cross section of the transplant site. Transplanted cells were present between the neural retina and the RPE. ONL, outer nuclear layer; PR, photoreceptors; RPE, retinal pigment epithelium. (**H**) Area of eGFP vector–, Axokine-, bFGF-, and BDNF cDNA–transduced RPE cells 7 days after transplantation. bFGF- and BDNF-cDNA–transduced RPE covered a wider area in eyes receiving transplants of BDNF- and bFGF-transduced RPE cells, although the difference was not significant. Scale bars: (**A**–**D**) 100 μm; (**F**) $\times$400 μm; (**G**) 50 μm.

Flow Cytometry of $CD4^+$ and $CD8^+$ T Cells

Mononuclear cells from the RPE-transplant recipients were isolated by placing the blood cells over a single-density gradient (Ficoll-Paque; BD Biosciences, Lincoln Park, NJ) and centrifuging at 400*g*. The mononuclear cells at the interface were collected and washed with Dulbecco's phosphate buffered saline (DPBS) and stored at −80°C. For analysis,

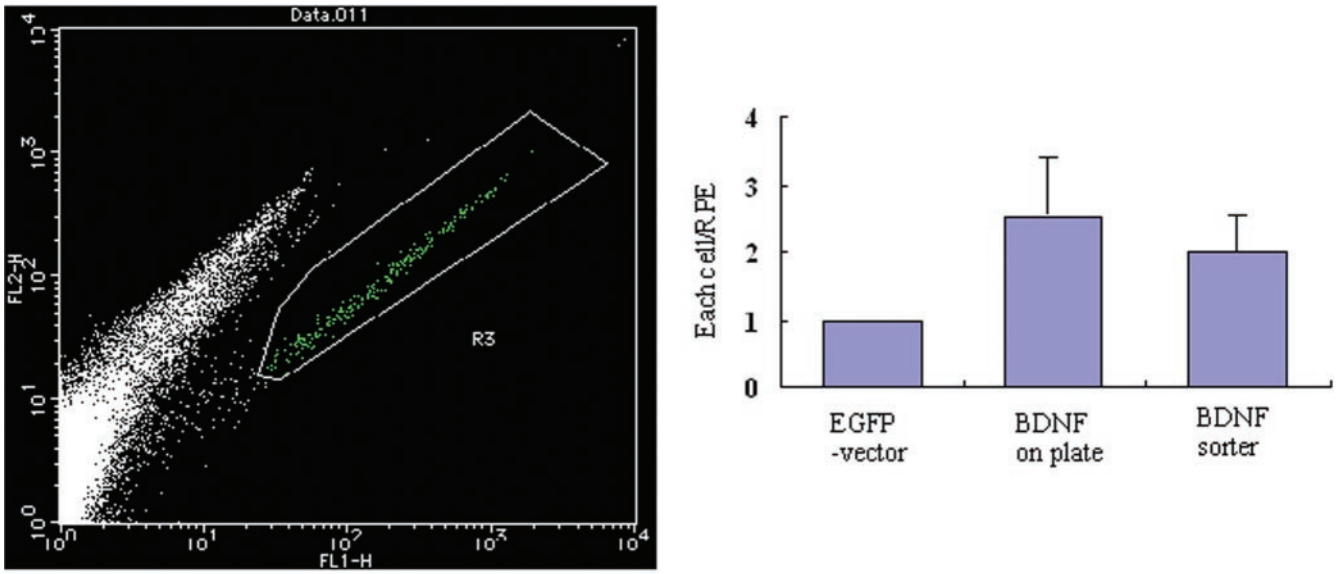

FIGURE 2. Transplanted cells with enhanced green fluorescence protein and BNDF genes (EGFP-BDNF) were collected by a cell-sorting system. (**A**) Dot plot representing gated cells recovered from rat eyecups is shown. EGFP-BDNF cells showing fluorescence (*green dots* in *white outline*) were collected by flow cytometry. (**B**) The expression of the BDNF gene was examined by real-time PCR, and the results are presented as the ratio against β-actin. The expression of BDNF gene in culture (BDNF on plate) and that recovered from the subretinal space (BDNF by sorter; $n = 3$) are compared with that of enhanced green fluorescence protein gene–transduced RPE (EGFP vector; $n = 4$).

the lymphocytes were washed with 3% fetal bovine serum in PBS (3% FBS-PBS) three times and exposed to phycoerythrin (PE)-conjugated mouse anti-rat CD8 monoclonal antibody (BD Biosciences) or fluorescein isothiocyanate (FITC)–conjugated mouse anti-rat CD4 monoclonal antibody (BD Biosciences) on ice for 30 minutes. The samples were then sorted on a flow cytometer (FACSCalibur Hg; BD Biosciences).

