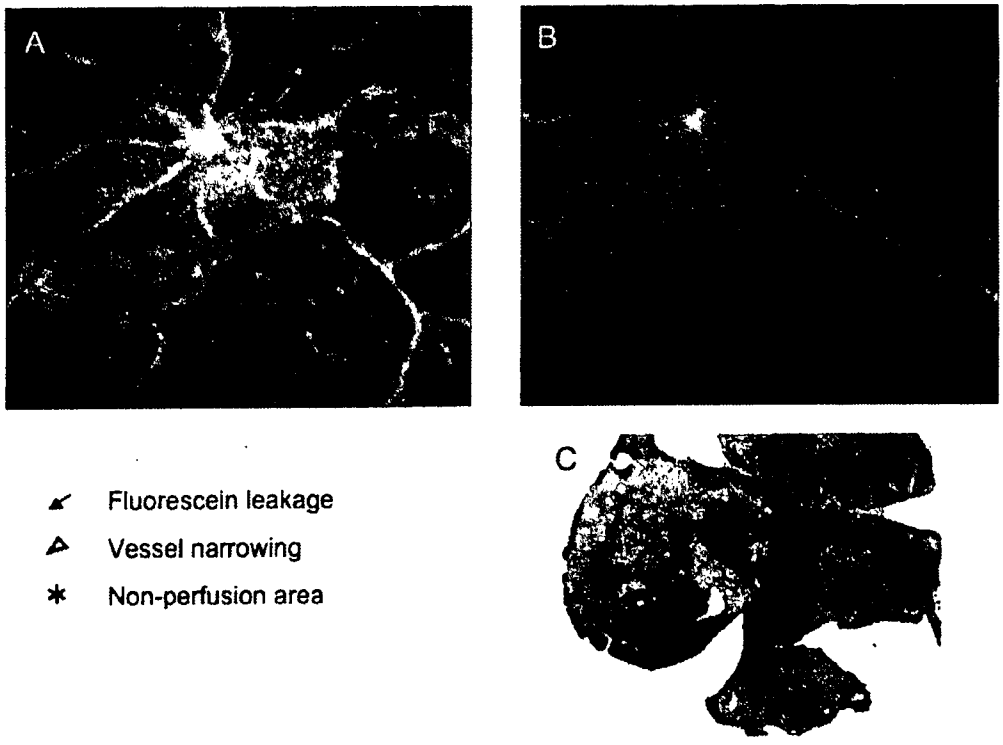

Figure 3. Fluorescein microangiography (FAG) of the rats 30 weeks after vector administration. (A) FAG from AAV5-CMV-*lacZ* injected rats. (B) FAG from rAAV5-CMV-*sflt-1* plus rAAV5-CMV-*lacZ* injected rats. The leakage from the fluorescein spot and avascular area are less extensive in B than in A, thus indicating that the progression of diabetic retinopathy is less marked in the rats treated with rAAV5-CMV-*sflt-1*. (C) A typical X-gal staining of the rat retina showing the distribution of the transduced tissue after subretinal injection of the rAAV-CMV-*lacZ* vector.

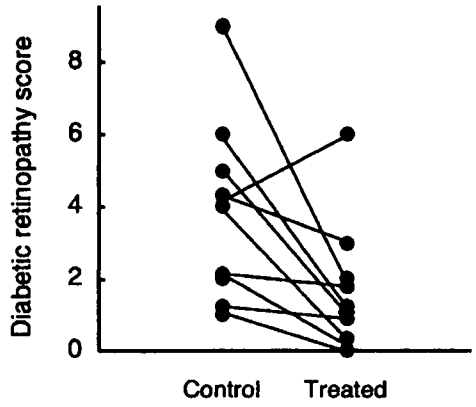

Figure 4. Diabetic retinopathy score of the rats evaluated at the end of the study. The Wilcoxon signed-ranks test demonstrates that the scores for the treated eyes are significantly less than those for the control eyes (n=8, $p<0.05$).

Corp, Foster City, CA) was applied over the retina and allowed to dry. Then the whole flat-mounted retina was examined by fluorescent microscopy (Nikon Labphoto, Nikon, Tokyo, Japan). Without providing information on the vectors injected, the status of DR was determined using the following three parameters: the presence of an avascular area, extensive hyperfluorescence, and arterial narrowing. Each parameter was scored from 0 (none) to 3 (severe) based upon the findings of FAG. The score for each eye was compared and analyzed using the Wilcoxon signed-ranks test. Rats that did not show DR in either of the eyes were excluded from the study. To confirm the subretinal injection of vector solution, X-gal staining of the eye was performed after FAG.

Results

Effect of sFlt-1 in vitro. To prove the biological activity of the protein produced from the *sflt-1* cDNA *in vitro*, we incubated HUVEC with different dilutions of media from 293 cells transfected with the plasmid. The conditioned media from transfected cells inhibited proliferation of HUVEC in a dose-dependent manner, whereas media from untransfected cells had no effect on HUVEC proliferation (data not shown).

Development of DM in SDT rats. All rats developed DM by 35 weeks of age and high blood glucose levels continued throughout the study (Fig. 2). At the end of the study, HbA1c levels in all rats were high (9.4±0.95%; means ± SD), and plasma sFlt-1 was not detected. No adverse effects of sFlt-1 gene therapy were observed throughout the study.

Evaluation of efficacy of gene transfer into the retina. Thirty weeks after the vector administration, FAG was performed to determine the progression of DR. DR was diagnosed using three parameters, arterial narrowing, pooling of fluorescein and a non-perfusion area, and the severity of these parameters were evaluated. The scores of DR in the treated eyes were significantly less than those in the control eyes (Fig. 3A and B; Fig. 4). X-gal staining demonstrated that the LacZ protein was produced in the retinal tissue after transduction with the

rAAV5 vector, and the transgene expression persisted for over 30 weeks after vector administration (Fig. 3C).

Discussion

VEGF is supposedly one of the most essential factors in retinal neovascularization during DR progression. The inhibition of retinal neovascularization by *sflt-1* gene transfer in animal models has been demonstrated in earlier reports (30,31). However, since the mechanisms underlying neovascularization in these models were not related to hyperglycemia, the effectiveness of sFlt-1 in inhibiting DR had not been estimated. To shed light on this issue, a more clinically relevant model has long been awaited. The recently developed SDT rat model is a candidate for this purpose. This model is unique because its diabetic status mimics human NIDDM rather than IDDM. The animals can live for 1 year after the onset of DM without insulin, with a gradual maturation of DR. Therefore, this model is a valuable tool because it can reflect mid- to late-stage human DR associated with NIDDM (32,33). However, this model has certain drawbacks as well. First, the disease progression is much slower than that in other 'conventional' animal models, and DR can be observed mainly after 55 weeks of age. Therefore, one series of experiments requires a long time period. Second, these animals are prone to death, probably due to the complications of DM. Unfortunately, the number of animals decreases before they develop sufficient disease severity. Therefore, to ensure valid results, the sample size of each group needs to be sufficiently large.

This study aimed to demonstrate the efficacy of gene therapy in preventing DR disease progression. For this purpose, we injected the vector soon after the onset of DM, and the efficacy was evaluated at the age of full-blown DR. Considering that a preventive action was significant in this study, a more precise examination of whether short-term *sflt-1* expression is sufficient to prevent DR progression or has an effect in reversing the DR status should be considered for future study. A more difficult task includes determining effective methods to develop this strategy into a clinically realized therapy. Generally, if the therapeutic efficacy is proven in small animals, additional experiments need to be performed on larger animals prior to conducting clinical trials. Regarding DR, no appropriate model has been found in species of large animals. Proliferative DR-like changes were observed in a galactose-fed dog model (44). Nevertheless, this model requires up to 7 years to establish mature DR, which is impractical in a preclinical study. Development of novel large animal models for this purpose is ideal but not practical due to the uncertainty of success in establishing such models during a defined time range. Resolving this problem may not be easy; however, we believe that before clinical trials are considered, further studies using large animals are essential.

In this study, the area of transgene expression was sufficiently wide to protect vision, and the expression continued for over 30 weeks after the injection, indicating that rAAV-mediated ocular gene transfer via a single injection of vector solution could lead to a long-term therapeutic effect. The area of *sflt-1* expression should be comparable to that of X-gal staining, although human sFlt-1 in the retina was undetectable by immunohistochemistry. This may have occurred probably due to technical difficulties in localizing the soluble antigen (31). Therefore, ocular gene transfer under the present experimental conditions is a practical approach. Nonetheless, DR progression was suppressed partially and not completely. At present, it is unclear whether the incomplete suppression was due to the residual actions of VEGF or the uninhibited activity of an alternative angiogenic factor (45). Regarding the latter, a combination of transgenes that act on different aspects of angiogenesis may increase the efficacy of gene therapy for DR prevention.

In patients with DM, VEGF is closely involved in the degree of complication. Elevated VEGF levels in the retina may worsen the DR status and cause visual loss (11,12), while high systemic VEGF levels induce neovascularization, improving ischemic conditions. If circulating sFlt-1 levels affect systemic VEGF levels, DM patients may develop ischemic heart disease, diabetic neuropathy, and diabetic gangrene. To avoid these adverse effects, local sFlt-1 delivery and VEGF inhibition is necessary. In this study, plasma sFlt-1 levels were not elevated after subretinal vector administration, and no adverse effects of *sflt-1* gene transfer were observed. Therefore, the subretinal administration of a vector solution and neutralization of the VEGF activity *in situ* appear to be appropriate measures that should be adopted to achieve our goal.

In conclusion, we demonstrated the successful prevention of DR in SDT rats by using an rAAV vector-encoding *sflt-1* gene. These findings strongly suggest the efficacy of sFlt-1 for DR and the usefulness of rAAV5 for ocular gene transfer. Further studies are necessary to develop and optimize ocular gene therapy for human DR.

Acknowledgements

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References

1. Hyman L: Epidemiology of eye disease in the elderly. Eye 1: 330-341, 1987.
2. Klein BE and Klein R: Ocular problems in older Americans with diabetes. Clin Geriatr Med 6: 827-837, 1990.
3. King H and Rewers M: Global estimates for prevalence of diabetes mellitus and impaired glucose tolerance in adults. WHO *Ad Hoc* Diabetes Reporting Group. Diabetes Care 16: 157-177, 1993.
4. Hotta N, Nakamura J, Sakakibara F, *et al*: Electroretinogram in sucrose-fed diabetic rats treated with an aldose reductase inhibitor or an anticoagulant. Am J Physiol 273: E965-E971, 1997.
5. Robison WG Jr, Nagata M, Tillis TN, Laver N and Kinoshita JH: Aldose reductase and pericyte-endothelial cell contacts in retina and optic nerve. Invest Ophthalmol Vis Sci 30: 2293-2299, 1989.

