HGF were determined by enzyme-linked immunosorbent assay (ELISA) using an IMMUNIS human HGF enzyme immunoassay kit (Institute of Immunology, Tokyo, Japan).

Experimental animal models

Liver fibrosis was induced by carbon tetrachloride (CCl_4) [22] or bile duct ligation (BDL) [23]. In the first model, 40 BALB/c mice received CCl_4 by intragastric administration of 5% CCl_4 at a dose of 2 ml/kg body weight (Wako, Tokyo, Japan) dissolved in olive oil, once a week for 16 weeks. After the establishment of liver fibrosis, AAV5-HGF ($n = 20$) or AAV5-LacZ ($n = 20$), at a dose of 10^{11} vector genomes were transfected into the portal vein through the splenic hilum. Mice were killed at 3, 6, 9, or 12 weeks after transfection ($n = 5$, each point). The second model was created by BDL. Mice were transfected with AAV5-HGF ($n = 5$) or AAV5-CMV-LacZ ($n = 5$) at day 0 by the same procedure described above. Seven weeks after transfection, BDL was performed on all mice. Briefly, the common bile duct was double-ligated using 4-0 silk through a midline abdominal incision. All mice were killed 2 weeks after BDL, and liver and blood were collected for histologic and protein analyses. All mice were anesthetized with ether during AAV transduction, BDL, and at death. The time schedule is shown in Fig. 1.

Histologic examination and immunostaining

Paraffin-embedded tissues were fixed and embedded in paraffin. The sections were stained with Azan-Mallory for collagen visualization. Hepatic fibrosis was assessed in a blinded manner by image analysis, using a planimetric method and the Automatic Image Analysis System (Carl Zeiss, Oberkochem, Germany). For immunohistochemical analysis, the sections were pretreated through deparaffinization, antigen unmasking, and blocking with 1% H_2O_2 for 10 min and 1.5% goat normal serum for 60 min. The specimens were incubated with mouse antihuman α-smooth muscle antibody (α-SMA) (1:100 dilution; Thermo Fisher Scientific, Waltham, MA, USA) for 60 min. After washing, the sections were incubated with biotin-conjugated anti-mouse IgG secondary antibody (1:5000) for 60 min, then with DAB for 1 min and counterstained with hematoxylin for 10 s.

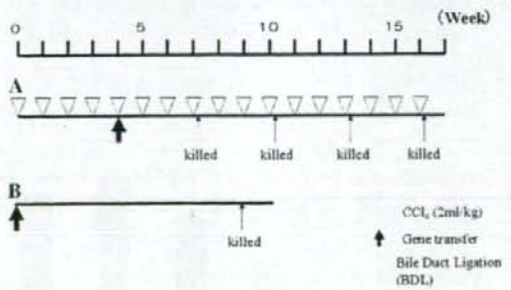

Fig. 1 Schedule of AAV5-HGF or AAV5-LacZ transduction (**a**: CCl_4 mice model, **b**: BDL mice model)

RNA isolation and cDNA synthesis

Total RNA was extracted from mice livers using ISOGEN (Nippon Gene, Tokyo, Japan). The concentration of RNA was spectrophotometrically determined, and the integrity of samples was confirmed by visualizing the 28S and 18S ribosomal RNA bands under ultraviolet light after agarose gel electrophoresis. One microgram of total RNA was reverse transcribed with random primers using a commercial cDNA kit (High-capacity cDNA Archive kit: Applied Biosystems, Foster City, CA, USA). The resulting synthesized cDNA was used for real-time polymerase chain reaction (PCR).

Real-time PCR

Serial dilutions of cDNA were made to determine the linear range for amplification. Real-time PCR was performed on the ABI PRISM 7900HT Sequence Detection System using TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression Assay (Collagen α1(I), α-SMA, TGF-β, TIMP, and MMP13; Applied Biosystems) for the PCR step. A standard curve for serial dilutions of 18S rRNA was similarly generated.

In situ zymography

At the final timepoint, mice livers were removed and embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). They were cut into 6-μm frozen sections, and put onto gelatin-coated film (MMP in situ Zymo-Film, Wako Chemical, Osaka, Japan) and incubated at 37°C for 17 h. Gelatin left undegraded on the film was stained with Biebrich Scarlet Stain Solution (Wako Chemical).

Statistical analysis

Data are expressed as mean ± SD, and the statistical significance of differences among groups was assessed

by Student's t test. P values < 0.05 were regarded as statistically significant.

Results

HGF expression in vitro

HepG2 cells (2×10^5) were infected with AAV5-LacZ (10^6 vector genomes) or AAV5-HGF (10^6 or 2×10^6 vector genomes) for 48 h. Cell lysates and culture medium were harvested at 48 h after infection, and human HGF expression was measured by ELISA. Dose-dependent expression of human HGF was detected in HepG2 cells with AAV5-HGF vector (0.10 ± 0.05 ng/ml in medium and 1.08 ± 0.12 ng/ml in cell lysates using 2×10^6 vector genomes), but no expression was observed in HepG2 cells transfected with AAV5-LacZ vector.

Human HGF expression by AAV5-HGF transduction in CCl_4-treated mice

The human HGF concentrations in the plasma or livers of CCl_4-treated mice were measured by ELISA up to 12 weeks after injection of AAV5-HGF vectors. Human HGF concentrations gradually increased and reached the peak values (25 ± 11 pg/ml in plasma and 250 ± 90 pg/g in the liver) 9 weeks after transduction. The level of HGF persisted for at least 12 weeks after transduction both in the serum and liver. No human HGF expression was found in mice that were transduced with AAV5-LacZ vectors (Fig. 2a and b). Endogenous HGF level was also measured by ELISA. At 12 weeks after AAV5-LacZ transduction, endogenous HGF level of AAV5-LacZ-transduced mice ($n = 5$) was 2.8 ± 0.29 ng/ml in plasma and 87.3 ± 13.7 ng/g in the liver. Endogenous HGF level of CCl_4-treated mice without gene transduction ($n = 5$) was 2.3 ± 0.51 ng/ml in plasma and 79.3 ± 4.72 ng/g in the liver.

Effects of HGF on CCl_4-induced hepatic fibrosis

In order to assess the effect of HGF on liver fibrosis, mice received CCl_4 by intragastric administration once a week for 16 weeks. Four weeks after CCl_4 administration began, mice were transduced once with AAV5-HGF or AAV5-LacZ. Azan-Mallory staining demonstrated that moderate bridging fibrosis was observed in the livers of both AAV5-HGF- and AAV5-LacZ-transduced mice up to 6 weeks after transduction (Fig. 3a, b, e, f). However, AAV5-HGF markedly attenuated fibrosis at 9 or 12 weeks after transduction (Fig. 3g and h) compared with control vector (Fig. 3c and d). Quantitative analysis of the fibrosis by image analysis showed a 50% reduction in fibrosis after AAV5-HGF transduction (Fig. 3i). Immunostaining for α-SMA was performed to detect activated HSC. Expression of α-SMA in the liver was increased in AAV5-LacZ-transduced mice (Fig. 4a–d). This expression was observed up to 6 weeks after AAV5-HGF transduction (Fig. 4e and f), but was suppressed after 9 and 12 weeks of transduction (Fig. 4g and h).

Effects of HGF on hepatic fibrosis induced by bile duct ligation

In order to assess the effect of HGF on liver fibrosis induced by BDL, mice were transduced with AAV5-HGF or AAV5-LacZ. Seven weeks after transduction, BDL was performed on all mice. Mice were killed 2 weeks after BDL to investigate the potential effect of HGF. At the time of death (9 weeks after AAV5-HGF transduction), plasma and liver human HGF concentrations were similar to those seen in the CCl_4 model (16 ± 5 pg/ml in plasma and 224 ± 76 pg/g in the liver). Histologic analysis showed extensive peribiliary and interstitial collagen deposition in mice transduced with AAV5-LacZ (Fig. 5a). However, AAV5-HGF transduction largely attenuated these findings after BDL (Fig. 5c). In AAV5-LacZ-transduced mice, α-SMA-positive cells were observed in the periportal areas

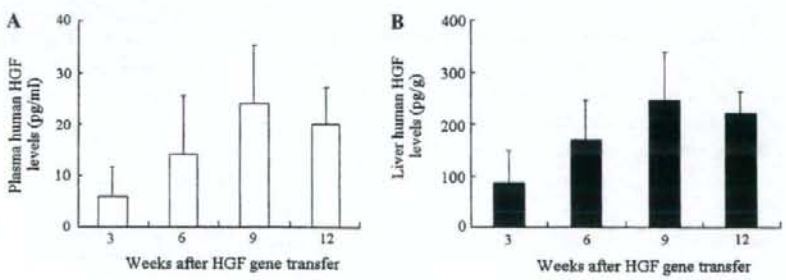

Fig. 2 Human HGF concentrations induced by AAV5-HGF transduction in CCl_4-treated mice (**a**: plasma, **b**: liver tissue). Human HGF concentrations gradually increased and reached a peak value both in the plasma and in the liver 9 weeks after transduction. Data represent the mean ± SD ($n = 5$)