Flow Cytometry of Serum

Rat serum was collected by venous puncture on the same day as the enucleation and centrifuged at 3500 rpm. RPE cells were collected in cold PBS, and 1×10^5 cells/mL were incubated with each serum examined, in 3% FBS-PBS on ice for 30 minutes. After the cells were washed with 3% FBS-PBS, they were incubated with phycoerythrin (PE)-labeled anti-rat IgG (anti-Rat IgG [H+L] PE conjugate [goat]; Jackson ImmunoResearch Laboratory, West Grove, PA) for 30 minutes on ice. Each sample was analyzed by flow cytometer. The cells that reacted with the serum showed stronger PE fluorescence and shifted to the intense fluorescence area when compared with that of control cells. The percentage of positive shift was calculated and compared.

Statistical Analysis

Statistical significance was determined by the Fisher protected least significant difference (PLSD) test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Rat RPE cells were successfully obtained and passaged (Fig. 1A, B). More than 99% of the cultured RPE cells were positive for anti-cytokeratin (Fig. 1C). Cells that did not incorporate the plasmids were killed within 7 days with 25 μg/mL of Zeocin and 400 μg/mL geneticin in the medium (data not shown).[21] The fluorescence from eGFP spread throughout the transduced cells (Fig. 1D), and the fluorescence pattern and the cell shapes were similar for each neurotrophic factor.

The transplant region of the rats was identified by indirect ophthalmoscopy and photographed during the successive follow-up examinations (Fig. 1E). The fluorescence of eGFP was clearly observed at the site of the transplantation (Fig. 1F) in the subretinal space (Fig. 1G). The size of the area showing fluorescence 7 days after transplantation covered a wider area in the eyes transplanted with BDNF- and bFGF-transduced RPE cells, although the difference was not significant (Fig. 1H).

One week after transplantation of RPE cells transduced with eGFP-BDNF, the transplanted cells were recovered by cell sorting. A dot plot representing the gated population of green eGFP-BDNF–positive cells (shown as green dots in white line in Fig. 2A) were collected by flow cytometry. In these eyes ($n = 3$), the expression of the BDNF gene was approximately 2.2 times higher than that of eyes injected with eGFP vector alone by real-time PCR ($P = 0.0302$, $n = 4$; Fig. 2B). However, the level of expression was somewhat lower than that of cells in culture.

To control for eGFP, RPE cells transduced with Topo TA vector alone, or with Axokine, bFGF, or BDNF were collected, and the degree of expression of each gene was determined by RT-PCR or real-time PCR. Axokine was detected only in the Axokine-transduced RPE cells because Axokine is not expressed in normal rat cells (Fig. 3A).

bFGF and BDNF are endogenously expressed in normal cultured rat RPE cells and were quantified by real-time PCR. The expression of BDNF by BDNF-transduced RPE cells was about three times higher than that of nontransduced or vector-transduced RPE cells. The expression of BDNF by Axokine or bFGF transduced cells was less than that of cultured cells or vector-transduced RPE cells (Fig. 3B). In contrast, bFGF-transduced RPE cells expressed an approximately 30 times higher level of bFGF than did nontransduced RPE cells. In addition, Axokine-transduced RPE cells expressed approximately nine times higher levels of bFGF than dud nontransduced RPE cells. Vector-transduced RPE cells also enhanced the expression of the bFGF gene (Fig. 3C).

The degrees of expression of the different neurotrophic factors by RPE cells transduced with vector, Axokine, BDNF, and bFGF genes were also determined by ELISA. The Axokine-transduced RPE cells expressed 373.9 pg CNTF/mg protein,

