Establishment of Effective Methods for Transducing Genes into Iris Pigment Epithelial Cells by Using Adeno-associated Virus Type 2

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Purpose. To establish an efficient method of transferring the human brain-derived neurotrophic-factor (hBDNF) gene into human iris pigment epithelial (hIPE) cells by using recombinant adeno-associated virus type 2 (rAAV2).

Methods. Cultured hIPE cells were treated with either hydroxyurea-sodium butyrate (HUSB; DNA synthesis inhibitor), or tyrphostin-1 (Tyr; epidermal growth factor receptor [EGFR] tyrosine kinase inhibitor), or a combination of HUSB and Tyr (HUSB-Tyr). After each treatment, cells were exposed to rAAV2 (rAAV-LacZ or rAAV-hBDNF). The levels of BDNF were measured by ELISA and also determined by Western blot analysis. Southern blot analysis was performed on each type of treated cell. The neuroprotective effect of BDNF on the retinal ganglion cells (RGCs) was quantitatively assessed by culturing rAAV-hBDNF-hIPE with RGCs.

Results. The infection of hIPE cells was significantly lower than ARPE and HT1080 cells, which are highly permissive cells for rAAV2. The treatment of HUSB-Tyr enhanced the transgene expression more than that after treatment with one of these agents in rAAV-hIPE cells. Southern hybridization revealed that the amount of replicative form monomer (RFm) was less in Tyr than in HUSB or HUSB-Tyr treatment and there was no difference in conversion of virus genome to double stranded form after HUSB and HUSB-Tyr treatment. However, adding Tyr treatment stimulated the JNK1/2 and p38 pathways and modified the target transgene expression. BDNF had a significantly greater rescue effect of RGCs with the HUSB-Tyr-treated rAAV-hBDNF-hIPE cells ($P < 0.01$) than that with the HUSB-treated rAAV-hBDNF-hIPE cells ($P > 0.05$) compared with noninfected hIPE cells.

Conclusions. The combined treatment of HUSB-Try is an effective method of increasing transgene expression with the AAV-mediated gene transfer. The role of HUSB and Tyr in the increase of gene expression may be different and related to the conversion of virus into the host genome and the enhancement of the transcription, respectively. (*Invest Ophthalmol Vis Sci.* 2005;46:3341–3348) DOI:10.1167/iovs.04-1351

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Transplantation of appropriate cells is an attractive therapeutic strategy to treat some retinal diseases such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD).[1,2] The use of autologous iris pigment epithelial (IPE) cells, instead of allogeneic retinal pigment epithelial (RPE) cells, prevents immunologic rejection by the host,[3,4] and some investigators are using autologous RPE cells for transplantation.[5] However, the removal of RPE cells from the patient presents some difficulties. IPE cells are derived embryonically from the same cells that give rise to RPE cells, and they have many functions in common with RPE cells, such as a specific phagocytosis system against photoreceptor outer segment (POS)[6–8] and the potential of forming tight junctions.[9]

We have reported that patients with AMD receiving a transplantation of autologous IPE cells showed no sign of rejection. More than 80% of the patients showed an improvement in visual acuity after the transplantation. However, the best visual acuity in these patients was still less than 0.3. These data suggest that there is a limitation in the use of autologous IPE cells for transplantation.[10–12]

Neurotrophic factors, cytokines, and growth factors have been shown to rescue photoreceptors from the degeneration induced by various conditions toxic to photoreceptors.[13,14] Transplantation of IPE cells that have been transduced with the brain-derived neurotrophic factor (BDNF) gene has been shown to rescue photoreceptors against phototoxicity in vivo and retinal neurons against *N*-methyl-D-aspartate (NMDA) toxicity in vitro.[15] These experiments suggest that the transplantation of IPE cells transduced with the BDNF gene (IPE-BDNF) may be an alternative method for protecting photoreceptor against various toxic conditions.

One of the methods used to transduce a gene into relevant cells is virus vectors, and among the different virus vectors available, the recombinant adeno-associated virus (rAAV) has been an efficient and effective vector.[16] rAAV has already been used for long-term gene transfer[17–19] into a variety of tissues, including lung,[20] muscle,[21–24] brain,[25] spinal cord,[26] retina,[27,28] dorsal root ganglia sensory neurons,[29] and liver.[30] Of the six AAV serotypes, serotype 2 (rAAV2) is best characterized and therefore is predominantly used in gene transfer studies,[31–34] but there are only a few reports on rAAV2-mediated gene transfer into IPE cells.

The purpose of this study was to determine the conditions that would increase the efficiency of transducing BDNF into IPE cells through rAAV2. The effects of the epidermal growth factor receptor (EGFR), a tyrosine kinase inhibitor, on the efficiency of transduction were also examined.

Materials and Methods

Cell Preparation

hIPE cells were obtained by peripheral iridectomy from patients who underwent filtration surgery for glaucoma and isolated as previously described.[35] The tenets of the Declaration of Helsinki were observed, and informed consent was obtained from all subjects who participated in the study. Human ARPE-19 (ARPE) cells were kindly given to us by

Leonard Hjelmeland (Department of Ophthalmology, Section of Molecular and Cellular Biology, University of California, Davis, CA). The hIPE and ARPE cells were maintained in a growth medium consisting of Ham's F-12 (Invitrogen-Gibco, Tokyo, Japan), 20% fetal bovine serum (FBS), and antibiotics (Invitrogen-Gibco).[35] The medium was changed every 3 days, and cultured cells were released by 0.125% trypsin-0.01% EDTA solution and passaged.

Human embryonic kidney cells (293T) were obtained from the American Type Culture Collection (Rockville, MD). HT1080 cells were supplied by the Cell Resource Center (Tohoku University, Sendai, Japan). The 293T and HT1080 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco) supplemented with 10% FBS and were passaged with 0.02% EDTA solution and 0.125% trypsin-0.01% EDTA solutions, respectively. hIPE cells at passages 1 to 3 were used in all experiments, and independent experiments were performed at least three times.

Construction of Recombinant Plasmids

The pAAV-MCS, pAAV-RC, and pHelper plasmids were obtained from a kit (AAV Helper-Free System; Stratagene, La Jolla, CA). The gene of interest, β-galactosidase or hBDNF, was introduced into the multicloning site (MCS) of pAAV-MCS. The hBDNF cDNA was generously supplied by Atsushi Takeda (Department of Neurology, Tohoku University).

Recombinant AAV Vector Production and Purification

Semiconfluent 293T cells on 15-cm plates were cotransfected with split-packaging plasmids (i.e., the recombinant pAAV expression plasmid pAAV-RC and pHelper), by a calcium phosphate–based protocol, according to the manufacturer's instructions (Stratagene). The rAAV vectors were purified by the single-step column purification (SSCP) method of Auricchio et al.[36–39]

Recombinant AAV Vector Titer Measurement

An infectious center assay[37] was performed with HT1080 cells to determine the infectious titer according to the manufacturer's instruction (Stratagene). The expression of LacZ was detected with an in situ β-galactosidase staining kit (Stratagene). The cells infected with rAAV were visually scored after 48 hours of culture and recorded as the number of viral particles (stained cells) per milliliter. This value was used to calculate the multiplicity of infection (MOI).