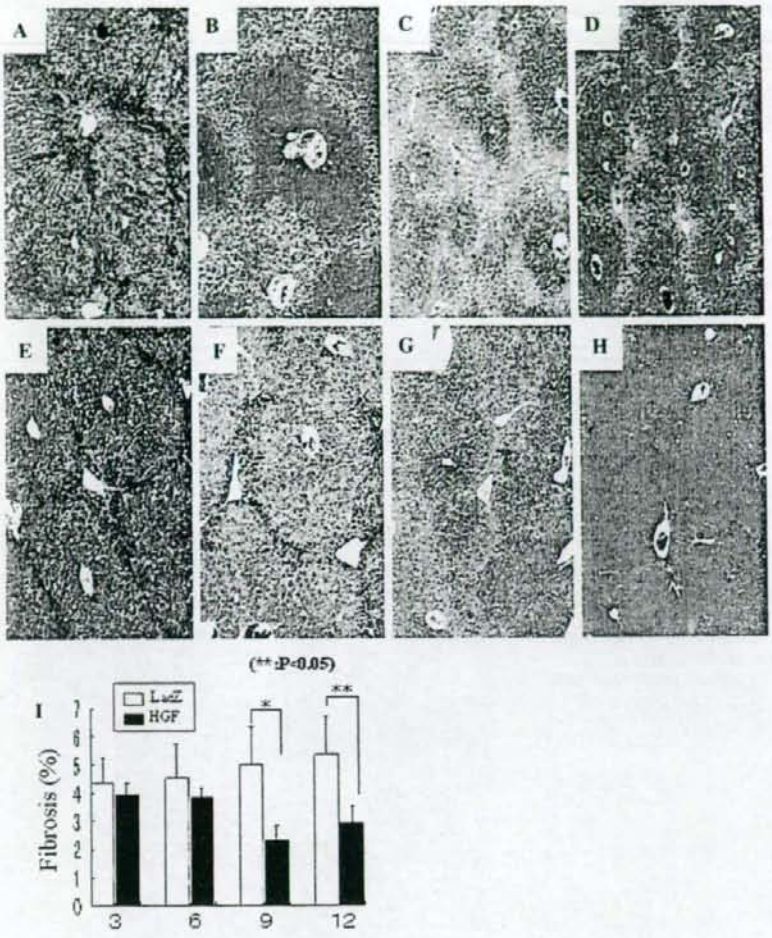

Fig. 3 a–h: Azan-Mallory staining of livers from mice treated with CCl_4 after gene transduction. Upper panels, AAV5-LacZ-transduced mouse livers (**a**: 3 weeks, **b**: 6 weeks, **c**: 9 weeks, and **d**: 12 weeks after AAV-5 LacZ transduction). Lower panels, AAV5-HGF-transduced mouse livers (**e**: 3 weeks, **f**: 6 weeks, **g**: 9 weeks, and **h**: 12 weeks after AAV-5 HGF transduction). Original magnification 40×. (**I**): Assessment of fibrosis using image analysis techniques, calculating the ratio of connective tissue to the whole area of the liver from mice transduced with AAV5-LacZ or AAV5-HGF. Data are presented as the mean ± SD (5 fields per mouse, $n = 5$)

(Fig. 5b). The number of these cells was reduced by AAV5-HGF transduction (Fig. 5d).

Fibrogenesis is suppressed in livers of AAV5-HGF-transduced mice

In order to investigate the role of HGF on liver fibrogenesis by CCl_4 or BDL, real-time PCR was performed (Fig. 6). TGF-β is a fibrogenic cytokine that plays a central role in regulating fibrosis. Expression of TGF-β mRNA was significantly suppressed in the liver of AAV5-HGF-transduced mice treated with CCl_4 (Fig. 6a) or BDL (Fig. 6e) mice. Collagen α1 (I) is the major form of collagen produced in fibrosis. The expression of Collagen α1 (I) mRNA was significantly suppressed in the liver of AAV5-HGF-transduced mice treated with CCl_4 (Fig. 6b) or BDL (Fig. 6f). Expression of α-SMA mRNA was significantly suppressed in the livers of AAV5-HGF-transduced mice treated with CCl_4 (Fig. 6c), although a significant difference was not seen in the livers of AAV5-HGF-transduced mice treated with BDL (Fig. 6g). Tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) inhibits collagen degradation by matrix metalloproteinase (MMP) and protects against apoptosis of HSC. The expression of TIMP-1 mRNA was significantly suppressed in the livers of AAV5-HGF-transduced mice treated with either CCl_4 (Fig. 6d) or BDL (Fig. 6h). These data suggest that AAV5-HGF transduction reduces liver fibrogenesis in 2 models of hepatic fibrosis.

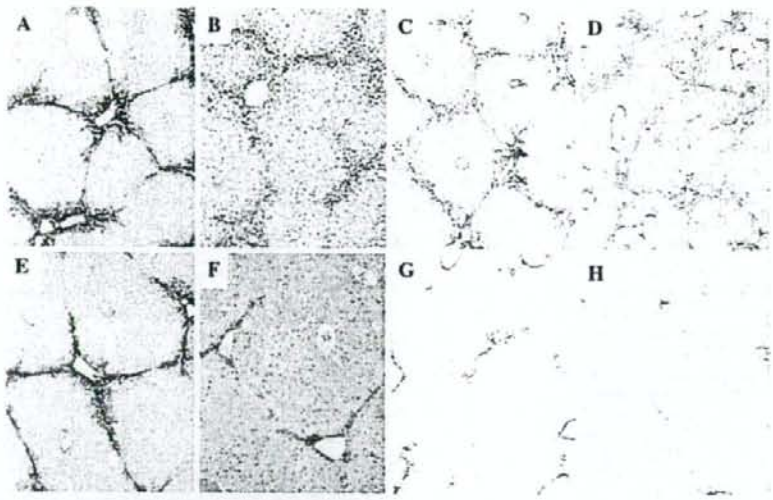

Fig. 4 α-SMA immunostaining of livers from mice treated with CCl_4 after gene transduction. Upper panels, AAV5-LacZ-transduced mouse livers (**a**: 3 weeks, **b**: 6 weeks, **c**: 9 weeks, and **d**: 12 weeks after AAV-5 LacZ transduction). Lower panels, AAV5-HGF-transduced mouse livers (**e**: 3 weeks, **f**: 6 weeks, **g**: 9 weeks, and **h**: 12 weeks after AAV-5 HGF transduction). Original magnification 40×

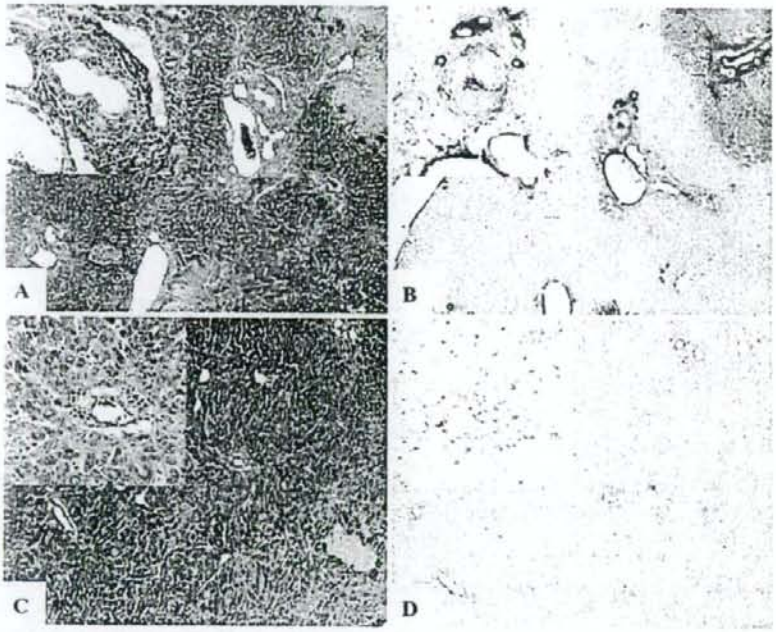

Fig. 5 Azan-Mallory staining (left panels) and α-SMA immunostaining (right panels) of BDL mouse livers after gene transduction (**a**, **b**: AAV-5 LacZ transduction; **c**, **d**: AAV-5 HGF transduction). Original magnification 40×, insets 100×

HGF transduction resolves liver fibrosis

Ets-1 has been shown to modulate transcription of several MMP genes [24, 25], and recent reports have shown that HGF increases collagenase expression in hepatic stellate cells via an Ets-1 transcription factor-dependent manner [26]. The MMP-13 is the interstitial collagenase in rodents that has the capability of degrading fibrillar collagens. Real-time PCR showed an increase in MMP-13 mRNA with HGF gene transduction in both CCl_4 mice (Fig. 7a) and BDL mice (Fig. 7b). In situ zymography showed extensive gelatin degradation in liver sections of HGF-transduced mice (Fig. 7c and d, right panels) compared with control mice (Fig. 7c and d, left panels). These data suggest that HGF gene transduction stimulates MMP expression followed by resolution of liver fibrosis.

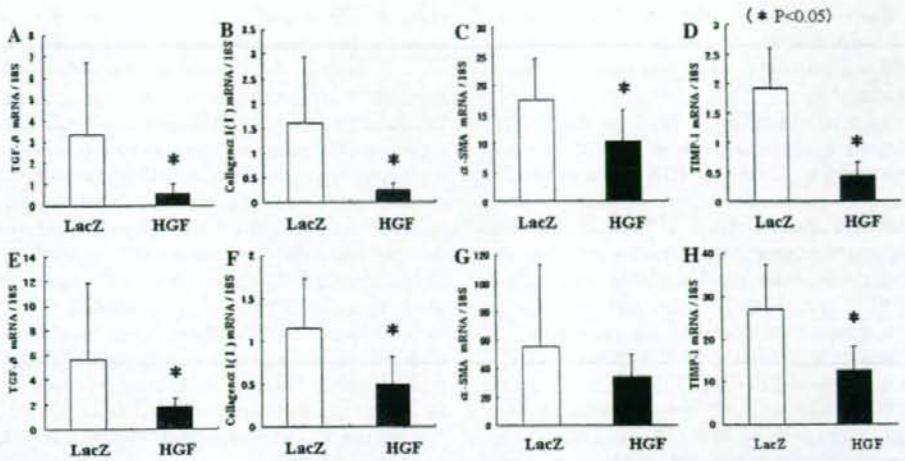

Fig. 6 HSC activation was suppressed by AAV5-HGF transduction in both CCl_4 mice (upper graphs) and BDL mice (lower graphs). At the final time point, total hepatic RNA was extracted. **a** and **e**: TGF-β; **b** and **f**: Collagen α1(I); **c** and **g**: α-SMA; **d** and **h**: TIMP. mRNA was quantified by real-time PCR. Data represent the mean ± SD ($n = 5$)