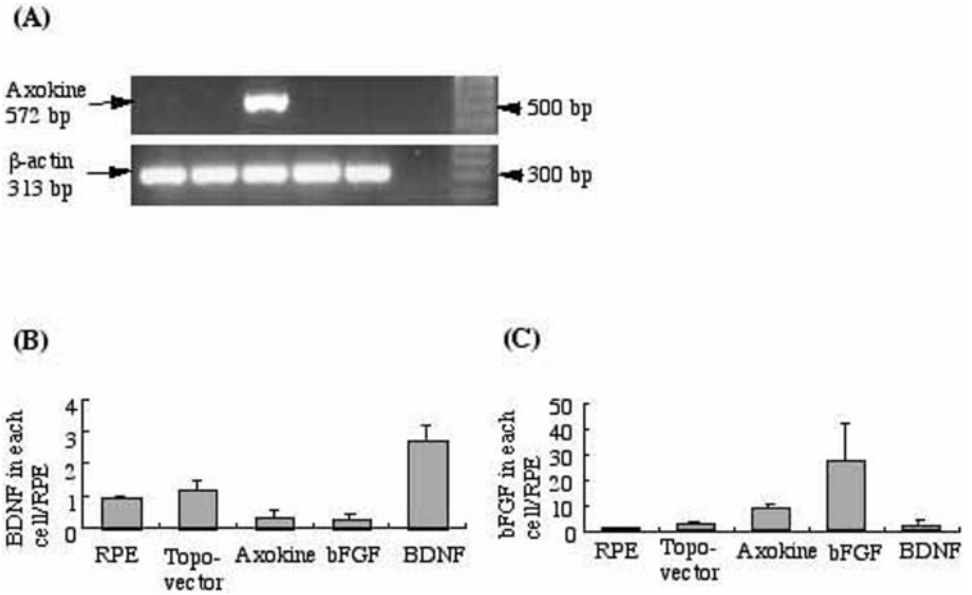

FIGURE 3. (**A**) RT-PCR of Axokine after each type of gene-transduced RPE cell is shown. *Bottom*: results of β-actin for gene-transduced RPE cells. *Lane 1*: results from nontransduced cultured RPE cells; *lane 2*: vector-; *lane 3*: Axokine-; *lane 4*: bFGF-; and *lane 5*: BDNF-transduced RPE cells; *lane 6*: negative control without reverse-transcription; and *lane 7*: 100-bp marker. Expression was observed only in Axokine-transduced RPE cells. (**B**) Real-time PCR to determine bFGF gene expression. The cells, after bFGF gene transduction, showed approximately 30 times higher bFGF gene expression than did normal cultured RPE cells. Axokine gene-transduced RPE cells also showed approximately nine times higher bFGF gene expression than did normal cultured RPE. The *x*-axis represents each cell type and the *y*-axis represents the ratio of bFGF gene expression to that in nontransduced cultured rat RPE cells (each cell/RPE). (**C**) Real-time PCR of BDNF. Cells after BDNF gene transduction showed approximately three times higher BDNF gene expression than nontransduced RPE or vector-transduced RPE cells. The *x*-axis represents each cell type, and the *y*-axis represents the ratio of BDNF gene expression to that of nontransduced cultured rat RPE.

and vector-transduced RPE cells expressed 28.6 pg CNTF/mg protein. bFGF-transduced RPE cells expressed 311.5 pg bFGF/mg protein, and vector-transduced RPE cells expressed 10.0 pg bFGF/mg protein. BDNF-transduced RPE cells expressed 95.2 pg BDNF/mg protein, and 32.0 pg BDNF/mg protein was expressed in vector-transduced RPE cells.

The effects of neurotrophic factor gene transduction on the expression of MHC-I and -II on RPE cells are shown in Figures 4A and 4B. The level of PCR products from the sample and spleen of Long-Evans rats (positive control) were the same by sequence analysis (model 310; Applied Biosystems, Foster City, CA; data not shown). Real-time PCR demonstrated that the Axokine-transduced RPE cells enhanced the expression of MHC-I 14 times more than did nontransduced RPE cells (Fig. 4C). RPE cells transduced with the bFGF gene enhanced the expression of MHC-II four times more than did nontransduced RPE (Fig. 4D). The vector alone or BDNF-transduced RPE cells did not enhance expression of MHC-I or -II.

The proportion of $CD8^+$ and $CD4^+$ T cells is presented as the $CD8^+$-to-$CD4^+$ T-cell ratio. The proportion of T cells in the rat after Axokine- and BDNF-transduced RPE transplantation is shown in Figures 5A and 5B, respectively. The average CD8-to-CD4 T-cell ratio for each neurotrophic factor is shown in Figure 5C. The ratio of $CD8^+$ to $CD4^+$ T cells was higher after Axokine-transduced RPE transplantation tha^n after the other types of neurotrophic factor- or vector-transduced RPE transplantation, but the differences were not statistically significant ($P = 0.0559$ in Axokine-transduced RPE against cultured rat RPE).

The percentage of the cells that reacted with each serum and shifted to the stronger fluorescein area by flow cytometer is shown in Table 3. No significant reaction was observed between the sera from age-matched normal SD rats and the nontransduced or neurotrophic factor gene-transduced rat RPE cells obtained by flow cytometer (Fig. 6A). No significant reaction was observed between the sera from the cell transplant recipients and those with the nontransduced RPE cells (Table 3). None of the sera obtained after transplantation of neurotrophic factor gene-transduced RPE cells showed a positive shift against the transplanted cells except Axokine-transduced RPE. A statistically significant positive shift (percentage of antibody-complexed cells) was observed from the sera after

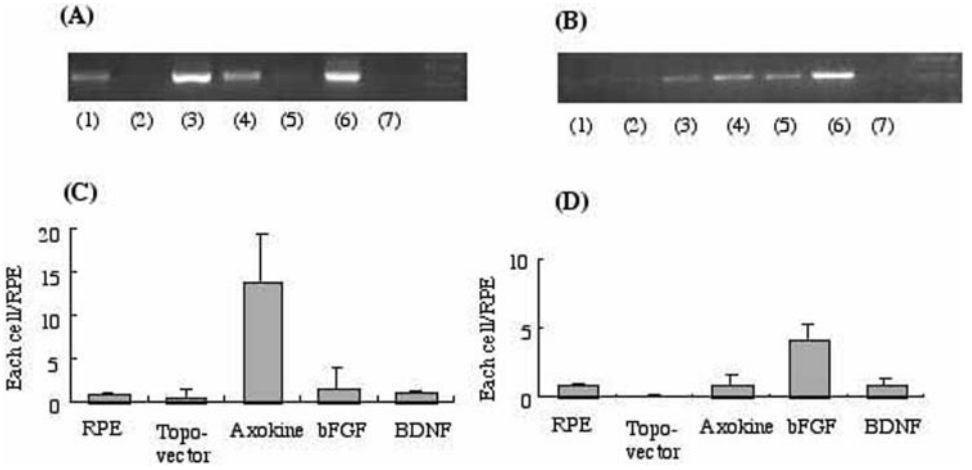

FIGURE 4. Results of RT-PCR of MHC-I (**A**) and -II (**B**) are shown. Contents of each lane are as described in Figure 3A. The results of real-time PCR of MHC-I (**C**) and -II (**D**) are also shown. Axokine appeared to enhance the MHC-I expression by approximately 14 times that of nontransduced RPE. bFGF seemed to enhance MHC-II expression by approximately four times that of noncultured RPE.