To determine the virus titer, the level of AAV2-specific capsid proteins was measured by enzyme-linked immunosorbent assay (ELISA; Progen Biotechnik, Heidelberg, Germany), and the virus titer was designated as capsids per milliliter.[40] rAAV-BDNF does not contain β-galactosidase and cannot be used for the infectious center assay.

Chemical Treatments and Viral Infection Assays

Hydroxyurea (HU), sodium butyrate (SB), and tyrphostin-1 (Tyr; an EGFR inhibitor) were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of HU (1 M) and SB (0.5 M) were made in phosphate-buffered saline (PBS), and Tyr (50 mM) was made in dimethyl sulfoxide (DMSO) and stored at −20°C. AAV-permissive medium was prepared to a final concentration of 40 mM HU plus 1 mM SB in culture medium. The concentration of hydroxyurea-sodium butyrate (HUSB) and the method of HUSB treatment were according to the manufacturer's instructions (Stratagene). Russell et al.[41] have also demonstrated that HUSB treatment results in dramatic stimulation of rAAV transgene expression in human fibroblast cells. They showed that the maximal concentration for transduction was 40 mM.

The working solution of Tyr was 500 μM, and it was prepared in culture medium by diluting the stock solution with ethanol and then adding it to the culture medium.[42] Cells were grown to subconfluence and maintained in culture medium.

To investigate the effects of HUSB, we treated cultures with AAV-permissive medium for 6 hours. To determine the effectiveness of a mixture of HUSB plus Tyr (HUSB-Tyr), we treated the cells with AAV-permissive medium for 4 hours. Then Tyr was added to a final concentration of 500 μM without removing the AAV-permissive medium and was incubated for 1.5 hours. For the Tyr treatment, cells were treated with 500 μM Tyr for 1.5 hours. After each treatment, cells were washed with DMEM supplemented with 2% FBS and infected with virus at 37°C for 2 hours. After the infection, the same volume of DMEM supplemented with 18% FBS was added to stop the infection, and the cells were then cultured.

Quantification of β-Galactosidase Expression

The expression of LacZ was quantified in the same way used for the rAAV infectious titer assay or was measured by the ELISA method (Promega, Madison, WI).

Quantification of BDNF Expression

Cells were maintained in serum-free medium for 8 h/d, and the culture medium was collected. After the culture medium was collected seven times, the combined culture media were concentrated with a filtering device (Biomax-10k NMWL; Millipore, Bedford, MA) by centrifugation. Cultured cells were lysed in lysis buffer (137 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, [pH 8.0], containing a protease inhibitor [Roche, Basel, Switzerland]). The cell lysates were sonicated and disrupted by the freeze–thaw method four times. The cell lysates and culture medium were centrifuged, and the supernatant was used for each analysis. The expression of BDNF in the lysates was measured by an immunoassay system (Emax; Promega).

Immunoblot Analysis of hIPE Cells

For Western blot analysis, 30 μg of cell lysates was separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The blots were first probed with antibodies to the phosphorylated form of proteins (anti-phosphorylated-JNK1/2 and anti-phosphorylated-p38; BD Biosciences, San Jose, CA). The blots were then stripped of the first antibody and reprobed with antibodies that detect the total protein (anti-JNK1/2 and anti-p38; BD Biosciences). For the detection of these antibodies, alkaline-phosphatase–conjugated anti-mouse IgG (Dako, Glostrup, Denmark) was used as a secondary antibody. Protein bands were detected by exposing the membranes to x-ray film (Fuji, Tokyo, Japan).

For the analysis of production of hBDNF, the blots were first probed with antibodies to BDNF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and second with alkaline-phosphatase–conjugated anti-rabbit IgG (Dako) and detected as previously described.

Southern Blot Analysis

IPE cells were grown to confluence in 10-cm plates. Cultures were treated with HUSB or HUSB plus 500 μM Tyr for 1.5 hours and infected with rAAV-BDNF at an MOI of 50 for 2 hours. After the infection, cells were cultured for 3, 24, or 72 hours. Nuclear fractions were obtained by a modification of the procedure of Sperinde and Nugent.[43] More specifically, cell pellets were resuspended in 200 μL of ice-cold HEPES-NP40 buffer (10 mM KC, 0.1 mM EDTA, 0.1 mM EGTA, and 0.625% Nonidet P40 [pH 7.4]) by vortexing for 10 seconds and incubating on ice for 10 minutes. The mixture was centrifuged at 10,000*g* for 10 seconds at 4°C, and the pellets of nuclear fraction were washed two times with HEPES-NP40 buffer. The centrifugation was repeated after each washing step. Total DNA was isolated from these nuclear fractions and digested with *Hin*dIII at 37°C overnight. Agarose gel electrophoresis and Southern hybridizations were performed with an alkaline-phosphatase–labeled (Amersham Biosciences, Buckinghamshire, UK) cytomegalovirus (CMV) DNA fragment from AAV virus.