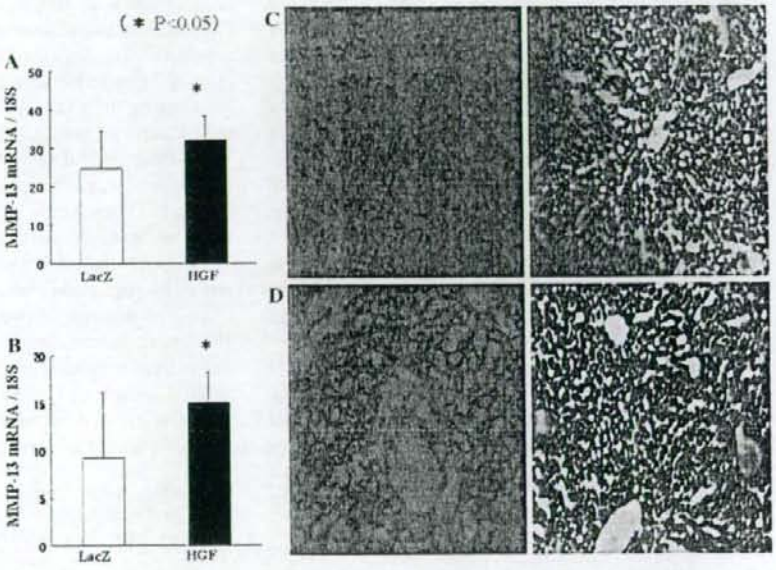

Fig. 7 HGF transduction increases resolution of liver fibrosis. Expression of MMP-13 mRNA in the livers from CCl_4-treated mice (**a**) or BDL mice (**b**). Data represent the mean ± SD ($n = 5$). In situ zymography from mouse livers from CCl_4 mice (**c**) or BDL mice (**d**). Left panels, AAV5-LacZ-transduced mouse livers. Right panels, AAV5-HGF-transduced mouse livers

Discussion

The present study demonstrates that AAV-mediated long-term HGF expression can be sustained up to 12 weeks by a single AAV5-HGF transduction, and significantly prevents liver fibrosis induced by CCl_4 and BDL. Up to our knowledge, this study is the first report that demonstrates the effect of AAV-mediated HGF gene therapy on liver fibrosis. CCl_4 induces acute liver injury that is attributed to inflammatory responses originating from CCl_4-derived free radical formation in the liver. Sustained hepatic inflammation by repetitive CCl_4 administration leads to liver fibrosis through the production of fibrogenic cytokines [27, 28]. In contrast, BDL causes biochemical stress and injury of the bile duct epithelium, which is followed by inflammation, epithelial cell proliferation, upregulation of fibrogenic cytokines that activate myofibroblasts in the periductal region, and finally leads to liver fibrosis [23].

Although the mechanisms resulting in liver fibrosis are different in these two animal models, a single round of AAV5-HGF transduction clearly suppressed liver fibrosis in both models.

With respect to the effect of HGF on fibrosis, two mechanisms are hypothesized. One is the suppression of fibrogenesis through inhibition of TGF-β gene expression. TGF-β has been implicated as a major cause of tissue fibrosis and is a potent inhibitor of hepatocyte growth. TGF-β induces the phenotypic transition of HSC into proliferating myofibroblast-like cells, which enhance production of ECM components [29]. Although the molecular mechanisms remain to be addressed, we previously demonstrated that HGF inhibits TGF-β expression [15] in dimethylnitrosamine (DMN)-treated rats. In this study, the mRNA of TGF-β was also significantly suppressed by HGF transduction in the livers of both CCl_4- and BDL-treated mice. Moreover, expression of α-SMA mRNA, a marker of HSC activation, also was suppressed by HGF transduction. Immunohistochemical staining demonstrated that HGF suppressed protein expression of α-SMA.

Another HGF-mediated mechanism is the resolution of liver fibrosis by increasing collagenase expression. MMPs are a family of extracellular zinc- and calcium-dependent proteases that promote degradation of the ECM components [30]. Ozaki et al. [26] reported that HGF increases MMP-1 promoter activity through increased expression and binding activity of Ets-1 in L190 cells, a human HSC. Ets-1 has been shown to modulate transcription of several MMP genes. In the present study, real-time PCR revealed an increase in MMP-13 mRNA by HGF gene transduction, and in situ zymography showed extensive gelatin degradation in liver sections of HGF-transduced mice. These data strongly suggest that HGF gene transduction stimulated MMP expression, resulting in the resolution of liver fibrosis.

As our and other investigators have reported, HGF has powerful therapeutic effects on liver fibrosis [12, 15, 31–33]. Matsuda et al. [12] reported that the continuous recombinant HGF injection accelerates the recovery from liver cirrhosis. However, a large amount of recombinant HGF is required using this method because HGF has a very short life. Recombinant HGF is very expensive, limiting such use in patients. Gene therapy has the advantage that it could sustain gene expression compared with the administration of recombinant protein. Recent studies have established several gene delivery systems for liver fibrosis. Since adenoviral vectors are capable of powerful gene expression, several reports have used adenoviral vector-mediated HGF gene therapy for liver fibrosis [31–33]. However, because it elicits a host immune response because of the highly immunogenic nature of the virus, it could not be used for repetitive transduction, limiting its clinical use [34, 35]. Nonviral vector systems, such as HVJ-liposome or naked plasmid administration, do not induce host immune response. However, these vectors require repetitive transduction to sustain gene expression because the expression is transient. Therefore, from a clinical viewpoint, a safe gene transfer method that achieves long-term expression is necessary. AAV vectors are single-stranded DNA viruses that are derived from a replication-deficient member of the parvovirus family. AAV vectors do not contain viral coding sequences and achieve efficient gene transduction in both dividing and nondividing target cells, while eliciting little immunogenicity. Moreover, they can achieve long-term gene expression in vivo [17, 36]. Davidoff et al. recently reported that AAV5-mediated gene expression stably maintained for 2 years in nonhuman primates [37]. In fact, a human clinical trial of an AAV vector has been conducted for the treatment of hemophilia B patients [38, 39]. Therefore, the use of AAV vectors for HGF gene therapy for liver fibrosis is feasible.

The influence of HGF on tumorigenicity should be considered, because the effect of HGF on the growth of HCC is still controversial. Although tumorigenicity has been reported in transgenic mice, overexpressing HGF, HGF expression level was 5000% higher in these mice compared with normal mice [40]. On the other hand, Shiota et al. [41] reported that growth of HCC cell lines was inhibited by the addition of recombinant HGF (50–200 ng/ml). Moreover, transgenic mice that express about 200–300% more HGF than normal mice demonstrate inhibition of neoplastic tumor development [42]. In our present study, human HGF level of HGF-transduced mouse was less than 40 pg/ml in serum, and less than 400 pg/g in liver tissue. We consider that such a low-level HGF concentration may not affect the tumorigenicity of HCC.

In conclusion, we demonstrated that AAV-mediated HGF gene therapy achieved long-term HGF expression, and markedly suppressed hepatic fibrosis induced by CCl_4 or BDL. These results suggest that AAV-HGF-mediated gene therapy may represent a novel strategy for the treatment of patients with progressive liver fibrosis.

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Protection Against Aminoglycoside-induced Ototoxicity by Regulated AAV Vector–mediated GDNF Gene Transfer Into the Cochlea

Yuhe Liu[1,2,3], Takashi Okada[1,4], Kuniko Shimazaki[5], Kianoush Sheykholeslami[6], Tatsuya Nomoto[1], Shin-Ichi Muramatsu[7], Hiroaki Mizukami[1], Akihiro Kume[1], Shuifang Xiao[3], Keiichi Ichimura[2] and Keiya Ozawa[1]

[1]Division of Genetic Therapeutics, Jichi Medical University, Tochigi, Japan; [2]Department of Otolaryngology, Jichi Medical University, Tochigi, Japan; [3]Department of Otolaryngology, Peking University First Hospital, Beijing, China; [4]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan; [5]Department of Physiology, Jichi Medical University, Tochigi, Japan; [6]Department of Neurobiology, Northeastern Ohio Universities College of Medicine, Rootstown, Ohio, USA; [7]Division of Neurology, Department of Medicine, Jichi Medical University, Tochigi, Japan

Since standard aminoglycoside treatment progressively causes hearing disturbance with hair cell degeneration, systemic use of the drugs is limited. Adeno-associated virus (AAV)-based vectors have been of great interest because they mediate stable transgene expression in a variety of postmitotic cells with minimal toxicity. In this study, we investigated the effects of regulated AAV1-mediated glial cell line–derived neurotrophic factor (GDNF) expression in the cochlea on aminoglycoside-induced damage. AAV1-based vectors encoding GDNF or vectors encoding GDNF with an rtTA2s-S2 Tet-on regulation system were directly microinjected into the rat cochleae through the round window at 5×10^{10} genome copies/body. Seven days after the virus injection, a dose of 333 mg/kg of kanamycin was subcutaneously given twice daily for 12 consecutive days. GDNF expression in the cochlea was confirmed and successfully modulated by the Tet-on system. Monitoring of the auditory brain stem response revealed an improvement of cochlear function after GDNF transduction over the frequencies tested. Damaged spiral ganglion cells and hair cells were significantly reduced by GDNF expression. Our results suggest that AAV1-mediated expression of GDNF using a regulated expression system in the cochlea is a promising strategy to protect the cochlea from aminoglycoside-induced damage.

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INTRODUCTION

Aminoglycoside antibiotics are frequently used in empiric therapy for serious infections, such as septicemia, complicated intra-abdominal infections, complicated urinary tract infections, and nosocomial respiratory tract infections. However, it is well known that aminoglycosides are associated with severe side effects, such as ototoxicity and nephrotoxicity, which attack the cochlea or vestibule and destroys the auditory and vestibular hair cells that pass information to the auditory nerve.[1] In addition, aminoglycosides predominantly destroy the outer hair cells by ototoxicity. Although the exact mechanism of damage is not well established,[2] aminoglycoside-induced hair cell loss results in a permanent hearing deficit[3] that can progressively occur 6 months to a year after exposure to these drugs. Therefore, the development of a strategy to prevent aminoglycoside-associated ototoxicity before adverse events occur is a critical issue in clinical settings.