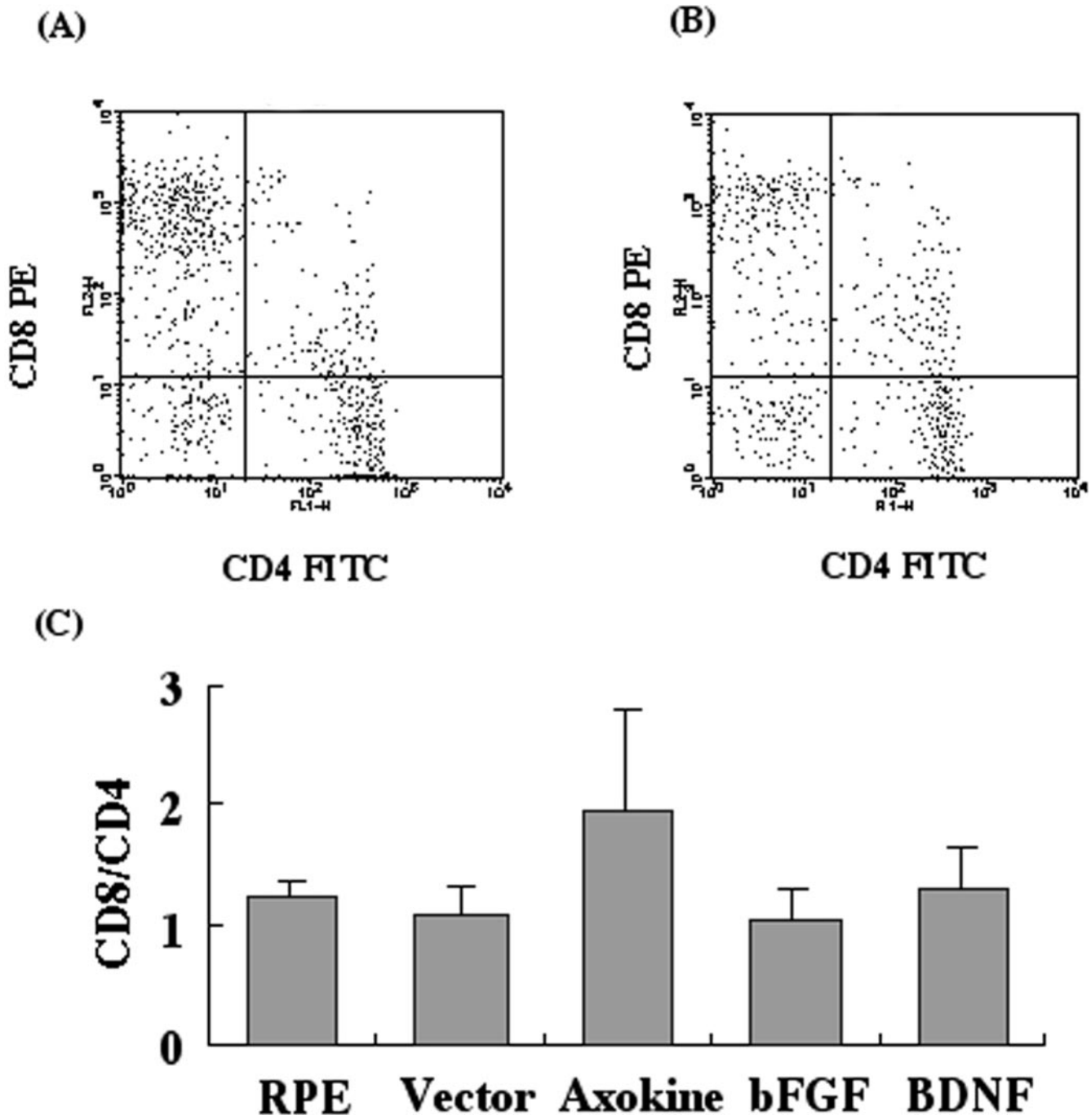

FIGURE 5. Ratio of $CD8^+$ to $CD4^+$ T cells determined by flow cytometry. Shown are results after (**A**) Axokine- and (**B**) BDNF-transduced RPE cells. The *x*-axis represents the $CD4^+$ T cells and the *y*-axis the $CD8^+$ T cells. (**C**) The difference was not statistically significant.

Axokine-transduced RPE transplantation (Fig. 6B) than after transplantation of nontransduced, vector-transduced, bFGF-, and BDNF-transduced RPE ($P = 0.0079$, 0.0131, 0.0120, and 0.0030, respectively; Fig. 6C, Table 3).