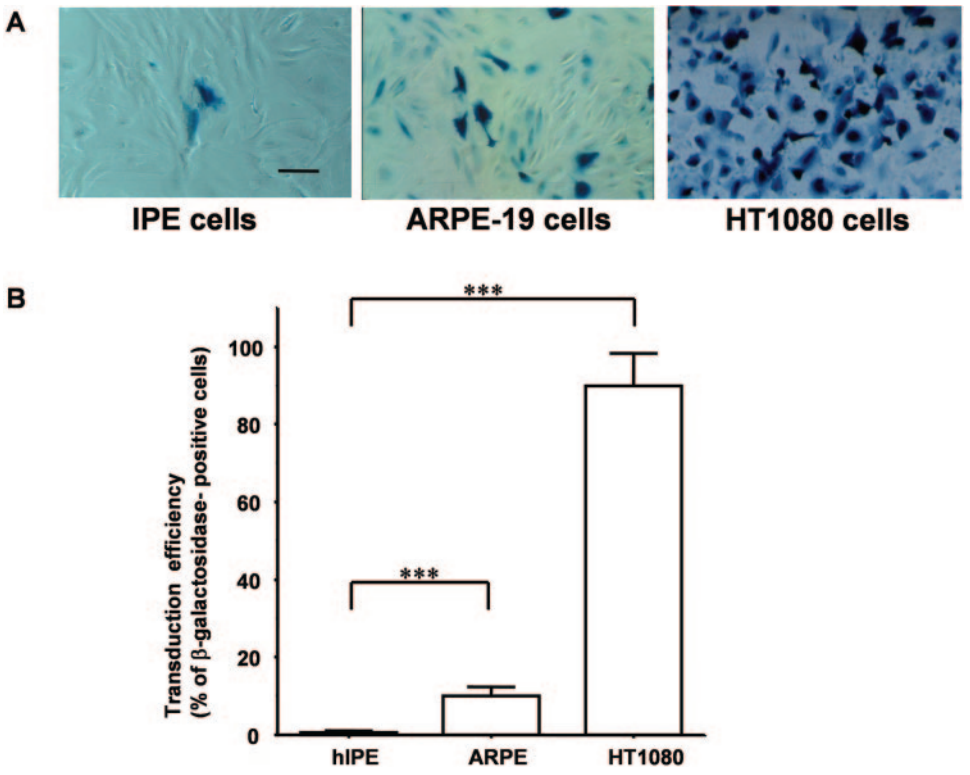

FIGURE 1. Transduction efficiency of cultured hIPE, ARPE, and HT1080 cells by rAAV2. Cells were treated with HUSB for 6 hours and exposed to rAAV-LacZ at an MOI of 5. After 48 hours of incubation, the cells transduced by rAAV were identified by β-galactosidase staining (**A**). Scale bar, 50 μm. The transduction efficiency was measured by counting the number of stained cells (**B**). Four independent experiments were performed, and all data were used for statistical analysis. Error bars, ±SD. $^{***}P < 0.001$.

Purification and Culture of Retinal Ganglion Cells

Retinal ganglion cells (RGCs) from 6- to 8-day-old Wistar rats were isolated and purified according to Goto et al.[44] Purified cells were cultured in enzyme-inhibiting medium (Neurobasal; Invitrogen-Gibco) containing 2 mM glutamine (Invitrogen-Gibco), penicillin-streptomycin (100 U/mL, 50 μg/mL; Invitrogen-Gibco), B-27 supplement (1:50; Invitrogen-Gibco), 50 ng/mL each of BDNF (Wako, Osaka, Japan) and ciliary neurotrophic factor (CNTF; Sigma-Aldrich), and 5 μM forskolin (Calbiochem, La Jolla, CA) on poly-L-lysine and laminin-coated coverslips (BD Biosciences) at a density of 1000 cells/cm^2.[45,46] Cultures were maintained at 37°C in humidified air containing 5% CO_2 and 95% air.

Immunocytochemistry

RGCs were fixed on coverslips with 2% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 60 minutes at 4°C. After pretreatment with 0.5% Triton-X for 5 minutes (except for detection of Thy-1.1) and 1% bovine serum albumin for 10 minutes, the cells were incubated with anti-Thy-1.1 (Chemicon, Temecula, CA), anti-HPC-1 (Sigma-Aldrich), or anti-glial fibrillary acidic protein (GFAP; Chemicon) overnight at 4°C. For an immunologic negative control, mouse IgG was applied instead of the first antibody. After they were washed, the slides were exposed to FITC-conjugated goat anti-mouse IgG (Dako) for 1 hour at room temperature. The samples were examined under a fluorescence microscope (Q550; Leica, Wetzlar, Germany) with L5 filter (excitation, band pass 480/40; emission, 527/30).

Evaluation of the Neuroprotective Effect of BDNF-Transduced hIPE Cells

The neuroprotective effects of BDNF on the RGCs were quantitatively assessed in combined cultures of rAAV-LacZ-infected hIPE (rAAV-LacZ-hIPE) cells, rAAV-BDNF-infected IPE (rAAV-BDNF-hIPE) cells, or non-infected hIPE (N-hIPE) cells combined with RGCs. They cells were treated with HUSB or HUSB-Tyr and then infected with rAAV. They were cultured on permeable membranes (Intercell TP substrate; Kurabou, Osaka, Japan), which consisted of 0.45-μm microporous cellulose filters, at 37°C until confluent. The cultures grew well, and the cells were cocultured with the RGCs in BDNF-free medium overnight. After cultivation, cell viability was determined by the metabolism of calcein-AM (Molecular Probes, Eugene, OR).[47] The total number of living RGCs in each well was counted in five fields (0.185 mm^2/field). The average of these fields was considered to be the total living cells in each well. Wells were counted in triplicate.

RESULTS

Transduction Efficiency of AAV2 in Human Cells

The transduction efficiency of rAAV into hIPE and ARPE cells was studied. For comparison, the human fibrosarcoma HT1080 cell line, which is known to be highly permissive of AAV2 transduction, was used. All cell types were treated with HUSB and exposed to rAAV-LacZ at an MOI of 5. After 48 hours of cultivation, the transduced cells were examined for LacZ staining (Fig. 1A). The hIPE cells showed substantially lower transduction than did the ARPE and HT1080 cells. In contrast, the efficiency of transduction into hIPE cells was 0.6% ± 0.5%, into ARPE cells was 10.0% ± 2.4%, and into HT1080 cells was 90.0% ± 9.6% ($P < 0.001$; Fig. 1B).

Stimulation of JNK1/2 and p38 Pathways in hIPE Cells

Stimulation of the JNK1/2 and p38 pathways in IB3 cells has been reported to increase the transduction efficiency of the vAVCMVluc vector.[42] hIPE cells were treated with HUSB, 500 μM Tyr, a mixture of HUSB+Tyr, or vehicle (0.5% DMSO+2.5% ethanol) alone and incubated. After incubation, the protein in the cells was extracted and analyzed by immunoblots against phosphorylated forms of the protein (phosphorylated-JNK1/2 or phosphorylated-p38α) and total protein (JNK1/2 or p38a; Fig. 2). Tyr treatment for 1.5 hours stimulated both pathways in the hIPE cells. HUSB-Tyr-treated cells also

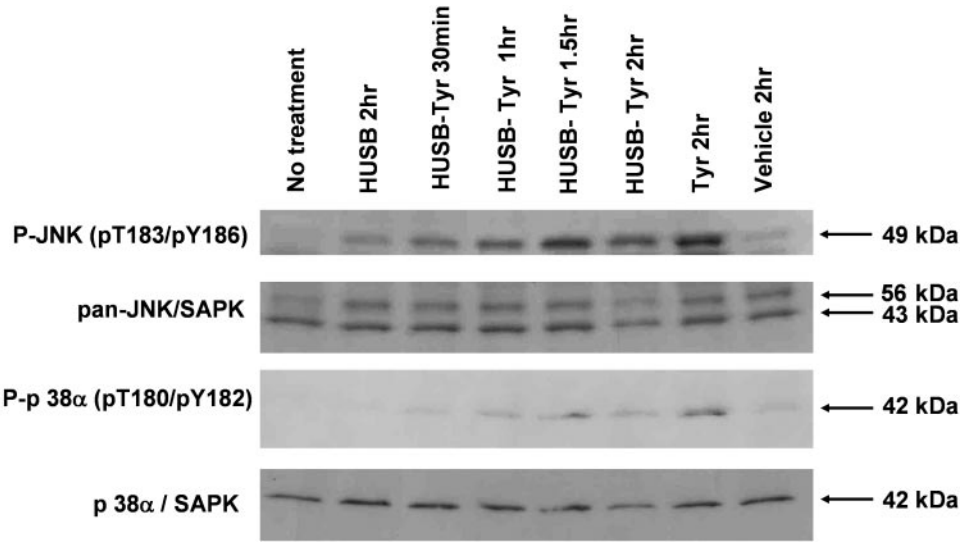

FIGURE 2. Immunoblot analysis of stress-activated protein kinases (SAPK), JNK, and p38. hIPE cells were treated with HUSB, Tyr (500 μM), a mixture of HUSB and 500 μM Tyr (HUSB-Tyr), or vehicle (0.5% DMSO and 2.5% ethanol) and incubated for the indicated times. After incubation, proteins were extracted from each cell. The blots were probed with antibodies to the phosphorylated form (α-P), anti-phosphorylated JNK1/2, or anti-phosphorylated p38 and detected by the chemiluminescence method. The blots were then stripped and reprobed with antibodies to total anti-JNK 1/2 or anti-p38.

stimulated both pathways after 1.5 hours of treatment, but no stimulation was detected with HUSB.