The expression of a transgene using viral vectors is a potential approach to introduce neurotrophic factors into the cochlea to prevent and treat aminoglycoside-induced hearing loss. However, most of the currently used vectors, such as adenovirus vectors or herpes simplex virus vectors, have an associated vector-related cytotoxicity.[4,5] Hence, adeno-associated virus (AAV) vectors may be good candidates for gene transfer into the cochlear cells because of their efficient transduction and their safety and potential in long-term expression.[6] We have previously demonstrated that an AAV1-based vector efficiently transduced the inner hair cells, the spiral ganglion cells, and many other types of cells.[7] Therefore, an AAV1-based vector should successfully introduce secretory proteins, such as glial cell line–derived neurotrophic factor (GDNF), into the cochlea to prevent aminoglycoside-induced ototoxicity.

GDNF, a member of the transforming growth factor β family, was initially identified as a survival factor for mid-brain dopaminergic neurons and for a wide range of neuronal populations in the central and peripheral nervous systems.[8–10] Although it is still unclear whether GDNF protects against ototoxicity, sustained infusion of recombinant GDNF protected the cochlear structure and function from noise- and drug-induced damage and stress,[11–18] although its half-life is very short. However, an overdose of GDNF was shown to enhance the sensitivity of the cochleae to insult and

Correspondence: *Takashi Okada, Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8551, Japan. E-mail: t-okada@ncnp.go.jp; Keiya Ozawa, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. E-mail: kozawa@jichi.ac.jp*

to destroy cochlear function.[12] Furthermore, it has been reported that testicular tumors are formed in GDNF-overexpressing mice.[19] Therefore, an appropriate regulation system is required to realize the therapeutic benefits of GDNF expression.

Regulated transgene expression has been successfully achieved in various gene therapy experiments using the Tet system.[20–23] Notably, tetracycline derivatives, such as doxycycline (Dox), activate the Tet-on system at doses 100-fold lower than tetracycline. Furthermore, the reverse Tet-responsive transcriptional activator (rtTA) series were improved through the generation of variants called rtTA2s-S2, which showed lower leakiness and better inducibility in HeLa cells and mice.[24,25]

In this study, we describe *in vivo* therapeutic experiments utilizing AAV1 vector–mediated tetracycline-regulated expression of GDNF in cochleae. We demonstrate that AAV1 vector–mediated GDNF expression protects sensory cells in the inner ear from drug-induced degeneration.

RESULTS

Expression and distribution of transgene in the cochlea

AAV1-EGFP or AAV1-GDNF containing either the enhanced humanized green fluorescent protein (*EGFP*) gene or the *GDNF* gene under the control of the CAG (human cytomegalovirus (CMV) immediate-early enhancer and Chicken β-actin promoter) promoter, and the Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Figure 1a), was injected into the cochlea. The GDNF protein level in the perilymph was measured by enzyme-linked immunosorbent assay (Figure 2a). There was a significant increase in GDNF concentration in the cochlea transduced with AAV1-GDNF. The widespread distribution of the GDNF expression was observed in the cochlea, including the spiral ganglion and the inner hair cells (Figure 2b).

To examine the possible transduction of the contralateral ear with the virus diffusion, we analyzed the AAV vector–meditated transgene expression of the rodents with this transduction approach using the optical bioluminescence imaging. Luciferase expression was mainly detected at the injected side of the cochleae (5,370.3 photons/sec/cm^2/sr, **Supplementary Figure S1**). Interestingly, the AAV vector also transduced the contralateral ear (876.8 photons/sec/cm^2/sr), along with the brain (792.9 photons/sec/cm^2/sr). Similar results were obtained with repeated experiments.

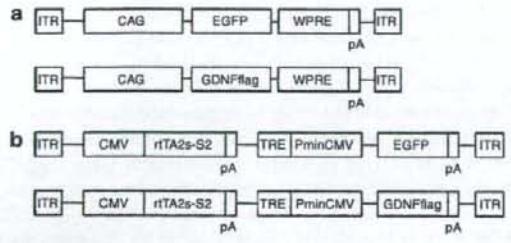

Figure 1 Schematic representation of the viral vectors used in this study. (**a**) An adeno-associated virus 1 (AAV1)-based vector was constructed using the CAG promoter to drive enhanced green fluorescent protein (EGFP) or mouse glial cell line–derived neurotrophic factor (GDNF) with a FLAG tag (GDNFflag). The Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was inserted into the 3′ end of the transgene cassette. (**b**) The transactivator rtTA2s-S2 is under the control of the CMV promoter. The minimal CMV promoter (PminCMV) induces transgene expression (EGFP or GDNFflag) in combination with the Tetracycline-responsive element (TRE) and transactivator. CAG, human cytomegalovirus immediate-early enhancer and Chicken β-actin promoter; CMV, cytomegalovirus immediate-early promoter; pA, the simian virus 40 polyadenylation sequences; ITR, inverted terminal repeat from AAV2.

Preservation of the hair cells and spiral ganglion cells in the cochlea

Hair cell loss in the whole-mount cochleae of all the tested rat groups was analyzed by F-actin staining with rhodamine–phalloidin. Figure 3a shows a representative dissection through the second turn of the rat cochlea, in which a full complement of hair cells is revealed by F-actin phalloidin staining. In the vehicle-treated control group, outer hair cells in the base and middle turn were drastically lost after kanamycin treatment (Figure 3b). In the AAV1-GDNF/kanamycin-treated group, successful protection of the hair cells in the cochlea was observed (Figure 3c). In the contralateral cochlea, some of outer hair cells were also protected (Figure 3d). The spiral ganglion cell loss in the basal turn of the cochlea was assessed using 4′,6-diamino-2-phenylindole dihydrochloride staining (Figure 4a and b). Survival of the spiral ganglion cells in the AAV1-GDNF injected cochlea was significantly improved compared to that in the AAV1-EGFP injected cochlea (Figure 4c).

Protection of cochlear function by GDNF

Auditory brain stem response (ABR) recordings of the aminoglycoside-treated animals were performed to examine hearing

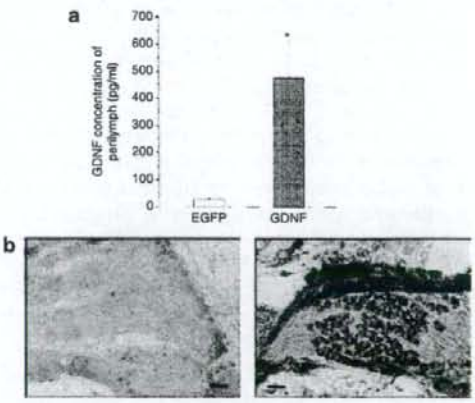

Figure 2 Expression and distribution of transgene in the cochlea. (**a**) Cochlear glial cell line–derived neurotrophic factor (GDNF) expression levels were measured by enzyme-linked immunosorbent assay in the transduced rats. GDNF expression level of the perilymph in the AAV1-GDNF/kanamycin group was significantly higher than that in the control group ($n = 5$, $P < 0.001$). (**b**) Immunohistochemistry was performed to analyze the expression of the GDNFflag in the rat cochlea. The AAV1-EGFP-transduced cochlea was used as a control (left). Cochlear sections were prepared after AAV1-GDNF injection and kanamycin administration. GDNflag expression was detected in the cochlea with an anti-FLAG antibody (right). Scale bar = 25 µm; ×400. AAV1, adeno-associated virus 1; EGFP, enhanced green fluorescent protein.

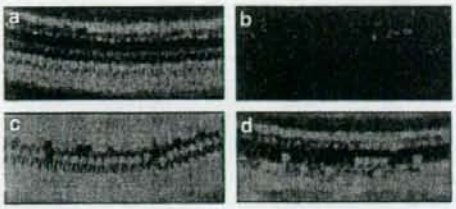

Figure 3 Preservation of the hair cells in the transduced cochlea. Hair cell loss in the cochlea of transduced rats was analyzed by F-actin staining. The dissected samples were dissected from the middle turn of the cochlea. The adeno-asssociated virus 1 (AAV1) vector was injected into the scala tympani of the cochlea prior to 12 days of kanamycin administration. (**a**) The normal cochlea. (**b**) The cochlea from the vehicle-treated ear; the many dark spaces represent the loss of outer hair cells. (**c**) The cochlea from the AAV1-GDNF-transduced ear. (**d**) The cochlea from the contralateral ear of AAV1-GDNF transduced rats. GDNF, glial cell line–derived neurotrophic factor.