DISCUSSION

Crafoord et al.[32] reported that autologous cells transplanted into the subretinal space of rats remain at the site, although another group reported that transplanted Schwann cells tend to migrate from the subretinal space.[33] In our preparations, fluorescence microscopy 1 week after transplantation demonstrated that the transplant area was not significantly different with the different types of transplanted cells and the transplanted RPE cells remained at the transplant site.

Our results also showed that the transplanted RPE cells expressed each type of neurotrophic factor for at least 7 days. The RPE cells transduced with eGFP-BDNF expressed higher levels of BDNF than RPE cells transduced with only the eGFP vector. The BDNF level, however, was somewhat less than that of RPE cells in culture, which suggests that the microenviron-

TABLE 3. Results of Analysis between the Sera from Rats and the RPE Cells by Flow Cytometry

Serum Source	RPE with or without Neurotrophic Factor Gene				
	Nontransduced	Vector Transduced	Axokine Transduced	bFGF Transduced	BDNF Transduced
Age-matched normal rats (n = 4)	1.07	0.97	1.14	1.55	0.81
Nontransduced RPE transplant (n = 7)	0.89	ND	ND	ND	ND
Vector-transduced RPE transplant (n = 7)	0.89	2.05	ND	ND	ND
Axokine-transduced RPE transplant (n = 7)	3.11	ND	11.41	ND	ND
bFGF-transduced RPE transplantation (n = 7)	1.41	ND	ND	2.21	ND
BDNF-transduced RPE transplantation (n = 7)	0.70	ND	ND	ND	1.14

Each number indicates the percentage of the RPE cells shifted to the stronger fluorescein area by flow cytometry. ND, examination not performed.