6. Aiello LP, Bursell SE, Clermont A, *et al*: Vascular endothelial growth factor-induced retinal permeability is mediated by protein kinase C *in vivo* and suppressed by an orally effective beta-isoform-selective inhibitor. Diabetes 46: 1473-1480, 1997.
7. Lee TS, MacGregor LC, Fluharty SJ and King GL: Differential regulation of protein kinase C and (Na,K)-adenosine triphosphatase activities by elevated glucose levels in retinal capillary endothelial cells. J Clin Invest 83: 90-94, 1989.
8. Nakamura J, Kato K, Hamada Y, *et al*: A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. Diabetes 48: 2090-2095, 1999.
9. Hammes HP, Wellensiek B, Kloting I, Sickel E, Bretzel RG and Brownlee M: The relationship of glycaemic level to advanced glycation end-product (AGE) accumulation and retinal pathology in the spontaneous diabetic hamster. Diabetologia 41: 165-170, 1998.
10. Murata T, Nagai R, Ishibashi T, Inomuta H, Ikeda K and Horiuchi S: The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas. Diabetologia 40: 764-769, 1997.
11. Aiello LP: Vascular endothelial growth factor and the eye: biochemical mechanisms of action and implications for novel therapies. Ophthalmic Res 29: 354-362, 1997.
12. Aiello LP, Avery RL, Arrigg PG, *et al*: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 331: 1480-1487, 1994.
13. Ozaki H, Seo MS, Ozaki K, *et al*: Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization. Am J Pathol 156: 697-707, 2000.
14. Ferrara N, Houck K, Jakeman L and Leung DW: Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocr Rev 13: 18-32, 1992.
15. Jakeman LB, Winer J, Bennett GL, Altar CA and Ferrara N: Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues. J Clin Invest 89: 244-253, 1992.
16. Senger DR, Perruzzi CA, Feder J and Dvorak HF: A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res 46: 5629-5632, 1986.
17. Thieme H, Aiello LP, Takagi H, Ferrara N and King GL: Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. Diabetes 44: 98-103, 1995.
18. Adamis AP, Miller JW, Bernal MT, *et al*: Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthalmol 118: 445-450, 1994.
19. Boulton M, Gregor Z, McLeod D, *et al*: Intravitreal growth factors in proliferative diabetic retinopathy: correlation with neovascular activity and glycaemic management. Br J Ophthalmol 81: 228-233, 1997.
20. Okamoto N, Tobe T, Hackett SF, *et al*: Transgenic mice with increased expression of vascular endothelial growth factor in the retina: a new model of intraretinal and subretinal neovascularization. Am J Pathol 151: 281-291, 1997.
21. Adamis AP, Shima DT, Tolentino MJ, *et al*: Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. Arch Ophthalmol 114: 66-71, 1996.
22. Robinson GS, Pierce EA, Rook SL, Foley E, Webb R and Smith LE: Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy. Proc Natl Acad Sci USA 93: 4851-4856, 1996.
23. Aiello LP, Pierce EA, Foley ED, *et al*: Suppression of retinal neovascularization *in vivo* by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proc Natl Acad Sci USA 92: 10457-10461, 1995.
24. Goldman CK, Kendall RL, Cabrera G, *et al*: Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. Proc Natl Acad Sci USA 95: 8795-8800, 1998.
25. Hasumi Y, Mizukami H, Urabe M, *et al*: Soluble FLT-1 expression suppresses carcinomatous ascites in nude mice bearing ovarian cancer. Cancer Res 62: 2019-2023, 2002.
26. Kendall RL and Thomas KA: Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA 90: 10705-10709, 1993.
27. Kong HL, Hecht D, Song W, *et al*: Regional suppression of tumor growth by *in vivo* transfer of a cDNA encoding a secreted form of the extracellular domain of the flt-1 vascular endothelial growth factor receptor. Hum Gene Ther 9: 823-833, 1998.
28. Shiose S, Sakamoto T, Yoshikawa H, *et al*: Gene transfer of a soluble receptor of VEGF inhibits the growth of experimental eyelid malignant melanoma. Invest Ophthalmol Vis Sci 41: 2395-2403, 2000.
29. Kendall RL, Wang G and Thomas KA: Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun 226: 324-328, 1996.
30. Bainbridge JW, Mistry A, De Alwis M, *et al*: Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptor sFlt-1. Gene Ther 9: 320-326, 2002.
31. Honda M, Sakamoto T, Ishibashi T, Inomata H and Ueno H: Experimental subretinal neovascularization is inhibited by adenovirus-mediated soluble VEGF/flt-1 receptor gene transfection: a role of VEGF and possible treatment for SRN in age-related macular degeneration. Gene Ther 7: 978-985, 2000.
32. Shinohara M, Masuyama T, Shoda T, *et al*: A new spontaneously diabetic non-obese Torii rat strain with severe ocular complications. Int J Exp Diabetes Res 1: 89-100, 2000.
33. Kakehashi A, Saito Y, Mori K, *et al*: Characteristics of diabetic retinopathy in SDT rats. Diabetes Metab Res Rev (Epub ahead of print March 30, 2006).
34. Dudus L, Anand V, Acland GM, *et al*: Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. Vision Res 39: 2545-2553, 1999.
35. Martin KR, Klein RL and Quigley HA: Gene delivery to the eye using adeno-associated viral vectors. Methods 28: 267-275, 2002.
36. Auricchio A, Behling KC, Maguire AM, *et al*: Inhibition of retinal neovascularization by intraocular viral-mediated delivery of anti-angiogenic agents. Mol Ther 6: 490-494, 2002.
37. Lotery AJ, Yang GS, Mullins RF, *et al*: Adeno-associated virus type 5: transduction efficiency and cell-type specificity in the primate retina. Hum Gene Ther 14: 1663-1671, 2003.
38. Rabinowitz JE, Rolling F, Li C, *et al*: Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J Virol 76: 791-801, 2002.
39. Yang GS, Schmidt M, Yan Z, *et al*: Virus-mediated transduction of murine retina with adeno-associated virus: effects of viral capsid and genome size. J Virol 76: 7651-7660, 2002.
40. Yoshimura I, Mizuguchi Y, Miyajima A, Asano T, Tadakuma T and Hayakawa M: Suppression of lung metastasis of renal cell carcinoma by the intramuscular gene transfer of a soluble form of vascular endothelial growth factor receptor I. J Urol 171: 2467-2470, 2004.
41. Zhang M, Volpert O, Shi YH and Bouck N: Maspin is an angiogenesis inhibitor. Nat Med 6: 196-199, 2000.
42. Hermens WT, ter Brake O, Dijkhuizen PA, *et al*: Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. Hum Gene Ther 10: 1885-1891, 1999.
43. Zolotukhin S, Byrne BJ, Mason E, *et al*: Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6: 973-985, 1999.
44. Kadof PF, Takahashi Y, Wyman M, Ferris F III: Diabetes-like proliferative retinal changes in galactose-fed dogs. Arch Ophthalmol 113: 352-354, 1995.
45. Casey R and Li WW: Factors controlling ocular angiogenesis. Am J Ophthalmol 124: 521-529, 1997.

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Adipose Tissue as a Novel Target for *In Vivo* Gene Transfer by Adeno-Associated Viral Vectors

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ABSTRACT

Traditionally, skeletal muscle and liver are the preferred target organs for gene transfer to supply a transgene product into the systemic circulation. In this respect, adipose tissue presents a number of attractive features. However, adipose tissue transduction *in vivo* has not been feasible by conventional methods. To solve this issue, we tested the utility of excipients in adeno-associated virus (AAV) vector-mediated gene transfer and found that Pluronics are suitable for this purpose. In a histological analysis of adipose tissue in *db/db* mice, Pluronic F88 showed the greatest augmentative effect on β-galactosidase expression in combination with the AAV1 vector. When the vector encoding mouse erythropoietin (Epo) was used in the same manner, increased plasma Epo concentrations were observed (230 ± 80 versus 58 ± 14 mU/ml). Moreover, the plasma Epo concentration returned to the normal level after the surgical removal of transduced adipose tissue. No damage was observed in the transduced tissue. Our results indicate that the proposed method is safe and efficient for gene transfer into adipose tissues, thus providing an alternative for supplemental gene therapy.

OVERVIEW SUMMARY

Adipose tissue holds promise as an alternative depot organ in gene transfer approaches. However, no efficient method of gene transfer into adipose tissue *in vivo* has been established. In this study, we explored the utility of excipients to augment gene transfer into the adipose tissue of mice and found that Pluronic F88 was useful for this purpose when combined with AAV serotype 1 vectors. The improvement was also demonstrated with vectors encoding murine erythropoietin, and the mice became polycythemic. Moreover, after removing transduced adipose tissue, plasma erythropoietin levels returned to normal, which suggests the unique advantage of this method.

INTRODUCTION

In SUPPLEMENTAL GENE THERAPY, skeletal muscle and the liver have been the preferred targets for gene transfer to supply transgene products into the systemic circulation. However, adipose tissue presents a number of attractive features. Adipose tissue can be found throughout the body and is easily accessible for vector injection. Increasing evidence supports the notion that adipocytes are designed to secrete numerous factors into the systemic circulation (Mohamed-Ali *et al.*, 1998). Further, the majority of adipocytes are considered to be nondividing, which is suitable for achieving long-term expression of transferred genes by the use of nonintegrating vectors such as adeno-associated virus (AAV) vectors (Russell and Kay, 1999). Moreover, the transduced tissue can be safely removed when unexpected events occur, thus adding a unique feature to safety considerations. However, adipose tissue transduction has not been feasible through conventional methods, and few studies have investigated its efficacy *in vivo* (Nagamatsu *et al.*, 2001; Ogata *et al.*, 2004). To overcome these limitations and develop a more practical method, we tested the usefulness of excipients for gene transfer. To achieve efficient and widespread gene transfer, it is essential to assure that the vectors stay within the target tissue for a certain period of time. For this purpose, nonionic surfactants are promising because they have low toxicity and unique features that help stabilize the membrane (American Pharmaceutical Association [AphA] and Royal Pharmaceutical Society of Great Britain [RPSGB], 1986). Moreover,

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improved gene delivery with Pluronic compounds was demonstrated in various applications (Kabanov and Alakhov, 2002; Kabanov *et al.*, 2005). Therefore, we tested the effects of these compounds on *in vivo* gene transfer targeting adipose tissue.

MATERIALS AND METHODS

Cells and plasmids for AAV vector preparation

HEK293 cells, a human embryonic kidney cell line, were maintained as described in a previous report (Fan *et al.*, 1998). Murine erythropoietin (Epo)-encoding plasmids have already been described by Mochizuki *et al.* (2004). AAV vectors of serotype 1 through 5, which encode LacZ or Epo and have a cytomegalovirus (CMV) promoter, were prepared by transient transfection, purified and quantitated as described previously (Matsushita *et al.*, 1998; Mochizuki *et al.*, 2004). Usually, the stocks of AAV vectors contained approximately 1-2 $\times$ 10^{10} genome copies/μl.

In vitro *assessment of potential toxicity of surfactants*

A panel of Pluronics was provided by Asahi Denka (Tokyo, Japan). Tween 80 was obtained from Sigma-Aldrich (St. Louis, MO). The surfactants were dissolved in distilled H_2O to prepare stock solutions at a concentration of 20%. To assess the potential compatibility, we added various concentrations of these surfactants to cultured 293 cells at the time of confluency. After 24 hr, the cells were examined microscopically. The highest concentrations of the surfactants that did not produce any deleterious effect on these cells were tested. The effect of the surfactants on AAV vector capsids was also examined by including a 10% surfactant in the vector stocks (AAV-LacZ with serotype 2 capsid, 2 $\times$ 10^{10} VG/μl) for 24 hr; subsequently, the vector solutions were added to the cultured 293 cells in a 96-well plate at a dose of 2 $\times$ 10^4 VG/cell. The final concentration of the surfactants within the culture medium was 0.05%. Two days later, the infectivity was assessed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining (Fan *et al.*, 1998).