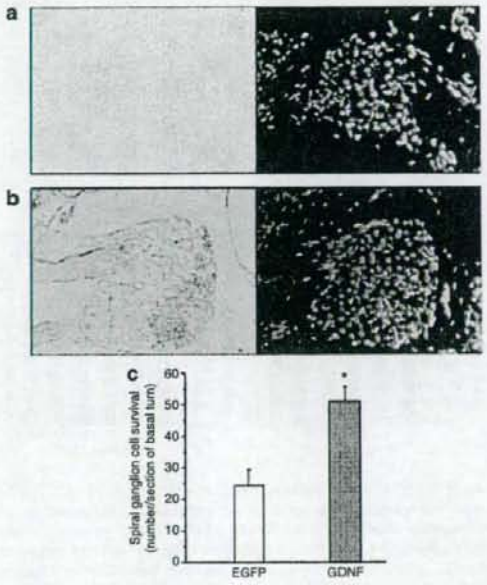

Figure 4 Survival of the spiral ganglion cells in the transduced cochlea. AAV1-GDNF-mediated rescue of the kanamycin-induced damage to the rat spiral ganglion neurons (SGNs): 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) staining was performed on the sections obtained from the rat pretreated with either AAV1-EGFP or AAV1-GDNF, followed by kanamycin injections. (**a, b**) Representative photomicrographs of the cryosections showing the basal turn of the cochlear spiral (**a**, AAV1-EGFP; **b**, AAV1-GDNF). (**c**) The number of DAPI-positive large-nucleus cells that exhibited SGN morphology was counted. An asterisk denotes a statistically significant difference between the AAV1-GDNF and AAV1-EGFP-transduced rats (*t*-test, $P < 0.001$). AAV1, adeno-associated virus 1; EGFP, enhanced green fluorescent protein; glial cell line–derived neurotrophic factor.

impairment. At all frequencies tested, both GDNF-transduced and contralateral, untreated ears showed a significant improvement in the threshold shifts compared to the ears transduced with EGFP ($n = 5$, $P < 0.05$) (Figure 5). In the EGFP group, there was

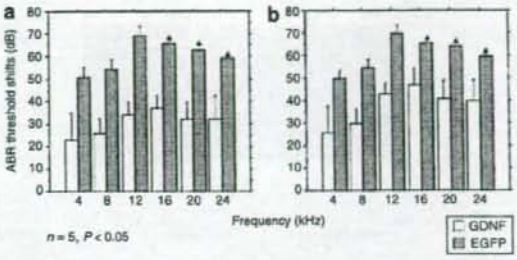

Figure 5 Protection of cochlear function by glial cell line–derived neurotrophic factor GDNF. Auditory brain stem response (ABR) threshold shifts (mean ± SD) of the (**a**) treated and (**b**) untreated ears at each tested frequency in the enhanced green fluorescent protein (EGFP) and GDNF transduced rats. The ABR threshold was measured twice in all the animals. The ears treated with AAV1-GDNF showed a significant improvement in the threshold shifts compared to the ears treated with AAV1-EGFP at all frequencies tested ($n = 5$, $P < 0.05$). Arrows indicate the average ABR thresholds that exceeded the output power of the ABR apparatus. AAV1, adeno-associated virus 1.

no significant difference in the ABR threshold shifts between the transduced and contralateral, untreated cochleae at all frequencies tested. Animals transduced with the AAV1-GDNF demonstrated lower ABR threshold shifts in the injected side compared to the contralateral side (at 12, 16, 20, 24 kHz; $P < 0.05$). These data indicate that both ears were protected even if the AAV1-GDNF was only injected into one ear.

Induced transgene expression

Two hundred and ninety three cells were transduced with the proviral plasmid harboring rtTA2s-S2 and the tetracycline-responsive element (TRE) to express the *EGFP* gene (Figure 1b). In the presence of Dox, a significant level of fluorescence was detected, suggesting that the rtTA2s-S2 system could switch on transcription following Dox treatment (Figure 6a). In contrast, reporter gene expression in the cultured cells was faint in the absence of Dox, indicating a low basal activity *in vitro*. Western blot analysis of GDNF showed that the transgene was induced in the presence of Dox, while no expression was detected in the absence of Dox (Figure 6b).

In vivo induction of GDNF expression and the dose–response to Dox

Enzyme-linked immunosorbent assay analysis of the GDNF expression level in muscle also showed low basal activity and induced expression after Dox treatment (Figure 6c). In the absence of Dox, the expression level of GDNF in the AAV2-S2-GDNF group was as low as that in the phosphate-buffered saline and AAV2-*LacZ* groups. On the other hand, significant increases in the GDNF level were observed in the muscle with increasing amounts of Dox, demonstrating that the rtTA2s-S2 system induces gene expression in a dose-dependent manner.

Inducible GDNF expression in the cochlea

Extensive inducible GDNF transgene expression was confirmed by immunohistochemistry using an anti-FLAG-antibody in the AAV1-S2-GDNF/kanamycin group in the presence of Dox (Figure 7).

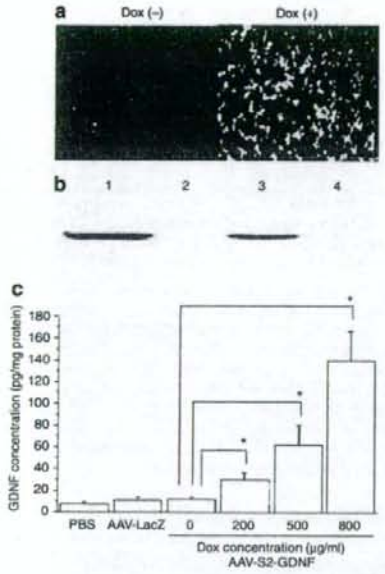

Figure 6 Induction of the transgene expression. (**a**) HEK293 cells were transfected with the proviral plasmid pAAV2-rtTA-S2-TRE-d2EGFP, and the expression of enhanced green fluorescent protein (EGFP) was induced by the doxycycline (Dox) (1 μg/ml). (**b**) Western blot analysis with an anti-FLAG antibody to detect the glial cell line–derived neurotrophic factor (GDNF) expression in the transduced 293 cells with the proviral plasmids. pAAV2-GDNF (lane 1), pAAV2-EGFP (lane 2), pAAV2-rtTA2s-S2-TRE-GDNF with Dox (lane 3), and pAAV2-rtTA2s-S2-TRE-GDNF without Dox (lane 4). (**c**) Dose–response of GDNF in the AAV2-S2-GDNF-injected muscle to the various concentrations of Dox. Mice were injected with phosphate-buffered saline (PBS), AAV2-LacZ, or AAV2-S2-GDNF followed by Dox administered in the drinking water. The mean muscle GDNF concentration in the animals treated with the AAV2-S2-GDNF in the absence of Dox was not significantly different compared to the animals treated with PBS or AAV2-*LacZ* ($P > 0.05$). The GDNF expression levels in the animals transduced with the AAV2-S2-GDNF significantly increased with increasing Dox concentration ($P < 0.05$). AAV1, adeno-associated virus 1.

In contrast, no detectable GDNF expression was observed in the cochlea of the AAV1-S2-GDNF/kanamycin group in the absence of Dox (data not shown).

Protection of cochlear function with induced GDNF expression

To evaluate the adverse effects of the transduction procedure, ABR recordings were performed on kanamycin-free rats after injection of the inducible AAV1-S2-GDNF vectors and Dox administration. At all frequencies tested, no significant increase in the ABR threshold was observed after virus injection (Figure 8a). This result indicates that AAV1 vector injection, transgene expression, and Dox administration did not affect the ABR threshold of the experimental rats. Interestingly, even if AAV1-S2-GDNF was injected into the cochlea of one ear, the cochleas of both ears were protected in the presence of Dox. In particular, the ABR threshold shifts were significantly improved in both the AAV1-S2-GDNF-injected cochlea of the kanamycin-treated rats in the presence of Dox (Figure 8b)

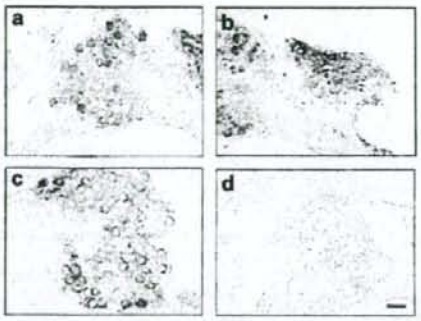

Figure 7 Expression of the GDNFflag in the rat cochlea. (**a**, **b**, **c**) The sections were sampled after the AAV1-S2-GDNF injection into the cochlea in the presence of doxycycline. GDNFflag expression was detected using an anti-FLAG antibody. (**d**) Samples from AAV1-EGFP-inoculated cochlea were analyzed as the negative control. Scale bar = 25 μm; ×400. AAV1, adeno-associated virus 1; GDNF, glial cell line–derived neurotrophic factor; EGFP, enhanced green fluorescent protein.

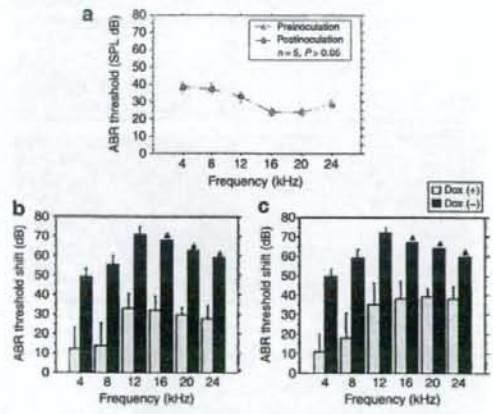

Figure 8 Protection of cochlear function with induced glial cell line–derived neurotrophic factor (GDNF) expression. (**a**) Auditory brain stem response (ABR) thresholds (mean ± SD) at each frequency tested in the AAV1-S2-GDNF-injected rat cochlea in the presence of doxycycline (Dox). No significant difference in the hearing thresholds was observed at each frequency between preinjection and postinjection. SPL, sound pressure level. ABR threshold shifts (mean ± SD) at each tested frequency in the (**b**) transduced or (**c**) nontransduced cochlea with or without Dox. Significant differences in the hearing threshold shifts were observed at each frequency between AAV1-S2-GDNF cochlea in the presence and absence of Dox ($n = 5$, $P < 0.05$). Arrows indicate the average ABR thresholds that exceeded the output power of the ABR apparatus.

and the cochlea of the noninjected contralateral ear (Figure 8c). However, the ABR threshold shifts at all frequencies were significantly lower in the treated group (AAV1-S2-GDNF/kanamycin plus Dox) than in the contralateral, untreated ear.

DISCUSSION

In this study, we showed that both sustained and regulated AAV1-mediated GDNF expression protected the cochlear function of rats from aminoglycoside-induced ototoxicity. Indeed, damaged spiral ganglion cells and hair cells were significantly reduced by

regulated GDNF expression. The ABR monitoring revealed that there was no loss of the cochlear function over the frequencies tested after AAV vector injection and Dox treatment. These data suggest that regulated expression of GDNF in the cochlea efficiently preserves the cochlea from kanamycin-induced ototoxicity.