Effect of Tyr on Conversion to the Double-Stranded Form

To examine the conformation of virus DNA, hIPE cells were infected with rAAV, with or without treatment. The cultures were harvested at 2, 24, and 72 hours and Southern hybridization was performed on the nuclear fraction of these cells (Fig. 3). At 2 hours of culture, there was no detectable virus DNA. At 24 and 72 hours, the RFm and dimmer (RFd) DNA were detected in the rAAV-infected cells. The conformation of RFd in chemical-treated cells was more than in nontreated cells. HUSB, HUSB-Tyr, or Tyr treatment increased the conformation of RFd. However, there was no difference between the combined treatment of HUSB-Tyr and individual treatment of these agents. The amount of RFm was less in Tyr than in HUSB or HUSB-Tyr treatment.

β-Galactosidase Expressions in rAAV-LacZ Transduced, Tyrphostin-Treated IPE Cells

To determine whether Tyr treatment can increase the transgene expression in hIPE cells, the cells were treated with HUSB, Tyr ,or HUSB-Tyr and infected with rAAV-LacZ at an MOI of 5. For the control, no treatment or vehicle (0.5% DMSO+2.5% ethanol)-treated cells were infected in the same way as the treated cells. The level of β-galactosidase was quantified after 72 hours of culture by ELISA (Fig. 4). HUSB treatment with the medium containing FBS dramatically increased the transgene expression. However, HUSB had no effect in serum-free conditions. Tyr treatment raised the transgene expression by treatment with or without FBS. In the presence of FBS, HUSB-Tyr treatment (31.9 $\pm$ 2.966 mU/μg protein) increased transgene expression more than HUSB treatment alone (18.49 $\pm$ 4.572 mU/μg protein).

To study the stability of transgene expression, the expression of β-galactosidase was monitored for 14 days. After each day of culture, transduced cells were identified by β-galactosidase staining, and the transduction efficiencies were calculated (Fig. 5). After 14 days of infection, transduction efficiency in HUSB-Tyr-treated cells (32.7% $\pm$ 5.9%) was significantly higher than in HUSB-treated cells (18.7% $\pm$ 0.6%).

BDNF Expression in rAAV-BDNF-Transduced, Tyrphostin-Treated IPE Cells

We compared the effects of HUSB and HUSB-Tyr treatment on rAAV-hBDNF-hIPE cells. The expression of BDNF in cell extracts or culture medium was measured by ELISA. For the control, nontreated and noninfected hIPE cells were analyzed. The BDNF expression in the cell extracts and in culture me-

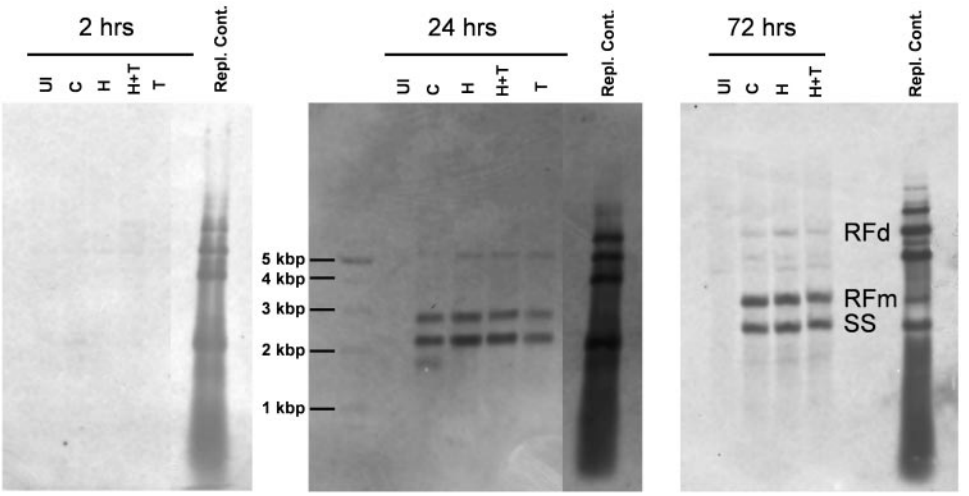

FIGURE 3. Southern hybridization analyses of RF virus DNA. hIPE cells were infected or not infected (*lane UI*), rAAV-BDNF-IPE (*lane C*), HUSB-treated rAAV-BDNF-IPE (*lane H*), HUSB-Tyr-treated rAAV-BDNF-IPE (*lane H+T*), or Tyr-treated rAAV-BDNF-IPE (*lane T*). Infection with rAAV-BDNF was performed at an MOI of 50. The cultures were harvested at 3, 24, and 72 hours, and the nuclear fraction was obtained. DNA (15 μg) was analyzed by agarose gel electrophoresis and hybridized to an alkaline-phosphatase–labeled CMV sequence probe. The replication control samples were prepared according to the genome of the rAAV vector. *Right*: locations of the RFm and RFd and single-stranded (SS) DNAs.

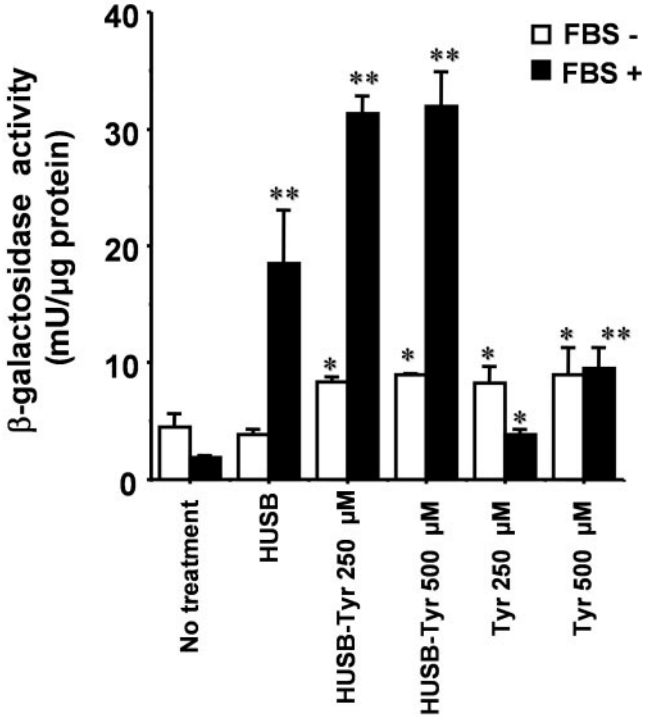

FIGURE 4. Change of β-galactosidase activity in rAAV-LacZ-hIPE cells induced by chemical treatments. hIPE cells were grown to confluence and placed in serum-containing or serum-free medium for 2 days. Cells were treated with HUSB, HUSB plus 250 μM Tyr, HUSB plus 500 μM Tyr, 250 μM Tyr, or 500 μM Tyr. After each treatment, cells were infected with rAAV-LacZ at an MOI of 5 for 2 hours. After 48 hours in culture, the β-galactosidase activity of each cell extract was measured. Nontreated hIPE cells were infected in the same way as treated cells (No treatment). Error bars, SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