Analysis of gene expression and of enhancing effect in vivo

For the *in vivo* experiments, the AAV vector solutions containing various concentrations of Pluronic F68, F88, and Tween 80 were injected into the subcutaneous adipose tissues of C57BLKS/J *db/db* mice (Japan SLC, Hamamatsu, Japan) at the age of 10 to 12 weeks. At the time of injection, the average weight of the animals was more than 50 g, and the subcutaneous adipose tissues were well developed. The AAV-CMV-LacZ vectors with various serotypes (serotype 1–5) were tested at a dose of 6 $\times$ 10^{10} VG/body. Two weeks after injection, the mice were killed, and their adipose tissues were enucleated, stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), cut into pieces, and then frozen with Tissue-Tek optimal cutting temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA) in dry ice–ethanol, as described (Ogata *et al.*, 2004). When the tissue blocks were sliced for microscopic examination, they were also evaluated macroscopically and photographed. A densitometric analysis of the blocks was performed in order to substantiate the difference in the efficiency of transduction of adipose tissue under various injection conditions. In practice, five areas of the corresponding blocks were chosen and quantified, using Image Gauge software (version 3.0; Fuji Photo Film, Tokyo, Japan). After subtracting the background value, these numbers were analyzed and the statistical significance was evaluated. Experiments were performed with AAV-Epo vectors under conditions optimized with the AAV-LacZ vectors. Later, experiments at a higher vector dose (2 $\times$ 10^{11} VG/body) were included. On the basis of the preliminary experiments, the volume of injection was optimized as 100 μl/body (50 μl per lobe) and kept constant thereafter. Whole blood was collected from the tail vein every 2 weeks. Plasma Epo concentrations were quantified with an enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mannheim, Germany). Complete blood counts were performed with a PC-608 particle counter (Erma, Tokyo, Japan).

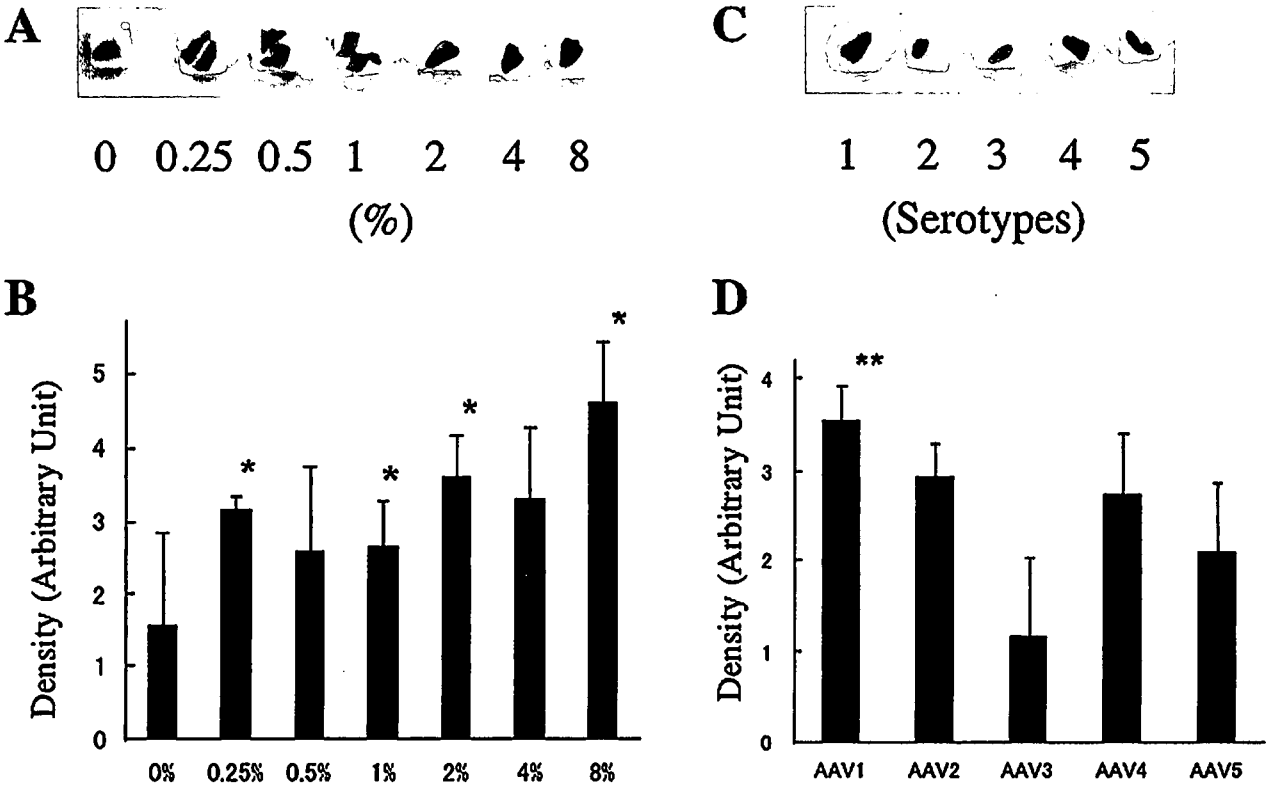
Detection of murine Epo within adipose tissue of db/db *mice*

Adipose tissues were obtained from *db/db* mice at the time of sacrifice or later by operation. For the immunofluorescence study, adipose tissues were fixed and frozen in the presence of the O.C.T. compound in dry ice–ethanol, following which they were reacted with goat anti-human Epo (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat IgG conjugated with Alexa 488 (Invitrogen Molecular Probes, Eugene, OR) at 4°C for 16 hr to visualize murine Epo by fluorescence microscopy, as described in a previous report (Ogata *et al.*, 2004).

Detection of murine Epo transcript within adipose tissue by reverse transcription-polymerase chain reaction

Total RNA was isolated from adipose tissue with an RNeasy lipid tissue kit (Qiagen, Hilden, Germany) and was converted to

FIG. 1. Distribution of LacZ expression within adipose tissues. AAV-CMV-LacZ vectors of various serotypes (serotypes 1–5) were tested (n = 3 for each serotype) at a dose of 6 $\times$ 10^{10} VG/body. Two weeks after injection, adipose tissues were removed, stained with X-Gal, and resected in pieces. Tissues were frozen with Tissue-Tek and then cut into 20-μm-thick slices for microscopic analysis. Blocks were also evaluated macroscopically, photographed, and analyzed by densitometry. (**A**) Representative blocks of adipose tissue injected with various concentrations of Pluronic F88. (**B**) Densitometric analysis of the corresponding blocks with various concentrations of Pluronic F88. Asterisks indicate statistical significance ($p < 0.05$) compared with values of tissues without F88 (0% data). (**C**) Blocks of adipose tissue injected with serotypes of AAV-LacZ vectors with 2% F88. (**D**) Densitometric analysis of blocks corresponding to the serotypes of AAV-LacZ vectors. Double asterisks indicate statistical significance ($p < 0.05$) relative to values obtained with the rest of the serotypes. Microscopic analysis of adipose tissue sections transduced with AAV1-LacZ without excipients is shown at low (**E**) and high (**F**) magnifications. Adipose tissues transduced with AAV1-LacZ in the presence of 2% Pluronic F88 are shown at low (**G**) and high (**H**) magnifications.


A
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(%)
C
1 2 3 4 5
(Serotypes)
B
Density (Arbitrary Unit)
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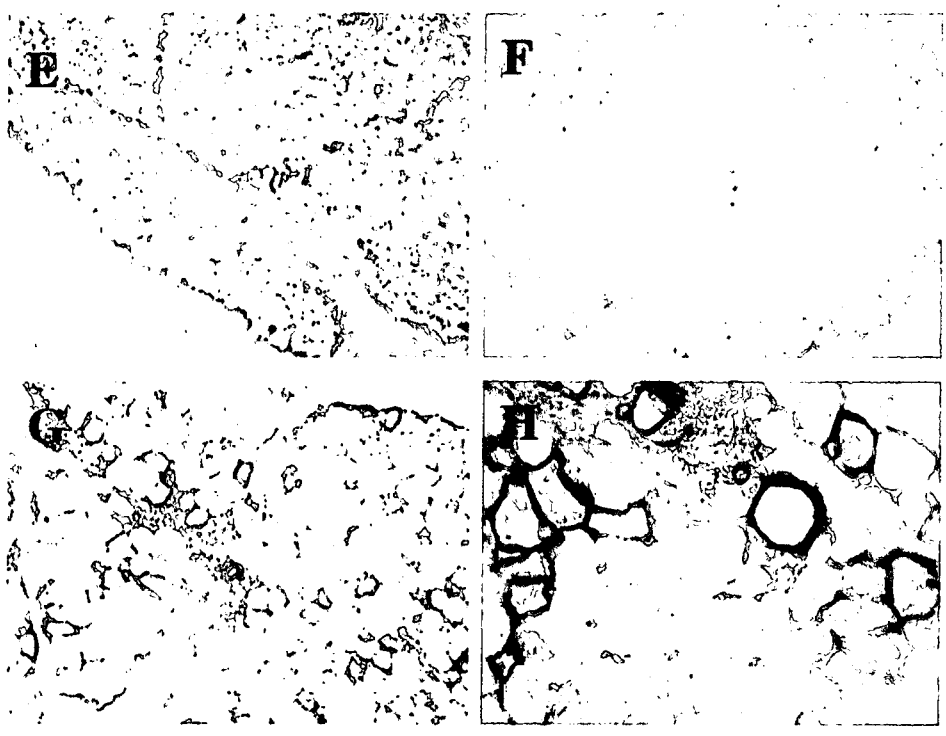
D
Density (Arbitrary Unit)
AAV1 AAV2 AAV3 AAV4 AAV5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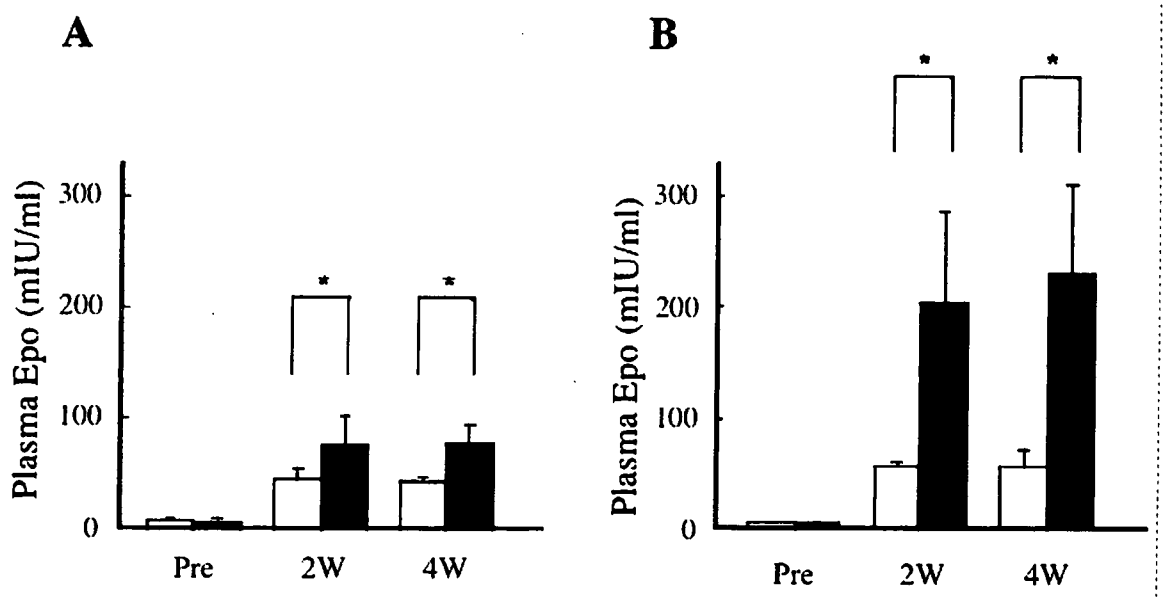