Among the various viral vector systems, the recombinant AAV-mediated gene transduction system offers several important advantages as a tool for direct somatic gene delivery into the cochlea. These include long-term stable expression of therapeutic genes in a wide variety of postmitotic tissues and minimal vector-related cytotoxicity.[26] In our previous report, we demonstrated the effective transduction of mouse cochleae with the AAV1-based vectors.[7] Generally the therapeutic effectiveness depends on an appropriate concentration and the half-life of the molecules. AAV vector–mediated gene transfer is a promising delivery technique to facilitate a long-term and chronic supply of therapeutic proteins that have a short half-life, such as GDNF. Furthermore, when the CAG promoter is used, efficient transduction activity is observed in the cochlear cells including the inner hair cells and spiral ganglion cells.[27,28]

Our data showed that AAV1-GDNF-mediated transduction of the rat cochleae provided significant protection of the cochlea against aminoglycoside-induced damage. This finding is consistent with previous studies that have used adenovirus-mediated GDNF expression[13,15,16] and demonstrates the feasibility of gene therapy with AAV1-based vectors for drug-induced hearing loss. Although the exact mechanism has not yet been elucidated, antioxidant pathways might be involved in the protective function of GDNF in the inner ear.[29] Free-radical formation following exposure to aminoglycoside is considered one of the major mechanisms to explain the aminoglycoside-related hair cell death.[1,30,31] It has been previously shown that GDNF is endogenously synthesized in the inner hair cells and spiral ganglion cells of the cochlea,[32] and the two known GDNF receptors are present in the spiral ganglion.[17,32,33] In the present study, we inoculated the cochlea with the AAV1 vectors via the round window membrane and detected a high level of transgene expression mainly in the inner hair cells and spiral ganglion cells. The AAV1-mediated GDNF expression pattern was similar to that of the endogenous protein; therefore GDNF supplemented *in situ* can play a substantial role in protection. Although the transduction of the *GDNF* gene was not observed in outer hair cells, GDNF levels in the perilymph of the manipulated cochleae was much higher than in the control cochleae. These cells may respond to the secretion of another growth factor that promotes hair cell survival. Upregulation of GDNF in inner hair cells and spiral ganglion cells following noise also support this concept.[34]

Compared to the vehicle controls, increased cochlear cell survival was observed in the contralateral ears of the AAV1-GDNF group, suggesting that the contralateral cochleae in treated rats were also moderately protected. Expression of the transgene was detected in the contralateral cochlea of the rats after injection with 5×10^{10} genome copies of AAV1 per cochlea (data not shown). AAV can diffuse from one ear to the other via the cerebrospinal fluid in rodents.[35] Therefore, secreted GDNF molecules may also diffuse and exert a protective effect in the opposite ear. Alternatively, GDNF might enhance the neuronal activity (either afferent or efferent) of both ears, protecting both the treated and the contralateral cochlear function. Moreover, since infusion of the vectors into the cochlea forces large amounts of the vectors into the cerebrospinal fluid, any functional effect might be associated with the transduction of the brain. In this context, it is of great interest to know whether the otoprotective effect was achieved by the simple diffusion of the transgene product or direct transduction of the cells in the contralateral ear. To answer this question, we analyzed the local expression of the nonsecretory protein marker in the rodents with this transduction approach. Consequently, we feel that the direct transduction of the cells in the contralateral ear might be involved in the neuroprotection.

The present results showed that AAV-mediated delivery of a Tet-on system was able to control transgene expression. This Tet-on system incorporates the mutant transactivator rtTA2s-S2 and the transgene in which messenger RNA transcription is activated in the presence of an inducer, leading to protein expression. As we showed, the inducible expression of GDNF efficiently protected the cochlear structure and function from kanamycin-induced damage. GDNF was overexpressed in the induced state with the rtTA2s-S2 system, whereas GDNF expression was nearly normal in the non-induced state. In our study, cochlear function was significantly protected from aminoglycoside-induced cochlear damage in the presence of Dox. Although intracochlear injections did not affect physiological cochlear function, intramuscular injections of the vectors expressing Dox-dependent activators may elicit a cellular and humoral response against the transactivator in nonhuman primates.[36–38] The use of tissue-specific promoters that restrict transgene expression to nonprofessional antigen-presenting cells, and the use of AAV vectors, may reduce the induction of a specific T-cell response.[39]

Another attractive feature of the Tet-on system is the high safety profile of the inducer. In our study, Dox was orally administered to the rats to induce GDNF expression. Transgene expression levels were dependent on the dose of Dox, and the dose range of this inducer was below the normal bactericidal treatment levels used in similarly sized animals.[40,41] Furthermore, a Dox regimen in mice that is proportional to a clinically accepted dose of the drug in humans causes a significant induction of transgene expression.

Sequences of antibiotic administration and withdrawal to reverse the Dox induction of therapeutic gene expression were demonstrated in previous studies.[25,42] However, the aminoglycoside-induced hearing impairment model is not an appropriate model for adding and removing the Dox diet because the insulting phase is too short to successively induce and repress the Tet-on system. Furthermore, treatment of age-related hearing loss or genetic hearing loss ideally needs long-term gene expression studies to exclude any adverse events associated with the therapeutic genes.

Efficient control of the tetracycline-regulatory system is based on the specificity of the TetR/tetO interaction and the efficiency and safety of its inducers, such as tetracycline or Dox.[43,44] Mutant tTA2s are composed of one TetR and three repeated oligonucleotides of the VP-16-derived minimal activation domain. In the Tet-on system, rtTA2s-S2 showed a high activating ratio because its background expression level was lower than that of other mutants, such as rtTA2s-M2, which despite having a higher activation potential had also a high initial background.[25] By using the mutant transactivator, Urlinger *et al.* demonstrated that stringent regulation of target genes could be achieved over a range of four to five orders of magnitude

in stably transfected HeLa cells.[24] These regulatory systems could be further optimized to offer several potential advantages. The tetracycline-dependent transcriptional silencer allows tight regulation of transgene expression by eliminating baseline leakage.[20,45] Gene regulation mediated by rtTA2s-S2 was substantially tighter when combined with active silencing by the tetracycline-dependent transcriptional silencer in the non-induced state.[41,46]

Our results show that AAV1-mediated gene transfer is a promising gene delivery approach for the inner ear apparatus. To become an efficient and safe therapeutic method, it will be necessary to improve vector technology to achieve long-term transduction in a fail-safe system. We presented data demonstrating successful AAV-mediated transfer and modulation of transgene expression in the cochlea using a modified Tet-on system. In addition to the need for dosage control of neurotrophic factors, the AAV1 and the Tet-on system maybe useful for the regulation of the expression of other therapeutic gene products in the cochlea. Following further improvements, the rAAV-mediated transduction system may be of potential use for cochlear gene therapy applications in humans.

MATERIALS AND METHODS

Construction and preparation of the plasmids. The AAV vector proviral plasmid pAAV2-CAG-EGFP-WPRE (pAAV2-EGFP) contained the *EGFP* gene under the control of the CAG promoter and the WPRE and was flanked by inverted terminal repeats. A *Bam*HI fragment containing the GDNFflag complementary DNA was subcloned into this plasmid to obtain the pAAV2-CAG-GDNF-WPRE (pAAV2-GDNF) cassette.

The pAAV2-CMV-GDNFflag plasmid with the CMV promoter, the first intron of the human growth hormone gene, and the simian virus 40 polyadenylation signal sequence, were inserted between the inverted terminal repeats of the AAV type 2 genome.[47] The transactivator rtTA2s-S2 complementary DNA in the pUHrT61-1 plasmid (BD Biosciences, San Jose, CA) and the TRE in the pTRE-d2EGFP plasmid (BD Biosciences, CA) were subcloned together into the pAAV2-CMV-GDNFflag plasmid to obtain the AAV vector proviral plasmid pAAV2-rtTA2s-S2-TRE-GDNF. A *Sac*II-*Eco*RI fragment containing the d2EGFP complementary DNA from the pTRE-d2EGFP plasmid (BD Biosciences, CA) was subcloned into this plasmid to create the pAAV2-rtTA2s-S2-TRE-EGFP plasmid (see **Supplementary Materials and Methods**).

Recombinant AAV vector production. The AAV1 vectors were produced as previously described by using a 293-cell transfection protocol[26] with the proviral plasmid pAAV2-EGFP, pAAV2-Luciferase,[48] pAAV2-GDNF, pAAV2-rtTA2s-S2-TRE-EGFP, or pAAV2-rtTA2s-S2-TRE-GDNF; the AAV packaging plasmid pAAV1RepCap; and the adenovirus helper plasmid pAdeno5 using an active gassing system.[49] The recombinant AAV2 expressing the *Escherichia coli* β-galactosidase gene under the control of the CMV promoter (AAV2-LacZ) was generated using the proviral plasmid pAAV-LacZ.[50] (see **Supplementary Materials and Methods**).

In vitro *expression of GDNF.* To detect the *in vitro* expression of the GDNFflag fusion protein, 293 cells were transduced with the AAV1-GDNFflag at 1×10^4 vector genome copies/cell. For the detection of the regulated expression, 293 cells were transduced with the AAV proviral plasmid pAAV2-rtTA2s-S2-TRE-d2EGFP or pAAV2-rtTA2s-S2-TRE-GDNF in the presence or absence of 1 μmol/l Dox-HCl (Sigma, St Louis, MO) (see **Supplementary Materials and Methods**).