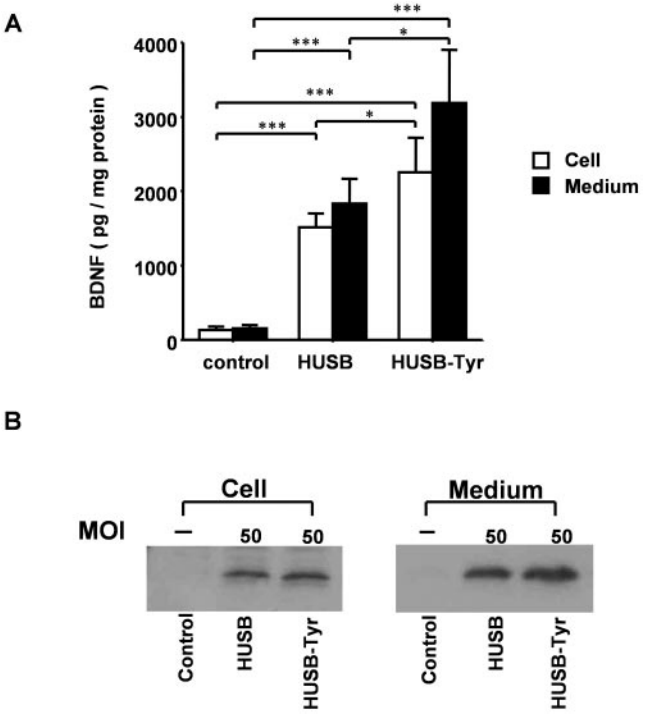

FIGURE 6. Measurement of the BDNF level in rAAV-hBDNF-hIPE cells with or without treatment. To evaluate the protein level of BDNF expression, hIPE cells were treated with HUSB or HUSB plus 500 μM Tyr. Then, the cells were infected at an MOI of 50. For the control, nontreated, and noninfected hIPE cells were analyzed. The BDNF level in the culture medium and cell lysates were measured by sandwich ELISA (**A**). Error bars, SD. $^{*}P < 0.05$, $^{***}P < 0.001$. Western blot analysis for BDNF is shown in (**B**).

dium of rAAV-hBDNF-hIPE cells was significantly higher than that in the control cells ($P < 0.001$; Fig. 6A). The combined HUSB-Tyr treatment enhanced the expression of the transgene more than HUSB treatment ($P < 0.05$). Western blot analysis confirmed these results (Fig. 6B).

To determine whether the MOI of rAAV affected the level of transgene expression, HUSB-Tyr-treated hIPE cells were infected with rAAV-hBDNF at various MOIs. Increasing the amounts of vector resulted in increased gene expression (Fig. 7). The degree of enhancement was more than 29-fold in cell extracts and 23-fold in culture medium (compared with the control cells) at an MOI of 100.

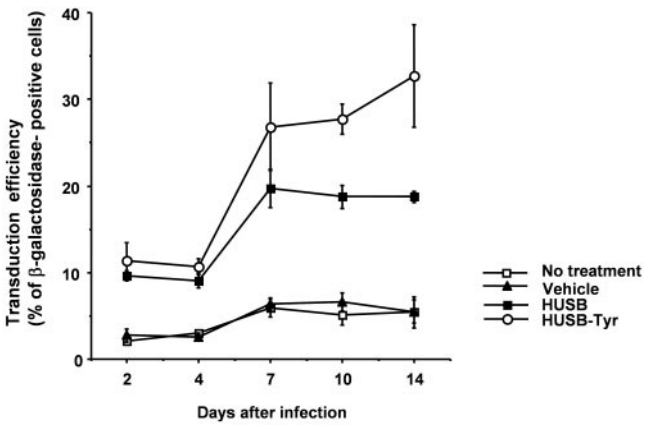

FIGURE 5. Time course of transduction efficiency in hIPE cells after infection with rAAV-LacZ. Treated and nontreated hIPE cells were infected with rAAV at an MOI of 5 and cultured. Cells were identified by β-galactosidase staining and counted at 2, 4, 7, 10, and 14 days after infection.

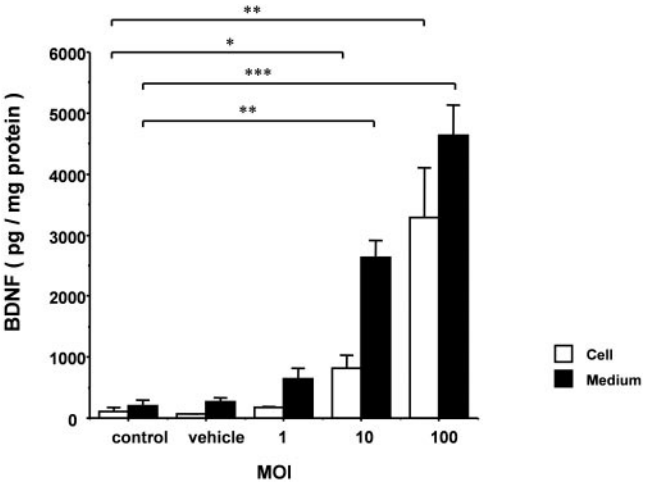

FIGURE 7. Changes of protein expression levels of BDNF in cells infected at different MOIs. hIPE cells were treated with a mixture of HUSB plus 500 μM Tyr and infected with the indicated amounts of rAAV-hBDNF. Control cells were not treated and not infected. Vehicle-treated cells were treated with vehicle of HUSB-Tyr (0.5% DMSO and 2.5% ethanol) and not infected. The BDNF level in the culture medium and cell lysates were measured by sandwich ELISA (Fig. 6). Error bars, SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