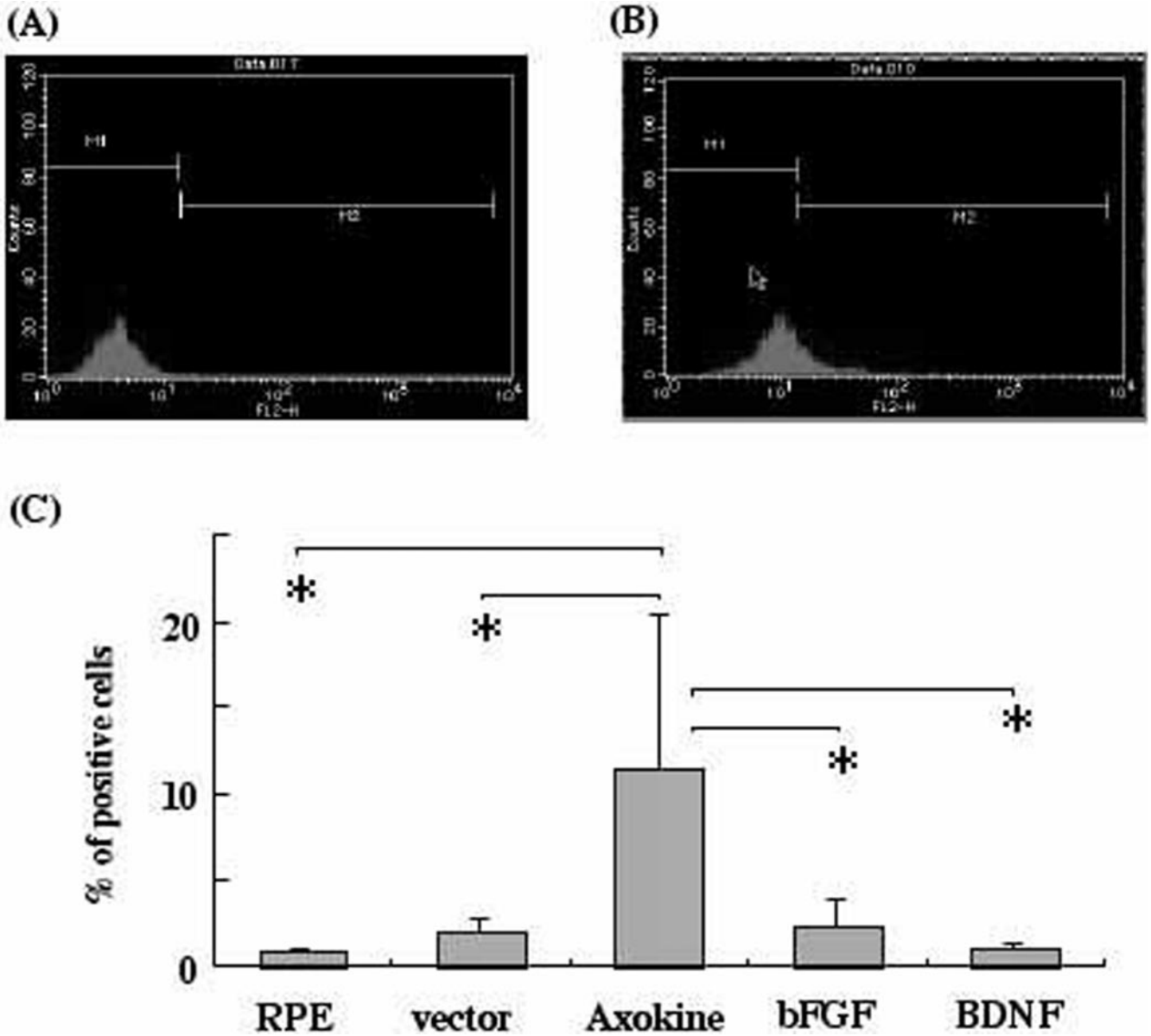

FIGURE 6. The results of serum reacted with the transplanted cells and analyzed by flow cytometry. (**A**) Normal Sprague-Dawley rat serum did not react with the cultured Long-Evans rat RPE. (**B**) Serum after Axokine gene–transduced rat RPE transplantation shows a shift of cells to a more intense area of fluorescence than that of control. (**C**) A statistically significant difference was observed in the serum after Axokine-transduced RPE transplantation when compared with those of serum of other gene-transduced transplanted cells. The *x*-axis represents the fluorescence intensity of PE (shown as log intensity) and the *y*-axis represents the number of cells.

ment of the subretinal space may influence the expression of this factor.

Because some investigators have reported that the eGFP reporter gene may also affect the results,[34,35] we also constructed neurotrophic-factor–transduced RPE cells without eGFP. These cells showed enhanced bFGF gene expression after transduction with bFGF or Axokine. BDNF-transduced cells expressed higher levels of BDNF than cells transduced by the other factors, but expressed less bFGF than nontransduced or vector alone–transduced cells. RPE cells transduced by the Axokine and bFGF genes also expressed less BDNF than that of nontransduced RPE cells. These results suggest that each neurotrophic factor has a synergistic effect on the expression of other genes. A synergistic effect between bFGF and Axokine was also reported by Chong et al.[36]

MHC-I and -II are major molecules expressed on grafted tissue,[37–39] and RPE cells have been reported to express these molecules when stimulated by IFN-γ[40] and lymphokine.[41] The expression of these molecules on RPE cells may explain the rejection of transplanted RPE cells[42] by T cells.[43]

Our results showed that the transduction of BDNF gene into RPE cells did not enhance the expression of MHC-I or -II. The expression of MHC-I and -II also was not enhanced on the cells recovered by cell sorting from eyes that had been transplanted with eGFP-BDNF–transduced RPE cells into the subretinal space.

Axokine-transduced RPE cells, on the other hand, enhanced the expression of MHC-I molecule on RPE cells. Although Axokine has been reported to delay photoreceptor degeneration in some animal models,[18,36] the side effects, such as subcapsular cataract, mild retinal folds,[36] and reduced amplitude of electroretinograms,[44] have also been reported.

Recognition of the MHC-I molecule may activate host T-cells,[39,43,45] although histologic examinations in our preparations did not show any inflammatory cells at the transplant site. However, when we transplanted human RPE cells that had undergone Axokine gene transduction, strong inflammation was observed at the transplant site (data not shown). The induction of MHC-I antigen and the higher ratio of $CD8^+$ to $CD4^+$ cells may also influence the effectiveness of Axokine-transduced RPE cell transplantation.

The effect of the mild enhancement of MHC-II expression in the bFGF-transduced RPE cells may also affect bFGF-transduced RPE cell transplantation. bFGF has also been reported to induce inflammation when delivered intravitreally.[46]

Although many researchers have reported the absence of acute immune rejection after allotransplantation,[25,47,48] the results of our evaluation of the transplant site at 7 days may not have been influenced by systemic immunity against the transplant. Although histologic examination showed no inflammation, our results may show that immunologic reaction of the host against some of the neurotrophic factor gene-transduced RPE could occur. A longer follow-up time than 1 week will be necessary to determine the effectiveness of the neurotrophic factor gene-transduced RPE transplantation.

Genetically modified RPE cells could present other antigens that are prerequisites for immune recognition by T lymphocytes.[40] Thus, we examined the antibody production against the transplanted cells using sera from rats after the cell transplantation. A statistically significant antibody production after Axokine-transduced RPE transplantation was found, and this may also affect the results of the Axokine-transduced RPE transplantation. We showed the results relatively early after transplantation, and further analysis at longer follow-up times is also important.[25]

In this experiment, we showed the results of a limited number of neurotrophic factor genes, selected host reactions, and short periods of observation after transplantation. From these experiments, we showed that the cDNAs of different neurotrophic factors can be transduced into RPE cells, and the transplantation of these cells will remain at the site of transplantation and express the neurotrophic factors. However, some of the neurotrophic factors can stimulate local and systemic immunologic reactions. Our results showed that RPE cells transduced with the BDNF gene did not stimulate MHC expression on the RPE and did not induce antibody production after 7 days of transplantation. Additional analysis of local and systemic reactions at later times after transplantation is ongoing in our laboratory.