Surgical procedures and cochlear perfusions. All animal studies were performed in accordance with the guidelines issued by the committee on animal research at Jichi Medical University. Twenty 5-week-old male Sprague–Dawley rats with normal Preyer's reflexes weighing 130–150 g were utilized (CLEA Japan, Tokyo, Japan). Five-week-old male C57BL/6J mice were utilized for optical bioluminescence imaging. The animals were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). A postauricular approach was performed to expose the tympanic bony bulla. A small opening (2 mm in diameter) to the tympanic bulla was made by carefully drilling through the bone of the bulla to gain access to the round window membrane. Subsequently, 5 μl of AAV vector solution (AAV1-EGFP, AAV2-Luciferase, or AAV1-GDNF, 5×10^{10} genome copies, $n = 5$ each) was microinjected into the cochlea through the round window for over 10 minutes using a glass micropipette (40 μm in diameter) fitted on a Univentor 801 syringe pump (Serial No. 170182, High Precision Instruments, Univentor Ltd., Malta). The rats were also injected with the AAV1-S2-GDNF in the presence ($n = 5$) or absence ($n = 5$) of Dox. A small plug of muscle was used to seal the cochlea, and the surgical wound was closed in layers and dressed with an antibiotic ointment.

***Transgene expression* in vivo.** The rats were deeply anesthetized and the perilymph was sampled from the inoculated cochlea through the round window. GDNF protein levels were measured using a GDNF Emax ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's instructions. The GDNF expression in the rat cochlea was determined by immunohistochemistry using an anti-FLAG antibody.

AAV2-LacZ or AAV2-S2-GDNF vector (1×10^{10} genome copies) was injected into the quadriceps of the C57BL/6J mice (6 weeks old, CLEA Japan, Tokyo, Japan). The mice were injected with phosphate-buffered saline ($n = 5$) or AAV2-*LacZ* ($n = 5$). Animals treated with various concentrations of Dox were injected with the AAV2-S2-GDNF ($n = 5$ per group). Two weeks after the transduction, animals were deeply anesthetized, and the injected muscle was sampled. The tissue levels of the GDNF protein were measured with an enzyme-linked immunosorbent assay kit (GDNF Emax ImmunoAssay System, Promega, WI), according to the manufacturer's instructions. The levels of GDNF were expressed as pg/mg protein. The assay sensitivity ranged from 16 to 1,000 pg/ml.

Two weeks after the injection of the AAV2-Luciferase, optical bioluminescence imaging was performed using the CCD camera (Xenogen, Alameda, CA). After intraperitoneal injection of reporter substrate D-Luciferin (375 mg/kg body weight), mice were imaged for scans.

Kanamycin administration and ABR assessment. A dose of 333 mg of kanamycin base/kg body weight was obtained by injecting 3 μl/g body weight. Seven days after virus injection, kanamycin was given subcutaneously twice daily for 12 consecutive days. The body weight of the animals was monitored daily to adjust the kanamycin dosages accordingly.

Auditory thresholds were determined by audiometry of evoked ABRs using Tucker-DAVIS Technologies and Scope software (Power Lab; ADInstruments, Coloado Springs, CO). Thresholds were evaluated for each animal prior to the start of the injection procedure and 2 days after the termination of kanamycin treatment. The ABRs were measured as previously described,[7] using a two-way repeated analysis of variance (see **Supplementary Materials and Methods**).

Histological evaluation of the cochlear preservation. Cochlear hair cell loss was determined by F-actin staining. One month after transduction, the presence of the cochlear spiral ganglion neurons was determined by 4′,6-diamino-2-phenylindole dihydrochloride staining to visualize nuclear chromatin. After decalcification, 6 μm mid-modiolus cryosections of the cochleae from each animal were histologically analyzed. The number of spiral ganglion neurons was determined in every third section of the cochlear basal turn from the AAV1-transduced and kanamycin-treated rats (see **Supplementary Materials and Methods**).

Statistical analyses. Results are presented as the means ± SD. Data were statistically analyzed using analysis of variance, paired student's *t*-test (injected versus contralateral sides) or unpaired student's *t*-test (therapy versus control groups) (StatView 5.0 software; SAS Institute, Cary, NC).

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SUPPLEMENTARY MATERIAL

Materials and Methods.

Figure S1. Bioluminescence of the transduced cochlea in living mice.

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Commentary E

AAV VECTORS PROVIDE A VALUABLE TOOL FOR NEUROSCIENCE

Shin-ichi Muramatsu
Division of Neurology, Jichi Medical University, Japan.

ABSTRACT

Recombinant adeno-associated virus (AAV) vectors provide efficient and long-term gene expression in the brain without significant toxicity. AAV vector-mediated expression of pathogenic genes can be used to create novel animal models of neurological diseases and spatially controlled delivery of Cre recombinase allows conditinal inactivation of target genes in adult transgenic mice. AAV vectors are faily useful in neuroscience.

Although the ultimate aim of developing new viral vectors is clinical application for safe and effective gene therapy, the expanding knowledge of vector biology make viral vector-mediated gene transfer a very efficient tool for studying complex features of the central nervous system and elucidating the pathogenesis of neurological diseases. In preclinical gene therapy experiments using animal models of neurodegenerative disorders, some of the most powerful gene delivery vehicles are recombinant adeno-associated virus (AAV) vectors, the only vectors derived from non-pathogenic and replication-defective viruses. To date, more than 100 different AAV sequences have been isolated from human and nonhuman primates, and recombinants have been investigated extensively for tissue tropism and transduction efficiency [1]. Vectors derived from AAV serotype 2 (AAV-2), a prototype of AAV, can provide long-term (>3 years) gene expression in mammalian brains without creating severe toxic effects or immune responses. Clinical trials for Parkinson's disease have been conducted using AAV-2 vectors [2] and the safety of these vectors has been confirmed [3]. While AAV-2 rarely transduces glial cells, transduction of oligodendrocytes is desirable for gene therapy of diseases that affect myelin, such as multiple sclerosis and leukodystrophy. Among the various new AAV serotypes that have been cloned recently, AAV serotype 8

(AAV-8) is of particular interest as a highly efficient gene transfer vehicle for many organs and the ability to transduce glial cells in the murine brain [4]. AAV-8 vector may be useful not only for enhancing the efficacy of therapies for dysmylinating or demylinating diseases of the brain, but also in studying development of oligodendrocytes and the myelination process.

Taking advantage of the efficient and non-toxic transduction of neurons, AAV vectors have already been successfully implemented to create novel animal models of neurological disorders by expressing pathogenic genes instead of therapeutic genes. Techniques for stereotaxic injection into the rodent brain have been established and modern magnetic resonance imaging navigation systems allow precise pin-pointing of infusion sites, even in non-human primates, offering an invaluable tool for studying regions of the brain. In addition, positron emission tomography with various tracers can be used to monitor the effects of transgene expression *in vivo*. Overproduction of α-synuclein in the substantia nigra or β-amyloid in the hippocampus recapitulates the neuronal degeneration in Parkinson's disease or Alzheimer disease, respectively. Viral vector-mediated expression of an α-synuclein model is valuable not only in providing better understanding of involvement in the disease process, but also to offer a closer semblance of the neurodegeneration found in Parkinson's disease than seen with the classical neurotoxin-induced model. Monkeys injected with AAV vectors expressing human mutant α-synuclein show gradual loss of nigral dopaminergic neurons, ubiquitin containing aggregates and motor impairments [5]. Roles of specific *amyloid-β peptide* (Aβ) can be studied in AAV vector-treated animals. Infusion of AAV vectors encoding fusion of *BRI*, a causative gene for familial British dementia, and Aβ42 cDNAs resulted in elevated levels of detergent-insoluble Aβ in the hippocampus [6]. Notably, *in vivo* transduction efficiency would vary due to vector batch differences. Despite this inherent weakness, viral vector-mediated overexpression offers a valuable tool for modeling aspects of neurodegeneration.

Use of AAV vectors is superseding conventional methods for *in vivo* delivery of molecular switches in the brain. Under conventional methods, conditional knock-out mice are made using bacteriophage P1 Cre recombinase (Cre) to shut down gene expression. Transgenesis is achieved by mating mice expressing Cre with transgenic mice expressing a target sequence flanked by loxP sequences, but because phenotypic changes may occur, interpreting the effects of molecular switches on adult brain function is difficult. In contrast, delivery of Cre using AAV vector allows specific inactivation of target genes in adult mice [7].

More recently, we have developed an AAV vector that expresses Cre only after induction by tamoxifen, a synthetic estrogen [8]. This vector was originally generated to increase the safety of gene therapy for neurodegenerative diseases. We have previously demonstrated that AAV vector-mediated gene transfer of 3 enzymes for synthesizing dopamine (DA), namely tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), and GTP cyclohydrolase I (GCH), results in behavioral recovery in animal models of Parkinson's disease. We also showed that gene delivery of glial cell line-derived neurotrophic factor (GDNF) in skeletal muscles slows down the degeneration of motoneurons in a mouse model of amyotrophic lateral sclerosis (ALS). To regulate gene expression and avoid overproduction of DA or GDNF, we generated a recombinant AAV vector that expresses an improved variant of tamoxifen-inducible Cre recombinase (CreERT2), where recombinase is

fused to the mutated ligand-binding domain of the human estrogen receptor. AAV vectors that express TH or GDNF were modified by adding flanking loxP sites to coding sequence of TH or GDNF (AAV-floxed TH or AAV-floxed GDNF). Hemiparkinsonian rats received a mixture of AAV-CreERT2, AAV-floxed TH, AAV-AADC and AAV-GCH, and half were further treated with intraperitoneal 4-OH tamoxifen (4 mg/kg, 5 days). Control rats were injected with AAV-LacZ alone, or AAV-Cre that expressed Cre recombinase with AAV-floxed TH/-AADC/-GCH. Behavioral recovery was observed in rats that received AAV vectors expressing DA-synthesizing enzymes, except for those that received AAV-Cre simultaneously. After tamoxifen treatment, AAV-CreERT2-injected rats again showed behavioral impairment. DA level in lesioned striatum was significantly lower and fewer TH-immunoreactive cells were seen in the tamoxifen-treated AAV-CreERT2 group compared with the tamoxifen-untreated group. AADC activity was unchanged after tamoxifen treatment, suggesting selective removal of the TH gene. Tamoxifen-induced reductions in GDNF expression were also observed in the muscles of mice that received both AAV-floxed GDNF and AAV-CreERT2. Inducible reduction of transgene using AAV-CreERT2 should represent not only a molecular switch for increasing the safety of gene therapy, but also a powerful experimental tool for neuroscience.