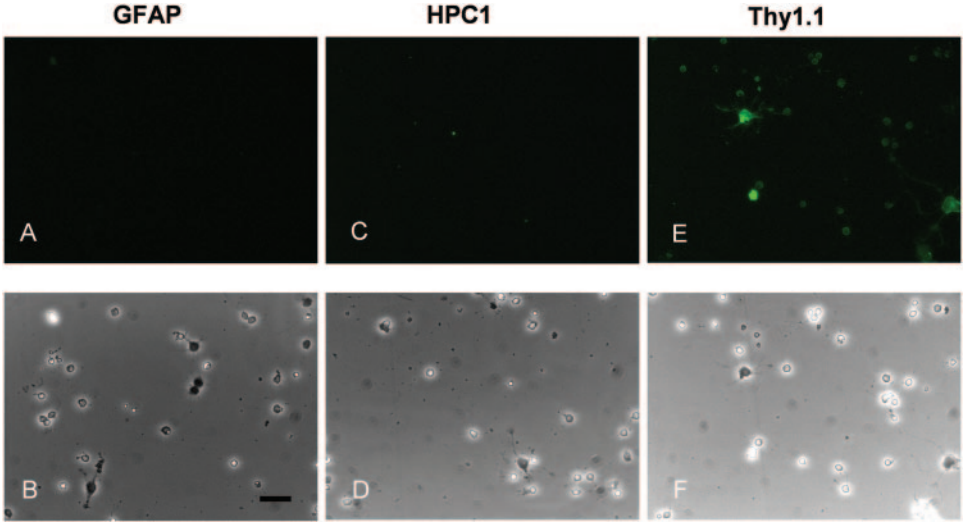

FIGURE 8. Immunocytochemistry of purified RGCs. Cells were treated with GFAP (**A**, **B**), HPC-1 (**C**, **D**), or Thy1.1 (**E**, **F**) primary antibodies. After the treatment, cells were exposed to FITC-conjugated goat anti-mouse IgG and examined under a fluorescence microscope. RGCs showed a diffuse expression of Thy1.1 antigen (**E**). Cells analyzed for GFAP (**A**) and HPC-1 (**C**) were negative. The configuration of the cells used in this immunocytochemistry was also observed under light microscope (**B**, **D**, **F**). Bar, 50 μm.

Purity of Isolated Ganglion Cells

The isolated RGCs were identified by the presence of the retrogradely transported 4-DiI-Asp fluorescent probe (Molecular Probes). Approximately 90% of the cells were labeled by 4-DiI-Asp (data not shown). Immunocytochemistry on purified RGCs using Thy1.1, a marker for RGCs; HPC-1, a marker for amacrine cells; and GFAP, a marker for glial cells, was performed to confirm the purity of the RGCs (Fig. 8). The RGCs showed a diffuse expression of Thy1.1 antigen and the expression of GFAP, but not with HPC-1.

Neuroprotective Effect against BDNF-Deprivation–Induced RGC Death

The neuroprotective effect of rAAV-hBDNF-hIPE cells against BDNF-deprivation–induced RGC death was quantitatively assessed by using combined cultures[15] and calcein-AM staining. rAAV-LacZ-hIPE and rAAV-BDNF-IPE cells were infected at an MOI of 100 after each treatment. Cells were cultured on 0.45-μm microporous cellulose filters. Cultured cells were combined with primary RGCs, which had been cultured for 4 days, and were incubated overnight with BDNF-deprived culture medium.

The percentage of viable RGCs in control (BDNF-free) medium was 62.7% ± 9.4% compared with normal culture (BDNF containing) medium (Fig. 9). In the RGCs exposed to BDNF-deprived culture medium, the viabilities of RGCs cocultured with N-IPE- or HUSB-Tyr-treated rAAV-hBDNF-hIPE cells were 136.2% ± 19.6% and 217.2% ± 27.65%, respectively. Statistical analyses demonstrated a significantly greater rescue effect in the HUSB-Tyr-treated rAAV-hBDNF-hIPE cells ($P < 0.01$) than in HUSB-treated rAAV-hBDNF-hIPE cells ($P > 0.05$) compared with N-IPE cells.

DISCUSSION

The inefficiency of transducing the rAAV vector prompted us to investigate different agents that might enhance the transduction.[48] Our results showed that we could enhance the transduction as manifested by the increased expression of rAAV2-mediated transgene in low permissive cells such as hIPE cells. The use of HUSB, which is a newly reported chemical reagent that increases gene transfer rates,[41] increased the transgene expression in hIPE cells. In addition, we found that a mixture of hIPE cells and HUSB-Tyr markedly improved transduction efficiency.

DNA-damaging agents including γ- and UV-irradiation and alkylating agents have been reported to be potent stimulators of transduction,[41,49] but these treatments are also cytotoxic. We challenged the HUSB treatment for effective gene transfer by rAAV. Russell et al.[41] reported that HU, which prevents DNA synthesis by inhibiting ribonucleotide reductase and depleting deoxynucleotide pools, increases the transduction rate of a targeted gene to stationary human fibroblast cultures based

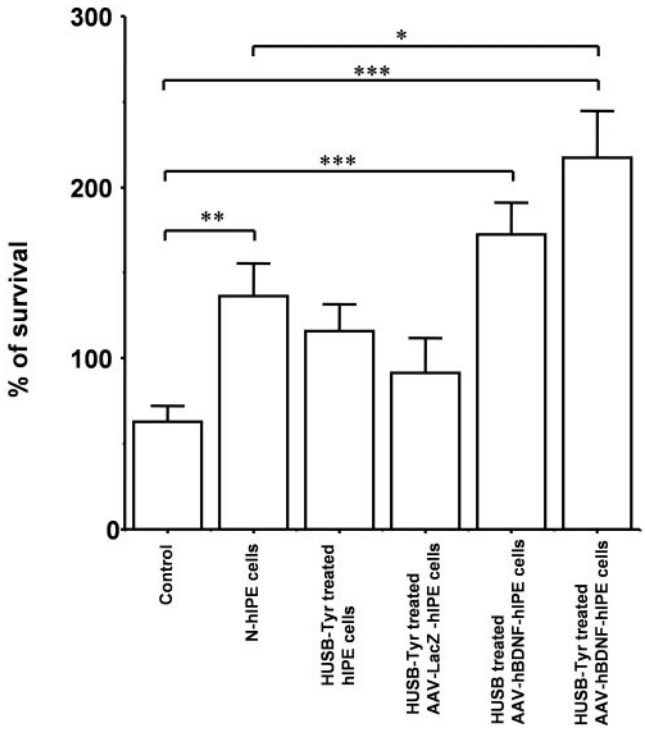

FIGURE 9. Neuroprotective effect against BDNF-deprivation–induced RGC death. The neuroprotective effect of rAAV-hBDNF-hIPE cells against BDNF-deprivation–induced RGC death was quantitatively assessed by the method of combined cultures. Viabilities were confirmed by calcein-AM staining. RGC cultures were incubated overnight with coculture of hIPE cells in the BDNF-deprivation culture medium. Control represents the viabilities of the primary RGCs without coculture of hIPE cells. RGCs cultured with BDNF-containing medium were considered to be 100% viable. Error bars, SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

on AAV vectors, with no decrease in cell viability or proliferative potential. Our results demonstrated that HUSB increased rAAV2-mediated transduction efficiency. The mechanism of stimulating transgene expression with HU treatment is reported to be the alteration of the chromosome structure, which can then induce similar DNA repair functions. During DNA repair synthesis, host DNA polymerases are abundant and are used for the conversion of single-stranded vector genomes to double-stranded molecules.[50] This process could then increase the transduction of nondividing cells, such as stationary cells.