FIG. 2. Plasma Epo concentrations in *db/db* mice after injection of AAV1-Epo vectors into adipose tissue at a dose of (**A**) 6×10^{10} or (**B**) 2×10^{11} VG/body. Open and solid columns represent groups without and with 2% Pluronic F88, respectively, at the time of vector injection into adipose tissue. Each column and error bar indicate, respectively, the mean and SD of the group ($n = 5$). Asterisks indicate significance ($p < 0.05$).

cDNA with reverse transcriptase (SuperScript; Invitrogen, Carlsbad, CA) and oligo(dT) primers in a 20-μl mixture after DNase I (amplification grade; Invitrogen) treatment according to the manufacturer's instructions. Subsequent polymerase chain reaction (PCR) amplification was carried out with 1 μl of cDNA solution in a 50-μl reaction mixture containing 5 units of *Taq* polymerase, 10 m*M* Tris-HCl (pH 8.5), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 100 μM dNTPs in the presence of specific primer pairs (200 n*M*) designed to amplify DNA fragments derived from the transcript of the mouse Epo transgene. Each PCR cycle involved denaturation at 94°C for 15 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec. The PCR products were analyzed by agarose gel electrophoresis. The authenticity of the PCR products was confirmed by observing their molecular sizes after agarose gel electrophoresis and by sequencing (ABI PRISM 310 genetic analyzer; Applied Biosystems, Foster City, CA). The primer sequences for mouse Epo were 5'-GTG CAG AAG GTC CCA GAC TGA GTG A-3' and 5'-TTG GCG TAG ACC CGG AAG AGC TTG-3'. The primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Clontech Laboratories (Palo Alto, CA).

Removal of transduced tissue

AAV1-Epo vector solution (2×10^{11} VG/body) including 2% Pluronic F88 was injected into the subcutaneous adipose tissues of *db/db* mice. After 4 weeks, the adipose tissues were removed by standard surgical techniques under anesthesia. Plasma Epo concentrations were followed up 2 weeks thereafter. Four animals were analyzed and monitored.

Data and statistical analysis

Data are presented as means ± SD and were analyzed by Student *t* test after confirming an insignificant difference in variance between groups. $p < 0.05$ was considered statistically significant.

RESULTS

Screening of excipients in vitro

First, *in vitro* screening experiments were performed using nonionic surfactants. We tested Pluronic F68, F88, L72, P85, and Tween 80 for their effects on cell culture. Pluronic F68 and F88 were innocuous to cultured 293 cells at concentrations of up to 10 and 0.3%, respectively. The rest of the surfactants showed deleterious effects on the cultured cells when included at concentrations of 0.1% or higher. Next, we incubated AAV2-LacZ vectors with up to 10% Pluronic F68 and F88 solutions for 1 hr and checked their infectivity in 293 cells at 1×10^5 VG/cell. There were no differences in the infectivity of the vectors treated with these excipients, as assessed by X-Gal staining (data not shown).

Assessment of LacZ expression by use of Pluronics

On the basis of general safety data and *in vitro* experiments, we selected Pluronic F68, Pluronic F88, and Tween 80 as candidates for vector injection into *db/db* mice. Because preliminary experiments indicated the usefulness of Pluronic F88 combined with the AAV1 vector, we tested the usefulness of F88 at various concentrations. Comparison of blocks showed that there appeared to be an augmentation of LacZ expression with increasing concentration (Fig. 1A). The enhanced expression was substantiated by densitometric analysis of the blocks (Fig. 1B). On the basis of the result, we compared the usefulness of serotypes 1 through 5 combined with 2% Pluronic F88. The result showed that AAV1 was the most suitable serotype to trans-

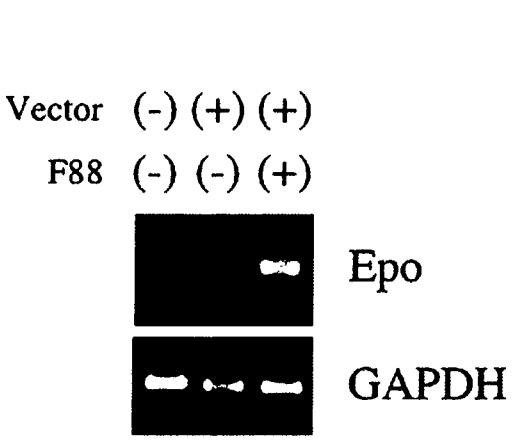

FIG. 3. Transgene expression from adipose tissue excised 2 weeks after vector injection. Immunofluorescence micrographs obtained with an antibody against Epo are shown. Adipose tissues without vector injection (**A**), or injected with AAV1-Epo vectors in the absence (**B**) or presence (**C**) of 2% Pluronic F88, were analyzed in the same fashion. (**D**) Results of RT-PCR, using mRNA extracted from adipose tissue, are shown. Lanes 1, 2, and 3 correspond to the adipose tissues shown in (**A**), (**B**), and (**C**), respectively.

duce adipose tissue *in vivo* (Fig. 1C and D). Enhancement of LacZ expression was also observed microscopically in the presence of Pluronic F88 (Fig. 1G and H) when compared with that of the vector alone (Fig. 1E and F).

Assessment of murine erythropoietin expression by use of Pluronic F88

On the basis of the findings obtained with LacZ vectors, we used vectors encoding murine erythropoietin (Epo) to demonstrate the enhancement of Epo concentration in a more quantitative manner. Increased plasma Epo concentration was observed 2 and 4 weeks after vector injection, and there was enhancement of Epo in the presence of 2% Pluronic F88 at a dose of 6×10^{10} VG/body (Fig. 2A). At a higher dose of 2×10^{11} VG/body, the enhancement effect was more prominent (230 ± 80 versus 58 ± 14 mU/ml at 4 weeks; Fig. 2B).

Assessment of transgene expression within adipose tissue

In the histological analysis of transduced tissues, a significant enhancement of Epo expression was observed by immunofluorescence when 2% F88 was included in the vector solution (Fig. 3A–C). Results of reverse transcription (RT)-PCR also showed enhanced expression of murine Epo in the presence of 2% F88 (Fig. 3D). Tissue damage or cellular infiltrates were not observed in the transduced adipose tissues throughout the histological evaluation (data not shown).

Effect of removal of transduced tissue

Bilateral lobes of abdominal adipose tissue were selected as a target for transduction. Plasma Epo concentrations were monitored after injection of AAV1-Epo vector (2×10^{11} VG/body) with 2% F88. After 4 weeks of observation, both lobes of the adipose tissue were removed *en bloc*, using standard surgical techniques. A significant decrease in plasma Epo concentration was observed 2 weeks after operation, with a return to the baseline level (Fig. 4). In addition, mice after removal of transduced tissue showed extended survival compared with "non-operated" mice. The survival period of *db/db* mice is summarized in Table 1.

DISCUSSION

In this study, we demonstrated the advantages of using excipients in adipose tissue transduction with AAV vectors. In practice, Pluronics have been widely used as excipients, including for administration to humans (APhA and RPSGB, 1986). Generally, one of the most frequent complications associated with administering a surfactant *in vivo* is hemolysis. With regard to this, it is noteworthy that Pluronics have a membrane-protecting effect on erythrocytes and that Pluronic F68 has long been used as a drug to prevent hemolysis and thrombotic events during extracorporeal circulation (Wright *et al.*, 1963). Further, their efficacy for use in the treatment of vasoocclusive disease in sickle cell anemia is currently being evaluated (Gibbs and Hagemann, 2004). Pluronics are also used in

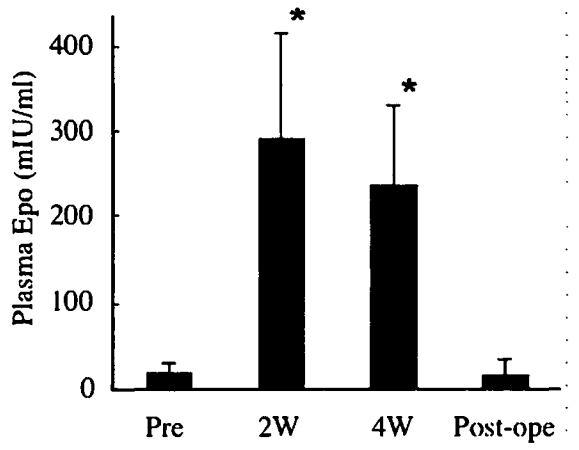

FIG. 4. Plasma Epo concentrations of *db/db* mice after injection and removal of adipose tissue. AAV1-Epo vectors were injected into adipose tissue at 2×10^{11} VG/body with 2% Pluronic F88. Four weeks after injection, transduced adipose tissues were surgically removed and monitored for an additional 2 weeks. Columns and bars indicate, respectively, mean ± SD of the groups ($n = 4$ each). Asterisks indicate significance ($p < 0.05$) relative to concentrations before injection. No significant differences in concentrations were observed 2 weeks after the operation relative to concentrations before injection.

a variety of applications in gene therapy; for example, they are used to augment gene transfer into cultured cells (Gebhart and Kabanov, 2001), to protect skeletal muscle membranes at the time of electroporation *in vivo* (Lee *et al.*, 1992; Hartikka *et al.*, 2001), and to enhance adenovirus-mediated gene transfer into the lungs (Croyle *et al.*, 2001). In addition, it has been postulated that adding a low concentration (0.01%) of Pluronic F68 into the stocks of AAV vectors prevents vector loss by reducing nonspecific attachment (Sommer *et al.*, 2003).

In the present study, the efficiency of gene transfer into adipose tissue was augmented by the use of Pluronic F88. The mechanisms behind this phenomenon are not clear; however, it is known that Pluronics improve the distribution of a solution and its content (APhA and RPSGB, 1986). In a previous study, one member of the Pluronics family (poloxamer 407) was shown to improve the efficiency of adenovirus-mediated gene transfer to arterial smooth muscle cells (Feldman *et al.*, 1997). A reduction in incubation time from 20 to 10 min to attain the same level of gene transfer by including this excipient was also demonstrated. The following two possible mechanisms were postulated for the increase in efficiency of gene transfer: (1) the formation of a transient local reservoir for the sustained release of adenoviral vectors, or (2) acceleration of the uptake of adenoviral vectors produced by the interaction between poloxamer 407 and the cell membrane. On the basis of our observations concerning LacZ expression within adipose tissues (Fig. 1), it can be said that vector distribution was improved and that uptake of the vectors was facilitated. The augmentation was most significant in the case of AAV1 vectors, as assessed on the basis of both LacZ and Epo. There may be a specific advantage in combining the AAV1 capsid with Pluronic F88 when transducing adipose tissue.