Induction of RNA interference (RNAi), a gene-silencing mechanism, by expressing target-complementary short hairpin RNA (shRNA) from viral vectors is another promising technique for investigating gene function *in vivo*. Persistent and functional shRNA expressions have been reported in therapeutic experiments including a mouse model of spinocerebellar ataxia type 1 [9]. Further improvements may be achieved by engineering upstream primary microRNAs with more appropriate controlled expression vectors.

With continued advances, AAV vectors will become more valuable tools for use in neuroscience.

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ARTICLE

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Viral-Mediated Temporally Controlled Dopamine Production in a Rat Model of Parkinson Disease

Xiao-gang Li,[1,2] Takashi Okada,[2] Mika Kodera,[1] Yuko Nara,[1] Naomi Takino,[1] Chieko Muramatsu,[1] Kunihiko Ikeguchi,[1] Fumi Urano,[3] Hiroshi Ichinose,[3] Daniel Metzger,[4] Pierre Chambon,[4] Imaharu Nakano,[1] Keiya Ozawa,[2,*] and Shin-ichi Muramatsu[1,†]

[1]*Division of Neurology, Department of Medicine, and* [2]*Divison of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi 329-0498, Japan*

[3]*Department of Life Science, Tokyo Institute of Technology, Kanagawa 226-8501, Japan*

[4]*Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Université Louis Pasteur, Collège de France, and Institut Clinique de la Souris, 67404 Illkirch Cedex, France*

*To whom correspondence and reprint requests should be addressed at the Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Tochigi 329-0498, Japan. Fax: +81 285 44 8675. E-mail: kozawa@ms.jichi.ac.jp.

†To whom correspondence and reprint requests should be addressed at the Division of Neurology, Department of Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Tochigi 329-0498, Japan. Fax: +81 285 44 5118. E-mail: muramats@ms.jichi.ac.jp.

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Regulation of gene expression is necessary to avoid possible adverse effects of gene therapy due to excess synthesis of transgene products. To reduce transgene expression, we developed a viral vector-mediated somatic regulation system using inducible Cre recombinase. A recombinant adeno-associated virus (AAV) vector expressing Cre recombinase fused to a mutated ligand-binding domain of the estrogen receptor α (CreER^{T2}) was delivered along with AAV vectors expressing dopamine-synthesizing enzymes to rats of a Parkinson disease model. Treatment with 4-hydroxytamoxifen, a synthetic estrogen receptor modulator, activated Cre recombinase within the transduced neurons and induced selective excision of the tyrosine hydroxylase (TH) coding sequence flanked by loxP sites, leading to a reduction in transgene-mediated dopamine synthesis. Using this strategy, aromatic L-amino acid decarboxylase (AADC) activity was retained so that L-3,4-dihydroxyphenylalanine (L-dopa), a substrate for AADC, could be converted to dopamine in the striatum and the therapeutic effects of L-dopa preserved, even after reduction of TH expression in the case of dopamine overproduction. Our data demonstrate that viral vector-mediated inducible Cre recombinase can serve as an *in vivo* molecular switch, allowing spatial and temporal control of transgene expression, thereby potentially increasing the safety of gene therapy.

Key Words: adeno-associated virus, gene therapy, gene regulation, tamoxifen, Cre recombinase, Parkinson disease, dopamine

INTRODUCTION

Advances in gene transfer methods, in particular the development of improved viral vectors, have expanded the potential of gene therapy to treat a wide range of genetic and acquired diseases. Efficient and long-term expression of therapeutic genes within the central nervous system has been demonstrated in preclinical studies aimed at treating neurodegenerative disorders, including Parkinson disease (PD) [1,2]. PD is a progressive movement disorder characterized by selective degeneration of dopaminergic neurons within the substantia nigra, which project to the striatum. As the dopamine content of the striatum decreases severely, its replacement becomes an important strategy to alleviate motor impairment of the disease. One such strategy is gene therapy to restore the local production of dopamine. Recombinant adeno-associated virus (AAV) vector-mediated gene transfer of dopamine-synthesizing enzymes, such as tyrosine hydroxylase (TH) and guanosine triphosphate cyclohydrolase I (GCH), with or without aromatic L-amino acid decarboxylase (AADC), has induced behavioral recovery in animal models of PD [3–5]. Before clinical trials examining this therapy can commence, however, it is desirable to have a mechanism by which

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dopamine synthesis can be controlled by regulation of gene expression.

Methods utilizing the properties of bacteriophage P1 site-specific Cre recombinase have been developed in recent years as a means of generating somatic mutations [6]. Regulation of Cre recombinase activity, achieved by fusing Cre with mutated hormone-binding domains of various steroid receptors, has been used in various transgenic applications [7,8]. A chimeric protein known as CreER^{T2}, obtained by fusing Cre to a mutated ligand binding domain of the human estrogen receptor α, is particularly useful. Cell-specific expression of CreER^{T2} in transgenic mice allows efficient tamoxifen-dependent Cre-mediated recombination at loci flanked by loxP sites, without background activity [9]. In the present study, we demonstrate that stereotaxic injection of recombinant AAV vectors expressing dopamine-synthesizing enzymes and CreER^{T2} enables spatiotemporal control of dopamine levels within the brains of rats of a PD model. Our results indicate that these vectors may have a number of applications in gene therapy.

RESULTS

Viral-Mediated Temporally Controlled Cre-Mediated Recombination

We generated AAV vectors expressing either Cre recombinase containing a nuclear localization signal (AAV-Cre) or tamoxifen-dependent Cre recombinase (AAV-CreER^{T2}). To engineer a reporter system, we designed an AAV-EGFP/Red vector to express a destabilized variant of red fluorescent protein (DsRed-Express DR) only after Cre-mediated recombination of a loxP-flanked DNA segment encoding a destabilized, red-shifted variant of green fluorescent protein (d2EGFP) (Fig. 1A). To determine the efficacy of viral-mediated recombination, we infected HEK293 cells with AAV-EGFP/Red and either AAV-Cre or AAV-CreER^{T2} (Fig. 1B). Co-infection of AAV-Cre and AAV-EGFP/Red resulted in expression of DsRed-Express-DR, while only d2EGFP was expressed in control cells infected with AAV-EGFP/Red alone. Co-infection with the reporter vector and AAV-CreER^{T2} induced DsRed-Express-DR expression in almost all 4-hydroxytamoxifen (4-OHT)-treated cells. Although we detected slight background expression of DsRed-Express-DR in the absence of 4-OHT, we observed only a limited number of these cells (<1%), indicating that CreER^{T2} activity is tightly regulated in these virally transduced cells. To test the potential use of AAV-CreER^{T2} *in vivo*, we used stereotaxic injections to deliver AAV-CreER^{T2} into the brains of reporter mice [10]. These mice were engineered to express a red-shifted variant of the wild-type green fluorescent protein (EGFP) only after Cre-mediated excision of a loxP DNA fragment. After 5 consecutive days of 4-OHT treatment (1 mg by intraperitoneal injection), we observed numerous EGFP-expressing cells in AAV-injected brains, all of which coexpressed Cre recombinase (Fig. 1C). In the absence of treatment with 4-OHT, only a few cells (<0.1% of Cre-positive cells) expressed EGFP in the vicinity of AAV-CreER^{T2}-injected sites (data not shown). These data indicate that floxed DNA segments are efficiently excised *in vivo* by combining AAV-CreER^{T2} injection with 4-OHT treatment.

Temporally Controlled Reduction of Dopamine Synthesis

We generated AAV vectors expressing each of the three dopamine-synthesizing enzymes (TH, AADC, and GCH). In the TH-expressing vector, two loxP sites flanked the TH coding sequence (AAV-floxed TH). We infected HEK293 cells with these dopamine-synthesizing vectors (AAV-floxed TH, AAV-AADC, and AAV-GCH) in combination

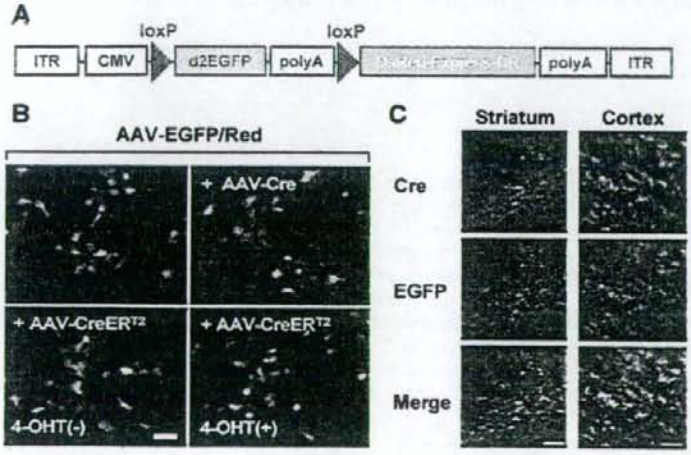

FIG. 1. Viral vector-mediated Cre-dependent floxed DNA excision. (A) Illustration of the AAV-EGFP/Red vector construct. A DsRed-Express-DR marker was placed downstream of the d2EGFP marker with a SV40 poly(A) sequence flanked by loxP sites. ITR, inverted terminal repeat; CMV, human cytomegalovirus immediate-early promoter followed by the first intron of human growth hormone. (B) 4-OHT-induced Cre-dependent recombination. HEK293 cells were infected with AAV-EGFP/Red and either AAV-Cre or AAV-CreER^{T2}. 4-OHT was added to the medium 5 h after infection. Fluorescence was observed 48 h after infection. Bar, 40 μm. (C) EGFP expression in the AAV-CreER^{T2}-injected striatum and cortex of transgenic mice. 4-OHT (1 mg) was administered intraperitoneally 1 week after vector injection every day for 5 days until the mice were killed. In these mice, the stop fragment was flanked by loxP sites and placed between the EGFP sequence and the *Gt(ROSA)26Sor* promoter. Bar, 40 μm.