References

1. Li LX, Turner JE. Inherited retinal dystrophy in the RCS rat: prevention of photoreceptor degeneration by pigment epithelial cell transplantation. *Exp Eye Res.* 1988;47:911–917.
2. Sheedlo HJ, Li L, Turner JE. Photoreceptor cell rescue in the RCS rat by RPE transplantation: a therapeutic approach in a model of inherited retinal dystrophy. *Prog Clin Biol Res.* 1989;314:645–658.
3. Yamaguchi K, Yamaguchi K, Gaur VP, Turner JE. Retinal pigment epithelial cell transplantation into aging retina: a possible approach to delay age-related cell death. *Jpn J Ophthalmol.* 1993; 37:16–27.
4. Kaplan HJ, Tezel TH, Berger AS, Wolf ML, Del Priore LV. Human photoreceptor transplantation in retinitis pigmentosa: a safety study. *Arch Ophthalmol.* 1997;115:1168–1172.
5. Das T, del Cerro M, Jalali S, et al. The transplantation of human fetal neuroretinal cells in advanced retinitis pigmentosa patients: results of a long-term safety study. *Exp Neurol.* 1999;157:58–68.
6. Radtke ND, Aramant RB, Seiler M, Petry HM. Preliminary report: indications of improved visual function after retinal sheet transplantation in retinitis pigmentosa patients. *Am J Ophthalmol.* 1999;128:384–387.
7. Humayun MS, de Juan E Jr, del Cerro M, et al. Human neural retinal transplantation. *Invest Ophthalmol Vis Sci.* 2000;41:3100–3106.
8. Peyman GA, Blinder KJ, Paris CL, et al. A technique for retinal pigment epithelium transplantation for age-related macular degeneration secondary to extensive subfoveal scarring. *Ophthalmic Surg.* 1991;22:102–108.
9. Algvere PV, Berglin L, Gouras P, Sheng Y, Kopp ED. Transplantation of RPE in age-related macular degeneration: observations in disciform lesions and dry RPE atrophy. *Graefes Arch Clin Exp Ophthalmol.* 1997;235:149–158.
10. Weisz JM, Humayun MS, De Juan E Jr, et al. Allogenic fetal retinal pigment epithelial cell transplant in a patient with geographic atrophy. *Retina.* 1999;19:540–545.
11. Abe T, Yoshida M, Tomita H, et al. Functional analysis after auto iris pigment epithelial cell transplantation in patients with age-related macular degeneration. *Tohoku J Exp Med.* 1999;189:295–305.
12. Thumann G, Aisenbrey S, Schraermeyer U, et al. Transplantation of autologous iris pigment epithelium after removal of choroidal neovascular membranes. *Arch Ophthalmol.* 2000;118:1350–1355.
13. Stanga PE, Kychenthal A, Fitzke FW, et al. Retinal pigment epithelium translocation after choroidal neovascular membrane removal in age-related macular degeneration. *Ophthalmology.* 2002;109: 1492–1498.
14. Abe T, Yoshida M, Tomita H, et al. Auto iris pigment epithelial cell transplantation in patients with age-related macular degeneration: short-term results. *Tohoku J Exp Med.* 2000;191:7–20.
15. Faktorovich EG, Steinberg RH, Yasumura D, Matthes MT, LaVail MM. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature.* 1990;347:83–86.
16. LaVail MM, Unoki K, Yasumura D, et al. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc Natl Acad Sci USA.* 1992; 89:11249–11253.