A novel action of Pluronics has been reported (Sriadibhatla *et al.*, 2006). In this literature, transcriptional activation of transgenes driven by the CMV promoter or nuclear factor-κB (NF-κB)-responsive elements was demonstrated in the presence of Pluronics. As all the vectors in our current study used the CMV promoter, transcriptional activation through this mechanism might be a concern. Therefore, this issue needs to be taken into consideration. In the literature, all the transcriptional activation was observed in *in vitro* experiments, and Pluronics were continuously present within the culture medium, typically at levels of 0.1% or higher. On the other hand, in our experiments, Pluronics were administered only at the time of vector injection, and the net amount constitutes 0.004% of the total body weight based on the volume and concentration of the vector solution. Moreover, the half-life of Pluronics *in vivo* is estimated as some hours and the majority of the administered material is known to be excreted from the urine within days (APhA and RPSGB, 1986; Gibbs and Hagemann, 2004). Therefore, it is unlikely that transcriptional activation is responsible for gene expression *in vivo* weeks after administration. Nonetheless, this mechanism of action may potentially be useful in order to enhance the outcome of gene therapy approaches *in vivo*. As all the known regulatable gene expression systems share the weakness of toxicity (Goverdhana *et al.*, 2005), safety profiles of Pluronics along with rapid clearance from the body may lead to the development of a novel system for regulatable gene expression *in vivo*. Further studies in this respect may extend the utility of Pluronics in future.

A relatively small number of studies have reported successful gene transfer into adipocytes. There are reports on gene transfer into cultured adipocytes by using viral vectors such as adenovirus (Meunier-Durmort *et al.*, 1996, 1997; Hertzel *et al.*, 2000), lentivirus (Morizono *et al.*, 2003; Carlotti *et al.*, 2004), and retrovirus (Ito *et al.*, 2005). Regarding efficacy *in vivo*, gene transfer into gonadal adipose tissues, using adenoviral vectors, demonstrated clinical efficacy in treating diabetic conditions (Nagamatsu *et al.*, 2001). Successful transduction of adipose tissue by using either simian immunodeficiency viral vector (Ogata *et al.*, 2004) or herpes simplex viral vector (Fradette *et al.*, 2005) was reported. To our knowledge, this is the first report that demonstrates the efficacy of adipocyte-mediated gene transfer by AAV vectors.

In terms of vector dose, adipocyte-mediated gene transfer required a higher vector dose to achieve the same plasma Epo

TABLE 1. LENGTH OF SURVIVAL OF *db/db* MICE

Group	*Survival (weeks after injection)*
AAV1-Epo (no Pluronic F88)[a]	4,[b] 4, 6, >8,[c] >8[c]
AAV1-Epo + 2% Pluronic F88[d]	4, 4, 5, 6, >8[c]
AAV1-Epo + 2% Pluronic F88 + operation[e]	>24, >24, >24, >24

[a]Reflects animals in Fig. 2B (open columns).
[b]This animal became paralyzed at the time of blood collection and subsequently died.
[c]These animals were killed at week 8 for tissue analysis.
[d]Reflects animals in Fig. 2B (solid columns).
[e]Reflects animals in Fig. 4.

concentration as that obtained by muscle- or liver-mediated gene transfer in our previous study (Mochizuki *et al.*, 2004). At a dose of 6×10^{10} VG/body, which was the standard dose for muscle- and liver-mediated gene transfer, the Epo concentration was less prominent; the plasma Epo concentration became comparable at a dose of 2×10^{11} VG/body. Therefore, even after the addition of F88, transduction efficiency was still low in adipose tissue. Whether there are any better methods to augment the efficiency of transduction, including the use of a higher vector dose or other serotype-derived vectors, needs to be investigated further.

In our series of experiments, all the transduced mice became polycythemic; therefore, transgene-derived Epo was functional (data not shown). Although the Epo concentration was augmented by the addition of Pluronic F88, there was no significant difference in blood hemoglobin levels or red blood cell counts among the groups. This is because the Epo concentrations in the transduced animals were far beyond the physiological dose–response window (Mochizuki *et al.*, 2004), and even modest Epo expression after injecting the vector without Pluronic F88 could result in polycythemic conditions. It is generally difficult to eliminate the possibility that the use of this excipient may alter the tropism of the vector and promote gene transfer to certain remote organ(s). Nonetheless, because removal of the transduced adipose tissue resulted in the elimination of the Epo (Fig. 4), we can exclude this possibility. Whether the tissue specificity of expression is common to all serotypes of AAV is yet to be confirmed. To test the tissue specificity, *db/db* mice are useful because they develop rich adipose tissues and a specific lobe can be completely removed by standard surgical procedures. On the other hand, the limitation of this model lies in the difficulty of long-term transgene expression: these animals were naturally diabetic and susceptible to thromboembolic events when they became polycythemic and eventually lost their lives after 4 weeks (Table 1). In this series of experiments, no clear threshold of Epo level on mortality was recognized, although all the "operated" animals attained long-term survival with normalized values of Epo and blood parameters. Therefore, in order to demonstrate long-term expression, a different transgene needs to be used.

Transducing adipose tissue may have another advantage with respect to immunology. Although the distribution and density of antigen-presenting cells within the adipose tissue remain unknown, it is possible that these cells are relatively scarce in the adipose tissue than in "standard" tissues such as muscle or liver. Therefore, the immune response against transgene product, which is a current hurdle in the field of gene therapy (Zaiss and Muruve, 2005), can partly be overcome by targeting adipose tissue. In our series of experiments, we did not observe any immunological responses to the transgene products or to the transduced adipose tissues. To test this hypothesis, a transgene product that is highly immunogenic to mice should be chosen and the outcome needs to be evaluated.

Adipose tissue is usually abundant in the body, can be easily transduced by simple vector injection, and can be removed safely. For these reasons, it is a potential depot organ for gene transfer. In this sense, there may be a wide range of applications of this method in supplemental gene therapy.

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REFERENCES

AMERICAN PHARMACEUTICAL ASSOCIATION and ROYAL PHARMACEUTICAL SOCIETY OF GREAT BRITAIN. (1986). *Handbook of Pharmaceutical Excipients*, 1st ed. Published jointly by the American Pharmaceutical Association (Washington, D.C.) and Pharmaceutical Press (London).

CARLOTTI, F., BAZUINE, M., KEKARAINEN, T., SEPPEN, J., POGNONEC, P., MAASSEN, J.A., and HOEBEN, R.C. (2004). Lentiviral vectors efficiently transduce quiescent mature 3T3-L1 adipocytes. Mol. Ther. **9,** 209–217.

CROYLE, M.A., CHENG, X., SANDHU, A., and WILSON, J.M. (2001). Development of novel formulations that enhance adenoviral-mediated gene expression in the lung *in vitro* and *in vivo*. Mol. Ther. **4,** 22–28.

FAN, D.S., OGAWA, M., FUJIMOTO, K.I., IKEGUCHI, K., OGASAWARA, Y., URABE, M., NISHIZAWA, M., NAKANO, I., YOSHIDA, M., NAGATSU, I., ICHINOSE, H., NAGATSU, T., KURTZMAN, G.J., and OZAWA, K. (1998). Behavioral recovery in 6-hydroxydopamine-lesioned rats by cotransduction of striatum with tyrosine hydroxylase and aromatic L-amino acid decarboxylase genes using two separate adeno-associated virus vectors. Hum. Gene Ther. **9,** 2527–2535.

FELDMAN, L.J., PASTORE, C.J., AUBAILLY, N., KEARNEY, M., CHEN, D., PERRICAUDET, M., STEG, P.G., and ISNER, J.M. (1997). Improved efficiency of arterial gene transfer by use of poloxamer 407 as a vehicle for adenoviral vectors. Gene Ther. **4,** 189–198.

FRADETTE, J., WOLFE, D., GOINS, W.F., HUANG, S., FLANIGAN, R.M., and GLORIOSO, J.C. (2005). HSV vector-mediated transduction and GDNF secretion from adipose cells. Gene Ther. **12,** 48–58.

GEBHART, C.L., and KABANOV, A.V. (2001). Evaluation of polyplexes as gene transfer agents. J. Control. Release **73,** 401–416.

GIBBS, W.J., and HAGEMANN, T.M. (2004). Purified poloxamer 188 for sickle cell vaso-occlusive crisis. Ann. Pharmacother. **38,** 320–324.

GOVERDHANA, S., PUNTEL, M., XIONG, W., ZIRGER, J.M., BARCIA, C., CURTIN, J.F., SOFFER, E.B., MONDKAR, S., KING, G.D., HU, J., SCIASCIA, S.A., CANDOLFI, M., GREENGOLD, D.S., LOWENSTEIN, P.R., and CASTRO, M.G. (2005). Regulatable gene expression systems for gene therapy applications: Progress and future challenges. Mol. Ther. **12,** 189–211.

HARTIKKA, J., SUKHU, L., BUCHNER, C., HAZARD, D., BOZOUKOVA, V., MARGALITH, M., NISHIOKA, W.K., WHEELER, C.J., MANTHORP, M., and SAWDEY, M. (2001). Electroporation-facilitated delivery of plasmid DNA in skeletal muscle: Plasmid dependence of muscle damage and effect of poloxamer 188. Mol. Ther. **4,** 407–415.

HERTZEL, A.V., SANDERS, M.A., and BERNLOHR, D.A. (2000). Adenovirus-mediated gene transfer in primary murine adipocytes. J. Lipid Res. **41,** 1082–1086.

ITO, M., BUJO, H., TAKAHASHI, K., ARAI, T., TANAKA, I., and SAITO, Y. (2005). Implantation of primary cultured adipocytes that secrete insulin modifies blood glucose levels in diabetic mice. Diabetologia **48,** 1614–1620.

KABANOV, A.V., and ALAKHOV, V.Y. (2002). Pluronic block copolymers in drug delivery: From micellar nanocontainers to biological response modifiers. Crit. Rev. Ther. Drug Carrier Syst. **19,** 1–72.

KABANOV, A., ZHU, J., and ALAKHOV, V. (2005). Pluronic block copolymers for gene delivery. Adv. Genet. **53,** 231–261.

LEE, R.C., RIVER, L.P., PAN, F.S., JI, L., and WOLLMANN, R.L. (1992). Surfactant-induced sealing of electropermeabilized skeletal muscle membranes *in vivo.* Proc. Natl. Acad. Sci. U.S.A. **89,** 4524–4528.

MATSUSHITA, T., ELLIGER, S., ELLIGER, C., PODSAKOFF, G., VILLARREAL, L., KURTZMAN, G.J., IWAKI, Y., and COLOSI, P. (1998). Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther. **5,** 938–945.

MEUNIER-DURMORT, C., FERRY, N., HAINQUE, B., DELATTRE, J., and FOREST, C. (1996). Efficient transfer of regulated genes in adipocytes and hepatoma cells by the combination of liposomes and replication-deficient adenovirus. Eur. J. Biochem. **237,** 660–667.

MEUNIER-DURMORT, C., GRIMAL, H., SACHS, L.M., DEMENEIX, B.A., and FOREST, C. (1997). Adenovirus enhancement of polyethylenimine-mediated transfer of regulated genes in differentiated cells. Gene Ther. **4,** 808–814.

MOCHIZUKI, S., MIZUKAMI, H., KUME, A., MURAMATSU, S., TAKEUCHI, K., MATSUSHITA, T., OKADA, T., KOBAYASHI, E., HOSHIKA, A., and OZAWA., K. (2004). Adeno-associated virus (AAV) vector-mediated liver- and muscle-directed transgene expression using various kinds of promoters and serotypes. Gene Ther. Mol. Biol. **8,** 9–18.