We also investigated other methods to enhance rAAV transgene transduction. Protein transfection of heparin sulfate on cell surface, which serves as a receptor for AAV2 and mediates viral attachments,[51–54] had only a nonsignificant increase. Prestimulation of the cells with a high-dose cytokine cocktail (IL-3, 20 ng/mL; IL-6, 50 ng/mL; and TNF-α, 10 ng/mL)[55] also did not change transduction efficiency significantly.

Recent studies have shown that Tyr, which is an EGFR tyrosine kinase inhibitor, increases the transduction efficiency of IB3 cells.[56] The effect of Try treatment on the transduction of rAAV2 has been reported to be related to the stimulation of stress-activated protein kinases.[57] Our study demonstrated that the stress-activated protein kinases (JNK and SAPK) and p38 kinase were activated by Tyr treatment but not by HUSB treatment in hIPE cells. The results of Southern blot analysis made clear the effect of HUSB and Tyr treatment, which increased the formation of RFd. There were no differences in the amount of RFd between that of single treated and HUSB-Tyr-treated cells, but HUSB increased the accumulation of RFm vector. These results indicate that the increased transgene expression in the HUSB-Tyr-treated cells, as shown in Figure 4, is due to mechanisms other than RFd formation. HUSB treatment led to an accumulation of RFm vector, which may lead to stable DNA conversion and increased gene expression.[42] Smith et al.[42] have shown the possibility that Tyr stimulates transcription from the CMV promoter mediated through the stimulation of stress-activated kinases. Our data also indicated that Tyr activated transcription by stimulation of stress-activated kinases and increased transgene expression. We suggest that the effects of HUSB or Tyr on transduction efficiency were mediated through different pathways, and the activation of both pathways enhanced transduction efficiency in low permissive cells such as IPE cells.

Combined cultures[15] of N-IPE cells and rAAV-hBDNF-hIPE cells rescued dissociated RGC cultures from BDNF deprivation. No significant protective effect was observed with the culture of HUSB-treated rAAV-hBDNF-hIPE cells compared with the culture of N-hIPE cells. However, a significant increase in the survival of RGCs was observed when they were cultured with HUSB-Tyr-treated rAAV-hBDNF-hIPE cells compared with the culture of N-hIPE cells. These results demonstrate that treatment by HUSB-Tyr showed the highest degree of RGC neuronal protection. Quantitative analysis by the BDNF-ELISA method also showed that transgene expression in cells mediated by HUSB-Try infection was the highest of any other treatment.

In summary, we have identified methods to enhance the efficiency of gene transfer to hIPE cells by using rAAV. Our studies indicated the optimal condition for transduction of the rAAV-mediated transgene into IPE cells and found that the downstream target of EGFR had a central role in regulating rAAV transduction of hIPE cells. Our long-term goal is to develop a treatment for patients with AMD or RP. Together with the previous reports that BDNF-expressed IPE cells support the survival of the retinal cells,[15,58] our report shows that this technique of transplanting autologous IPE cells transfected with an appropriate growth factor gene via rAAV2 is a useful method of treating retinal degenerative diseases.

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References

1. Rezai KA, Kohen L, Wiedemann P, Heimann K. Iris pigment epithelium transplantation. *Graefes Arch Clin Exp Ophthalmol*. 1997; 235:558–562.
2. Rezai KA, Lai WW, Farrokh-Siar L, et al. A new method of culturing and transferring iris pigment epithelium. *Invest Ophthalmol Vis Sci*. 1997;38:2255–2260.
3. Rezai KA, Semnani RT, Patel SC, Ernest JT, van Seventer GA. The immunogenic potential of human fetal retinal pigment epithelium and its relation to transplantation. *Invest Ophthalmol Vis Sci*. 1997;38:2662–2671.
4. Abe T, Takeda Y, Yamada K, et al. Cytokine gene expression after subretinal transplantation. *Tohoku J Exp Med*. 1999;189:179–89.
5. Binder S, Krebs I, Hilgers RD, et al. Outcome of transplantation of autologous retinal pigment epithelium in age-related macular degeneration: a prospective trial. *Invest Ophthalmol Vis Sci*. 2004; 45:4151–4160.
6. Rezai KA, Lappas A, Farrokh-Siar L, et al. Iris pigment epithelial cells of long evans rats demonstrate phagocytic activity. *Exp Eye Res*. 1997;65:23–29.
7. Sakuragi M, Tomita H, Abe T, Tamai M. Changes of phagocytic capacity in basic fibroblast growth factor-transfected iris pigment epithelial cells in rats. *Curr Eye Res*. 2001;23:185–191.
8. Sugano E, Tomita H, Abe T, Yamashita A, Tamai M. Comparative study of cathepsins D and S in rat IPE and RPE cells. *Exp Eye Res*. 2003;77:203–209.
9. Rezai KA, Lappas A, Kohen L, Wiedemann P, Heimann K. Comparison of tight junction permeability for albumin in iris pigment epithelium and retinal pigment epithelium in vitro. *Graefes Arch Clin Exp Ophthalmol*. 1997;235:48–55.
10. Abe T, Yoshida M, Kano T, Tamai M. Visual function after removal of subretinal neovascular membranes in patients with age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol*. 2001; 239:927–936.
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. Abe T, Tomita H, Kano T, et al. Autologous iris pigment epithelial cell transplantation in monkey subretinal region. *Curr Eye Res*. 2000;20:268–275.
13. Faktorovich EG, Steinberg RH, Yasumura D, Matthes MT, LaVail MM. Basic fibroblast growth factor and local injury protect photoreceptors from light damage in the rat. *J Neurosci*. 1992;12:3554–3567.
14. LaVail MM, Yasumura D, Matthes MT, et al. Protection of mouse photoreceptors by survival factors in retinal degenerations. *Invest Ophthalmol Vis Sci*. 1998;39:592–602.
15. 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.
16. Cashman SM, Sadowski SL, Morris DJ, Frederick J, Kumar-Singh R. Intercellular trafficking of adenovirus-delivered HSV VP22 from the retinal pigment epithelium to the photoreceptors: implications for gene therapy. *Mol Ther*. 2002;6:813–823.
17. Madry H, Cucchiarini M, Terwilliger EF, Trippel SB. Recombinant adeno-associated virus vectors efficiently and persistently transduce chondrocytes in normal and osteoarthritic human articular cartilage. *Hum Gene Ther*. 2003;14:393–402.

18. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol*. 1996;70:8098–8108.
19. Clark KR, Sferra TJ, Johnson PR. Recombinant adeno-associated viral vectors mediate long-term transgene expression in muscle. *Hum Gene Ther*. 1997;8:659–669.
20. Flotte TR, Afione SA, Conrad C, et al. Stable in vivo expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. *Proc Natl Acad Sci USA*. 1993;90: 10613–10617.
21. Kessler PD, Podsakoff GM, Chen X, et al. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc Natl Acad Sci USA*. 1996;93:14082–14087.
22. Fisher KJ, Jooss K, Alston J, et al. Recombinant adeno-associated virus for muscle directed gene therapy. *Nat Med*. 1997;3:306–312.
23. Chao H, Samulski R, Bellinger D, et al. Persistent expression of canine factor IX in hemophilia B canines. *Gene Ther*. 1999;6: 1695–1704.
24. Herzog RW, Hagstrom JN, Kung SH, et al. Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. *Proc Natl Acad Sci USA*. 1997;94:5804–5809.
25. Klein RL, Meyer EM, Peel AL, et al. Neuron-specific transduction in the rat septohippocampal or nigrostriatal pathway by recombinant adeno-associated virus vectors. *Exp Neurol*. 1998;150:183–194.
26. Peel AL, Zolotukhin S, Schrimsher GW, Muzyczka N, Reier PJ. Efficient transduction of green fluorescent protein in spinal cord neurons using adeno-associated virus vectors containing cell type-specific promoters. *Gene Ther*. 1997;4:16–24.
27. Flannery JG, Zolotukhin S, Vaquero MI, et al. Efficient photoreceptor-targeted gene expression in vivo by recombinant adeno-associated virus. *Proc Natl Acad Sci USA*. 1997;94:6916–6921.
28. Lewin AS, Drenser KA, Hauswirth WW, et al. Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa. *Nat Med*. 1998;4:967–971.
29. Fleming J, Ginn SL, Weinberger RP, et al. Adeno-associated virus and lentivirus vectors mediate efficient and sustained transduction of cultured mouse and human dorsal root ganglia sensory neurons. *Hum Gene Ther*. 2001;12:77–86.
30. Snyder RO, Miao CH, Patijn GA, et al. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat Genet*. 1997;16:270–276.
31. Flotte T, Carter B, Conrad C, et al. A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. *Hum Gene Ther*. 1996;7:1145–1159.
32. Rabinowitz JE, Samulski J. Adeno-associated virus expression systems for gene transfer. *Curr Opin Biotechnol*. 1998;9:470–475.
33. Bennett J, Maguire AM, Cideciyan AV, et al. Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina. *Proc Natl Acad Sci USA*. 1999;96:9920–9925.
34. Kay MA, Manno CS, Ragni MV, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet*. 2000;24:257–261.
35. Abe T, Tomita H, Ohashi T, et al. Characterization of iris pigment epithelial cell for auto cell transplantation. *Cell Transplant*. 1999; 8:501–510.
36. Auricchio A, Hildinger M, O'Connor E, Gao GP, Wilson JM. Isolation of highly infectious and pure adeno-associated virus type 2 vectors with a single-step gravity-flow column. *Hum Gene Ther*. 2001;12:71–76.
37. Zolotukhin S, Byrne BJ, Mason E, et al. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther*. 1999;6:973–985.
38. Zolotukhin S, Potter M, Zolotukhin I, et al. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods*. 2002;28:158–167.
39. Qiu J, Handa A, Kirby M, Brown KE. The interaction of heparin sulfate and adeno-associated virus 2. *Virology*. 2000;269:137–147.
40. Grimm D, Kern A, Pawlita M, et al. Titration of AAV-2 particles via a novel capsid ELISA: packaging of genomes can limit production of recombinant AAV-2. *Gene Ther*. 1999;6:1322–1330.
41. Russell DW, Alexander IE, Miller AD. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. *Proc Natl Acad Sci USA*. 1995;92:5719–5723.
42. Smith AD, Collaco RF, Trempe JP. Enhancement of recombinant adeno-associated virus type 2-mediated transgene expression in a lung epithelial cell line by inhibition of the epidermal growth factor receptor. *J Virol*. 2003;77:6394–6404.
43. Sperinde GV, Nugent MA. Heparan sulfate proteoglycans control intracellular processing of bFGF in vascular smooth muscle cells. *Biochemistry*. 1998;37:13153–13164.
44. Goto W, Ota T, Morikawa N, et al. Protective effects of timolol against the neuronal damage induced by glutamate and ischemia in the rat retina. *Brain Res*. 2002;958:10–19.
45. Otori Y, Wei JY, Barnstable CJ. Neurotoxic effects of low doses of glutamate on purified rat retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 1998;39:972–981.
46. Yasuyoshi H, Kashii S, Zhang S, et al. Protective effect of bradykinin against glutamate neurotoxicity in cultured rat retinal neurons. *Invest Ophthalmol Vis Sci*. 2000;41:2273–2278.
47. Levin LA, Clark JA, Johns LK. Effect of lipid peroxidation inhibition on retinal ganglion cell death. *Invest Ophthalmol Vis Sci*. 1996; 37:2744–2249.
48. Mah C, Fraites TJ, Jr, Zolotukhin I, et al. Improved method of recombinant AAV2 delivery for systemic targeted gene therapy. *Mol Ther*. 2002;6:106–112.
49. Alexander IE, Russell DW, Miller AD. DNA-damaging agents greatly increase the transduction of nondividing cells by adeno-associated virus vectors. *J Virol*. 1994;68:8282–8287.
50. Muzyczka N. Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr Top Microbiol Immunol*. 1992;158:97–129.
51. Pajusola K, Gruchala M, Joch H, et al. Cell-type-specific characteristics modulate the transduction efficiency of adeno-associated virus type 2 and restrain infection of endothelial cells. *J Virol*. 2002;76:11530–11540.
52. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol*. 1998;72:1438–1445.
53. Summerford C, Bartlett JS, Samulski RJ. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med*. 1999;5:78–82.
54. Opie SR, Warrington KH Jr, Agbandje-McKenna M, Zolotukhin S, Muzyczka N. Identification of amino acid residues in the capsid proteins of adeno-associated virus type 2 that contribute to heparan sulfate proteoglycan binding. *J Virol*. 2003;77:6995–7006.
55. Nathwani AC, Hanawa H, Vandergriff J, et al. Efficient gene transfer into human cord blood CD34+ cells and the CD34+CD38- subset using highly purified recombinant adeno-associated viral vector preparations that are free of helper virus and wild-type AAV. *Gene Ther*. 2000;7:183–195.
56. Mah C, Qing K, Khuntirat B, et al. Adeno-associated virus type 2-mediated gene transfer: role of epidermal growth factor receptor protein tyrosine kinase in transgene expression. *J Virol*. 1998;72: 9835–9843.
57. Bruening W, Giasson B, Mushynski W, Durham HD. Activation of stress-activated MAP protein kinases up-regulates expression of transgenes driven by the cytomegalovirus immediate/early promoter. *Nucleic Acids Res*. 1998;26:486–489.
58. Hojo M, Abe T, Sugano E, et al. Photoreceptor Protection by Iris Pigment epithelial transplantation transduced with AAV-mediated brain-Derived neurotrophic factor gene. *Invest Ophthalmol Vis Sci*. 2004;45:3721–3726.