17. Unoki K, LaVail MM. Protection of the rat retina from ischemic injury by brain-derived neurotrophic factor, ciliary neurotrophic factor, and basic fibroblast growth factor. *Invest Ophthalmol Vis Sci.* 1994;35:907–915.
18. LaVail MW, Yasumura D, Matthes MT, et al. Protection of mouse photoreceptors by survival factors in retinal degenerations. *Invest Ophthalmol Vis Sci.* 1998;39:592–602.
19. Abe T, Sato M, Tamai M. Dedifferentiation of the retinal pigment epithelium compared to the proliferative membranes of proliferative vitreoretinopathy. *Curr Eye Res.* 1998;17:1103–1109.
20. Takeda A, Onodera H, Sugimoto A, et al. Coordinated expression of messenger RNAs for nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 in the rat hippocampus following transient forebrain ischemia. *Neuroscience.* 1993;55:23–31.
21. Kano T, Abe T, Tomita H, et al. Protective effect against ischemia and light damage of iris pigment epithelial cells transfected with the BDNF gene. *Invest Ophthalmol Vis Sci.* 2002;43:3744–3753.
22. Abe T, Takeda Y, Yamada K, et al. Cytokine gene expression after subretinal transplantation. *Tohoku J Exp Med.* 1999;189:179–189.
23. Schnerr H, Niessen L, Vogel RF. Real time detection of the tri5 gene in Fusarium species by light cycler-PCR using SYBR Green I for continuous fluorescence monitoring. *Int J Food Microbiol.* 2001;71:53–61.
24. Hiratsuka M, Kishikawa Y, Narahara K, et al. Detection of angiotensin-converting enzyme insertion/deletion polymorphisms using real-time polymerase chain reaction and melting curve analysis with SYBR Green I on a GeneAmp 5700. *Anal Biochem.* 2001; 289:300–303.
25. Zhang X, Bok D. Transplantation of retinal pigment epithelial cells and immune response in the subretinal space. *Invest Ophthalmol Vis Sci*. 1998;39:1021–1027.
26. Negro A, Corsa V, Corona G, et al. Structure-function studies of human ciliary neurotrophic factor. *Neurochem Res.* 1994;19:223–227.
27. Kurokawa T, Seno M, Igarashi K. Nucleotide sequence of rat basic fibroblast growth factor cDNA. *Nucleic Acids Res.* 1988;16:5201.
28. Kastern W. Characterization of two class I major histocompatibility rat cDNA clones, one of which contains a premature termination codon. *Gene.* 1985;34:227–233.
29. Syha-Jedelhauser J, Wendling U, Reske K. Complete coding nucleotide sequence of cDNA for the class II RT1: B beta I chain of the Lewis rat. *Biochim Biophys Acta.* 1991;1089:414–416.
30. Abe T, Durlu YK, Tamai M. The properties of retinal pigment epithelial cells in proliferative vitreoretinopathy compared with cultured retinal pigment epithelial cells. *Exp Eye Res.* 1996;63: 201–210.
31. Panayotatos N, Radziejewska E, Acheson A, et al. Exchange of a single amino acid interconverts the specific activity and gel mobility of human and rat ciliary neurotrophic factors. *J Biol Chem.* 1993;268:19000–19003.
32. Crafoord S, Geng L, Seregard S, Algvere PV. Experimental transplantation of autologous iris pigment epithelial cells to the subretinal space. *Acta Ophthalmol Scand.* 2001;79:509–514.
33. Lawrence JM, Sauve Y, Keegan DJ, et al. Schwann cell grafting into the retina of the dystrophic RCS rat limits functional deterioration. *Invest Ophthalmol Vis Sci.* 2000;41:518–528.
34. Shen WY, Lai MC, Beilby J, et al. Combined effect of cyclosporine and sirolimus on improving the longevity of recombinant adenovirus-mediated transgene expression in the retina. *Arch Ophthalmol.* 2001;119:1033–1043.
35. Doi K, Hargitai J, Kong J, et al. Lentiviral transduction of green fluorescent protein in retinal epithelium: evidence of rejection. *Vision Res.* 2002;42:551–558.
36. Chong NH, Alexander RA, Waters L, et al. Repeated injections of a ciliary neurotrophic factor analogue leading to long-term photoreceptor survival in hereditary retinal degeneration. *Invest Ophthalmol Vis Sci.* 1999;40:1298–1305.
37. Zinkernagel RM, Doherty PC. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv Immunol.* 1979;27:51–177.
38. Batchelor JR, Welsh KI, Burgos H. Transplantation antigens per se are poor immunogens within a species. *Nature.* 1978;273:54–56.
39. Molajoni ER, Cinti P, Orlandini A, et al. Mechanism of liver allograft rejection: the indirect recognition pathway. *Hum Immunol.* 1997; 53:57–63.
40. Percopo CM, Hooks JJ, Shinohara T, Caspi R, Detrick B. Cytokine-mediated activation of a neuronal retinal resident cell provokes antigen presentation. *J Immunol.* 1990;145:4101–4107.
41. Liversidge JM, Sewell HF, Forrester JV. Human retinal pigment epithelial cells differentially express MHC class II (HLA, DP, DR and DQ) antigens in response to in vitro stimulation with lymphokine or purified IFN-gamma. *Clin Exp Immunol.* 1988;73:489–494.
42. Kohen L, Enzmann V, Faude F, Wiedemann P. Mechanisms of graft rejection in the transplantation of retinal pigment epithelial cells. *Ophthalmic Res.* 1997;29:298–304.
43. Enzmann V, Hollborn M, Wiedemann P, Kohen L. Molecular and cellular evidence for T-cell stimulation by allogeneic retinal pigment epithelium cells in vitro. *Graefes Arch Clin Exp Ophthalmol.* 2001;239:445–451.
44. Liang FQ, Aleman TS, Dejneka NS, et al. Long-term protection of retinal structure but not function using RAAV: CNTF in animal models of retinitis pigmentosa. *Mol Ther.* 2001;4:461–472.
45. Hudrisier D, Riond J, Burlet-Schiltz O, et al. Structural and functional identification of major histocompatibility complex class I-restricted self-peptides as naturally occurring molecular mimics of viral antigens: possible role in CD8+ T cell-mediated, virus-induced autoimmune disease. *J Biol Chem.* 2001;276:19396–19403.
46. Borhani H, Peyman GA, Rahimy MH, Beuerman RW. Vitreoretinal toxicity of basic fibroblast growth factor. *Int Ophthalmol.* 1993; 17:195–199.
47. Li L, Turner JE. Optimal conditions for long-term photoreceptor cell rescue in RCS rats: the necessity for healthy RPE transplants. *Exp Eye Res.* 1991;52:669–679.
48. Jiang LQ, Hamasaki D. Corneal electroretinographic function rescued by normal retinal pigment epithelial grafts in retinal degenerative Royal College of Surgeons rats. *Invest Ophthalmol Vis Sci.* 1994;35:4300–4309.