MOHAMED-ALI, V., PINKNEY, J.H., and COPPACK, S.W. (1998). Adipose tissue as an endocrine and paracrine organ. Int. J. Obes. Relat. Metab. Disord. **22,** 1145–1158.

MORIZONO, K., DE UGARTE, D.A., ZHU, M., ZUK, P., ELBARBARY, A., ASHJIAN, P., BENHAIM, P., CHEN, I.S., and HEDRICK, M.H. (2003). Multilineage cells from adipose tissue as gene delivery vehicles. Hum. Gene Ther. **14,** 59–66.

NAGAMATSU, S., NAKAMICHI, Y., OHARA-IMAIZUMI, M., OZAWA, S., KATAHIRA, H., WATANABE, T., and ISHIDA, H. (2001). Adenovirus-mediated preproinsulin gene transfer into adipose tissues ameliorates hyperglycemia in obese diabetic KKA^{y} mice. FEBS Lett. **509,** 106–110.

OGATA, K., MIMURO, J., KIKUCHI, J., TABATA, T., UEDA, Y., NAITO, M., MADOIWA, S., TAKANO, K., HASEGAWA, M., OZAWA, K., and SAKATA, Y. (2004). Expression of human coagulation factor VIII in adipocytes transduced with the simian immunodeficiency virus agmTYO1-based vector for hemophilia A gene therapy. Gene Ther. **11,** 253–259.

RUSSELL, D.W., and KAY, M.A. (1999). Adeno-associated virus vectors and hematology. Blood **94,** 864–874.

SOMMER, J.M., SMITH, P.H., PARTHASARATHY, S., ISAACS, J., VIJAY, S., KIERAN, J., POWELL, S.K., MCCLELLAND, A., and WRIGHT, J.F. (2003). Quantification of adeno-associated virus particles and empty capsids by optical density measurement. Mol. Ther. **7,** 122–128.

SRIADIBHATLA, S., YANG, Z., GEBHART, C., ALAKHOV, V.Y., and KABANOV, A. (2006). Transcriptional activation of gene expression by Pluronic block copolymers in stably and transiently transfected cells. Mol. Ther. **13,** 804–813.

WRIGHT, E.S., SARKOZY, E., DOBELL, A.R., and MURPHY, D.R. (1963). Fat globulemia in extracorporeal circulation. Surgery **53,** 500–504.

ZAISS, A.K., and MURUVE, D.A. (2005). Immune responses to adeno-associated virus vectors. Curr. Gene Ther. **5,** 323–331.

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A Histone Deacetylase Inhibitor Enhances Recombinant Adeno-associated Virus-Mediated Gene Expression in Tumor Cells

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The transduction of cancer cells using recombinant adeno-associated virus (rAAV) occurs with low efficiency, which limits its utility in cancer gene therapy. We have previously sought to enhance rAAV-mediated transduction of cancer cells by applying DNA-damaging stresses. In this study, we examined the effects of the histone deacetylase inhibitor FR901228 on tumor transduction mediated by rAAV types 2 and 5. FR901228 treatment significantly improved the expression of the transgene in four cancer cell lines. The cell surface levels of alpha v integrin, FGF-R1, and PDGF-R were modestly enhanced by the presence of FR901228. These results suggest that the superior transduction induced by the HDAC inhibitor was due to an enhancement of transgene expression rather than increased viral entry. Furthermore, we characterized the association of the acetylated histone H3 in the episomal AAV vector genome by using the chromatin immunoprecipitation assay. The results suggest that the superior transduction may be related to the proposed histone-associated chromatin form of the rAAV concatemer in transduced cells. In the analysis with subcutaneous tumor models, strong enhancement of the transgene expression as well as therapeutic effect was confirmed *in vivo*. The use of this HDAC inhibitor may enhance the utility of rAAV-mediated transduction strategies for cancer gene therapy.

Key Words: histone deacetylase inhibitor, AAV vector, cancer

INTRODUCTION

Recombinant adeno-associated virus (rAAV) has been of considerable interest to developers of clinical gene therapies [1,2]. This is because, unlike adenoviruses, the introduction of AAV vectors has not been associated with significant inflammation either experimentally or clinically [3]. Furthermore, diseases associated with AAV have not been found in human or animal populations. However, the transduction of cancer cells using rAAV occurs with very low efficiency, which limits its utility in gene therapy. Consequently, we have sought to enhance rAAV-mediated transduction of cancer cells by applying DNA-damaging stresses such as γ-rays or anticancer agents [4–6].

An alternative approach to improving the rAAV-mediated transduction of tumor cells may be to enhance transcription in the target cells. One technique to bring about this event may be to apply a histone deacetylase (HDAC) inhibitor, since HDAC inhibitors are known to regulate the transcription of various genes. Significantly, an HDAC inhibitor increases adenovirus-mediated transduction of cancer cell lines because it enhances the levels of the viral receptor on the cell surface [7]. On the other hand, the effects of HDAC inhibitors on rAAV-mediated transduction of tumor cells have not yet been fully elucidated. Treatment with an HDAC inhibitor causes gene expression from a silenced rAAV genome that has been integrated into the host's genome to recover [8]. However, rAAV exists mostly as an extrachromosomal genome rather than as an integrated genome, and this extrachromosomal form is the primary source of rAAV-mediated gene expression [9]. Therefore, the HDAC inhibitor-mediated recovery of expression from the integrated and silenced genome does not reflect a typical situation of rAAV-mediated transduction. Whereas no

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clear mechanism has been determined for the effect on the episomal vector-mediated expression, the histone deacetylase inhibitor should also contribute to the enhanced transcription before integration occurs.

Here we show that HDAC inhibitors markedly enhance the transgene expression immediately after rAAV-mediated transduction of tumor cells *in vitro* as well as *in vivo*. Our data also suggest that the vector genome in the cells is in the histone-associated chromatin form, which is capable of superior transcription. HDAC inhibitors may improve tumor cell transduction by enhancing the acetylation of the histone-associated chromatin of the rAAV genome.

RESULTS

Effects of FR901228 Treatment on the Transduction of U251MG Cells with rAAV

To analyze whether an HDAC inhibitor can also improve rAAV-mediated gene expression soon after the infection,

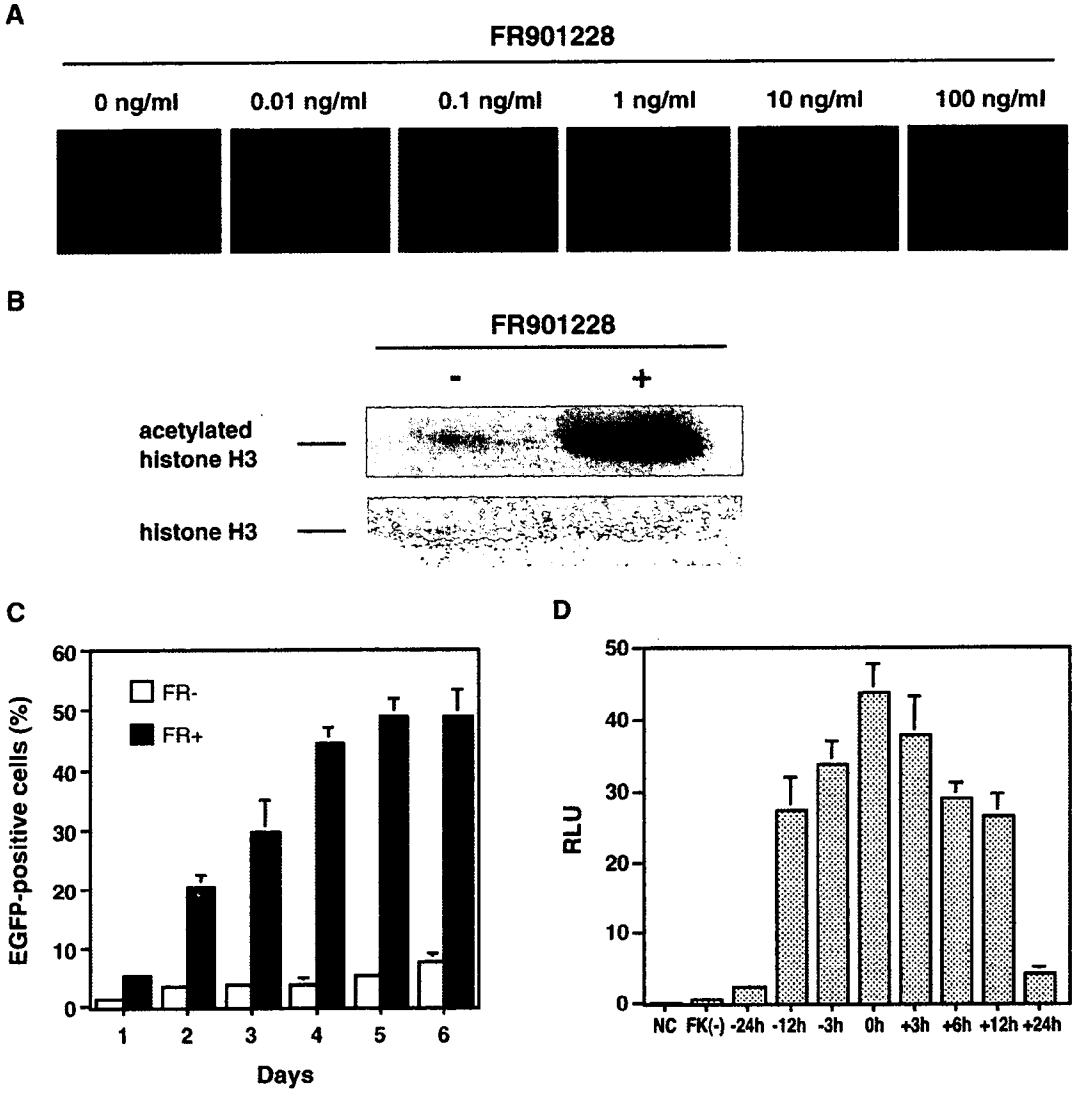

FIG. 1. (A) Effects of FR901228 treatment on the transduction of U251MG cells with rAAV. U251MG cells were infected with 1×10^4 genome copies/cell of AAV2EGFP in the presence of various concentrations of FR901228. EGFP expression was observed 24 h after infection. (B) Detection of the histone acetylation in U251MG cells caused by FR901228 treatment. Cells were incubated in the presence or absence of FR901228 for 24 h. The levels of acetylated histone H3 and histone H3 were determined by Western blot analysis. Histone H3 serves as a loading control. (C) The percentage of EGFP-positive cells at various time points after transduction with AAV2EGFP in the presence (FR+) or absence (FR−) of 1 ng/ml FR901228 was determined by FACS. Cells were infected with AAV2EGFP at 1×10^3 genome copies/cell. The data shown are the means and standard deviations of three independent experiments. (D) The kinetics of the effect on the FR901228-assisted transduction of U251MG cells. Cells were treated with FR901228 at various time points around the transduction with rAAV expressing luciferase as indicated. Luciferase assay was performed on the luminometer 48 h after the transduction.

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we transduced U-251MG human glioma cells with EGFP-expressing rAAV (AAV2EGFP) in the presence of the HDAC inhibitor FR901228. We found that FR901228 treatment improved the AAV2EGFP-mediated gene expression in a dose-dependent manner early after the infection (Fig. 1A). The fact that FR901228 also enhanced the acetylation of the histones in the cells was confirmed by Western blot analysis (Fig. 1B). To assess when gene expression was maximal, we transduced U251MG cells with AAV2EGFP in the presence or absence of 1 ng/ml FR901228 and assessed EGFP expression at various time points after transduction (Fig. 1C). This revealed that the enhancement of gene expression depended on the incubation period and required 4 days before the expression reach a plateau. To analyze the kinetics of the effect on the FR901228-assisted transduction of U251MG cells, cells were treated with FR901228 at various time points around the transduction with luciferase-expressing rAAV type 2 (AAV2-Luc) (Fig. 1D). As a result, the transduction efficiency peaked when cells were treated with FR901228 at the time of virus transduction.

TABLE 1: Relative expression of FGF-R1 and PDGF-R in U251MG cells treated with recombinant AAV alone (1×10^4 genome copies/cell) or together with FR901228 (0.3 or 3 ng/ml) for 24 h as analyzed by quantitative PCR

	$2^{\text{correctedΔCt (GAPDH − target)}}$	
FR901228 (ng/ml)	FGF-R1	PDGF-Rα
0	1.00	1.00
0.3	1.28	1.77
3	1.60	2.30

The relative expression of the target mRNA was determined as the ratio of the expression in U251MG cells treated with recombinant AAV and FR901228 to that in U251MG cells treated with recombinant AAV alone. Data are means ($n = 5$).

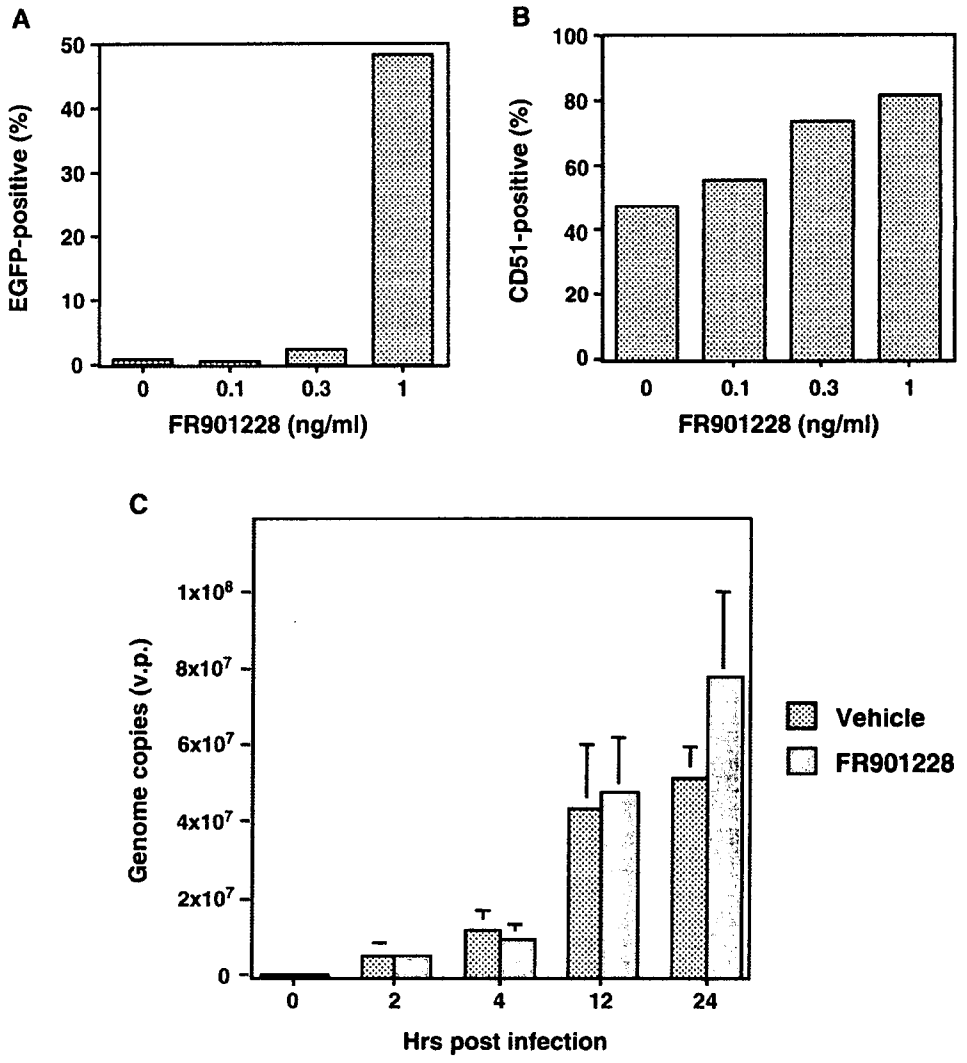

FIG. 2. (A) Percentage of EGFP-positive U251MG cells after transduction with 1×10^4 genome copies/cell of AAV2EGFP in the presence of various concentrations of FR901228. The cells were analyzed 24 h after the transduction for EGFP expression by FACS. The data shown are the average percentages of EGFP-positive cells after three independent transductions. (B) Integrin expression in transduced cells is only modestly enhanced by FR901228 treatment. The cells were stained 24 h after the transduction with monoclonal antibodies to CD51 (integrin v chain, clone 13C2) and analyzed by FACS. The data shown are the average percentages of positive cells after three independent transductions. (C) Transgene copy number in U251MG cells transduced with 1×10^4 genome copies/cell of AAV2EGFP in the presence of 1 ng/ml FR901228. The copy number of the transgene was estimated by real-time PCR at 0, 2, 4, 12, and 24 h after the rAAV infection.

Effects on Receptor Expression and Viral Entry

To determine if FR901228 acted by enhancing the entry of rAAV, we infected U251MG cells with AAV2EGFP in the presence of various concentrations of FR901228 and then analyzed the EGFP and alpha v integrin levels in the cells by fluorescence-activated cell sorting (FACS). This analysis showed that 24 h after AAV2EGFP infection with 1 ng/ml FR901228, 48% of the U251MG cells were EGFP-positive, whereas at lower concentrations of FR901228 only very few cells were EGFP-positive (Fig. 2A). However, this FR901228 concentration range (0.3–1 ng/ml) only modestly enhanced the levels of AAV2 coreceptor, alpha v integrin (Fig. 2B). In addition, when we estimated the amount of the rAAV genome in the transduced cells by real-time quantitative PCR analysis, we found that FR901228 treatment did not significantly affect the copy number of the rAAV (Fig. 2C). Furthermore, we also estimated the effect of FR901228 on the expression of coreceptors for the AAV. FR901228 moderately

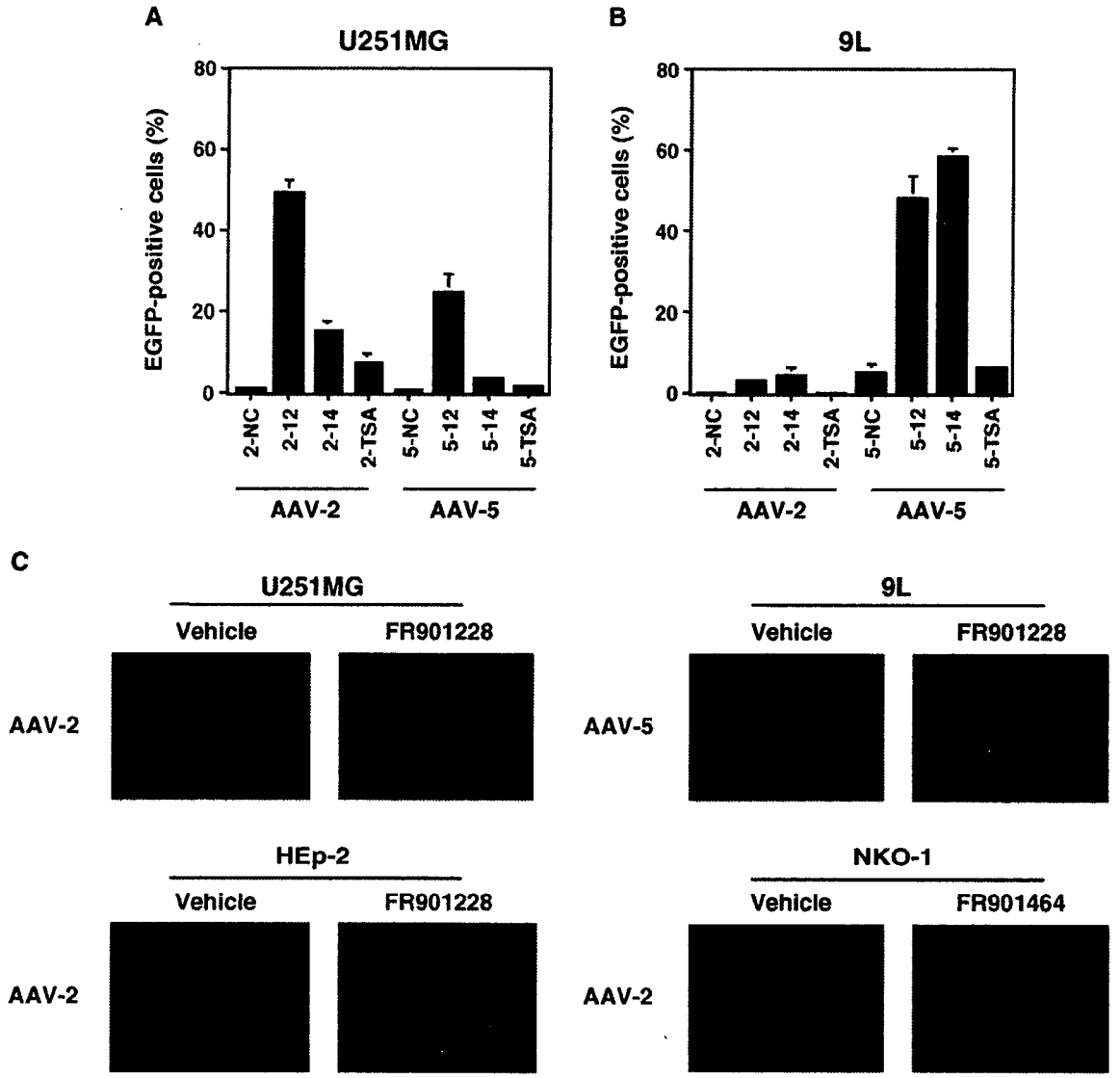

FIG. 3. (A, B) EGFP expression by AAV2EGFP and AAV5EGFP differs depending on the tumor cell being transduced. U251MG or 9L cells were infected with 1 × 10^4 genome copies/cell of AAV2EGFP (2) or AAV5EGFP (5) in the presence of vehicle (NC) or 1 ng/ml of various HDAC inhibitors, FR901228 (12), FR901464 (14), or TSA. The cells were analyzed by FACS 24 h after the infection. The data show the average percentages of EGFP-positive cells after three independent transductions + SD. (C) Representative data of the enhanced transgene expression by HDAC inhibitors in various cell lines infected with AAV vectors. Twenty-four hours after the AAV2EGFP or AAV5EGFP infection at 1 × 10^4 genome copies/cell with 1 ng/ml of the FR901228 or FR901464, cells were examined under the fluorescence microscope.

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increased mRNA levels of fibroblast growth factor receptor 1 (FGF-R1) and platelet-derived growth factor receptor (PDGF-R), although the augmentation was not enough to explain the drastic increase of the expression (Table 1).

Transduction of Tumor Cells with AAV Vectors Derived from Distinct Serotypes

Type 2 and type 5 rAAV differed from each other in the efficiency of their transduction of U251MG and the 9L glioma cells. Although FR901228 and other HDAC inhibitors (FR901464 or trichostatin A (TSA)) remarkably enhanced the transduction of both rAAVs in general, AAV2EGFP-mediated transduction of U251MG cells was more efficient than AAV5EGFP-mediated transduction while AAV5EGFP-mediated transduction of 9L cells was better than AAV2EGFP-mediated transduction (Figs. 3A and 3B). FR901228 and FR901464 also had promoting effects on AAV2EGFP- and AAV5EGFP-mediated transduction of the head and neck cancer cell lines HEp-2 and NKO-1 (Fig. 3C).

Chromatin Modification with FR901228

We characterized chromatin composition of the episomal AAV vector genome by using the chromatin immunoprecipitation (ChIP) assay. ChIP is a technique to test for the presence of certain DNA-binding proteins that might modulate chromatin structure and/or transcriptional characteristics of the specific region of DNA with which they are associated. We made use of polyclonal antibodies generated against histone H3 as well as acetylated histone H3, which have been linked to chromatin modification and regulation of transcription. The primers for the CMV promoter region in the AAV vector genome gave a higher level of PCR product when used on templates from FR901228-treated cells compared to those from cells without FR901228 treatment. Higher levels of acetylated histone H3 were found on the CMV promoter region of the AAV vector versus the GAPDH promoter region of the cellular DNA (Table 2A). In contrast, enrichment of acetylated histone H3-associated DNA was not significant on plasmid vector genome irrespective of the presence of the ITR (Table 2B).

FR901228-Assisted Enhancement of Tumor Transduction *in Vivo*

In the analysis using optical bioluminescence imaging of the subcutaneous tumors, we confirmed drastic enhancement of the luciferase gene expression *in vivo* (Fig. 4A). The signal intensity in animals treated with FR901228 (n = 5, [1.5 ± 0.9] × 10^6 photons/s/cm^2/sr) was 37.4-fold higher than in control animals (n = 3, [4.0 ± 2.4] × 10^4 photons/s/cm^2/sr). A subcutaneous

TABLE 2: PCR amplification of immunoprecipitated DNA

(A) Chromatin composition of episomal AAV vector genome was characterized by using the chromatin immunoprecipitation assay

Ab of interest	FR901228	$2^{\text{correctedΔCt (GAPDHprom – CMVprom)}}$
Rabbit IgG	–	<0.001
Rabbit IgG	+	<0.001
Anti-histone H3	–	1.0 ± 1.8
Anti-histone H3	+	7.3 ± 1.4
Anti-acetyl histone H3	–	1.0 ± 0.4] <0.0001
Anti-acetyl histone H3	+	22.0 ± 0.8]

(B) Cells were transfected with a plasmid harboring the EGFP expression cassette under the CMV promoter (pEGFP) or a plasmid carrying an identical EGFP expression cassette flanked by ITR regions (pITR-EGFP)

Plasmid	Ab of interest	FR901228	$2^{\text{correctedΔCt (GAPDHprom – CMVprom)}}$
pEGFP	Rabbit IgG	–	<0.001
	Rabbit IgG	+	<0.001
	Anti-acetyl histone H3	–	1.0
	Anti-acetyl histone H3	+	1.3
pITR-EGFP	Rabbit IgG	–	<0.001
	Rabbit IgG	+	<0.001
	Anti-acetyl histone H3	–	1.0
	Anti-acetyl histone H3	+	1.2

U251MG cells were transduced with AAV vector at 1 × 10^4 genome copies/cell in the presence or absence of 1 ng/ml FR901228. Twenty-four hours after the transduction, chromatin proteins of interest were cross-linked to DNA by formaldehyde. Shared DNA was immunoprecipitated with histone H3 antibody or acetylated histone H3 antibody to enrich for the CMV promoter region or GAPDH promoter region. Relative differences in the levels of immunoprecipitated DNA, which are reflective of the levels of the chromatin protein of interest occupying a particular island, between different promoter regions and cell treatment with FR901228 were quantified by quantitative PCR.

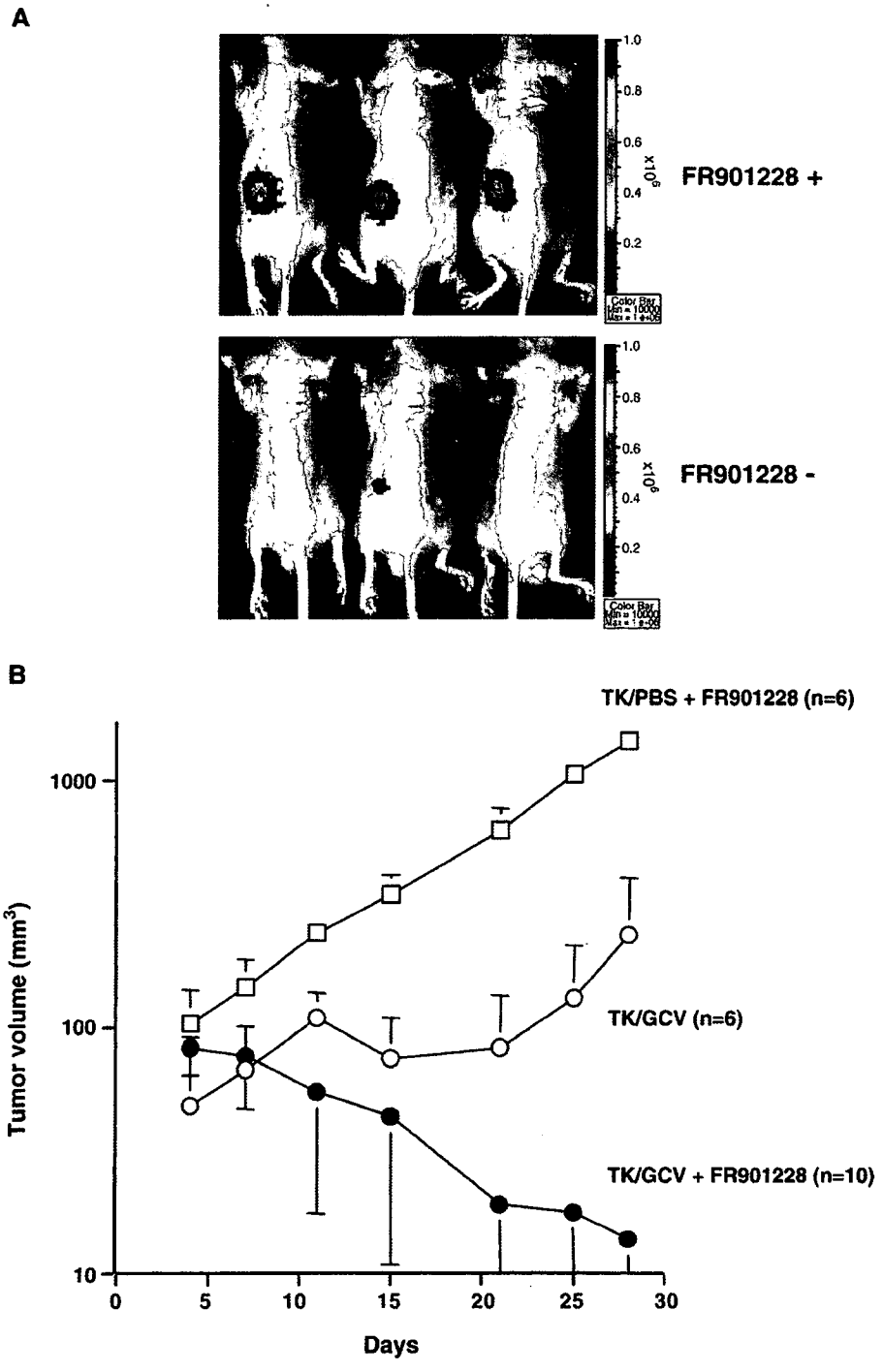

FIG. 4. (A) FR901228-assisted enhancement of tumor transduction *in vivo*. U251MG cells were mixed with PBS (FR901228–, $n = 3$) or transduced with a recombinant AAV2 expressing luciferase (AAV2Luc) at 1×10^4 genome copies/cell for 1 h (FR901228+, $n = 5$), and then 3×10^6 of the transduced cells in 100 μl PBS were inoculated subcutaneously into the BALB/c mice along with the intraperitoneal injection of FR901228 at 1 mg/kg. Twenty-four hours after administration of the FR901228, optical bioluminescence imaging was performed using the CCD camera. (B) The effects of FR901228 on the rAAV-mediated transduction for 9L tumor elimination *in vivo*. Cells were transduced with AAV5TK at 1×10^4 genome copies/cell for 1 h, and then 3×10^6 of the transduced cells in 100 μl PBS containing 25% (v/v) basement membrane matrix were inoculated subcutaneously into the BALB/c mice. The tumor-bearing animals received intraperitoneal injection of FR901228 at 3 mg/kg (group 1, $n = 6$; group 3, $n = 10$) or PBS (group 2, $n = 6$). The animals were also exposed to ganciclovir (GCV) at 100 mg/kg per day (groups 2 and 3) or PBS (group 1) for 14 consecutive days by intraperitoneal placement of the miniosmotic pumps.

tumor model with athymic nude mice demonstrated that the combination of AAV-mediated transduction for HSV-*tk*/GCV therapy and FR901228 treatment ($n = 10$) resulted in statistically significant reduction of tumor growth relative to HSV-*tk*/GCV therapy without FR901228 treatment (unpaired t test, $P < 0.05$, $n = 6$; Fig. 4B). When the tumor-bearing animals were treated with GCV and FR901228, 8 of 10 tumors were eliminated at 4 weeks after transduction.

Discussion

HDAC inhibitors significantly improved the expression of the transgene in cancer cells. The enhancement of the coreceptor level was modest and copy number of the

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rAAV in the transduced cells was also modestly affected by the FR901228 treatment. Furthermore, association of the acetylated histone H3 in the episomal AAV vector genome was demonstrated by using the chromatin immunoprecipitation assay. In the analysis with the subcutaneous tumor models, strong enhancement of the transgene expression as well as therapeutic effect was confirmed *in vivo*.

Treatment with an HDAC inhibitor is known to cause the recovery of the gene expression of a rAAV vector genome that has been integrated and silenced after long-term selection [8]. However, rAAV occurs mostly as extrachromosomal genomes rather than as integrated genomes, and these extrachromosomal forms are the primary source of rAAV-mediated gene expression early after transduction [9]. There has been no direct investigation of the effects of HDAC inhibitors on the rAAV-mediated transient gene expression. We examined whether the HDAC inhibitor could contribute to the enhanced transcription before integration occurs.

FR901228 treatment significantly improved the transient expression of the transgene in four cancer cell lines. The FR901228 treatment improved the rAAV-mediated gene transfer in a dose-dependent manner, and the highest enhancement was observed in the U251MG cells with AAV2EGFP. In the U251MG cells, the cell surface levels of alpha v integrin, FGF-R1, and PDGF-R were only modestly enhanced by the presence of FR901228. These observations contrast with a previous report that suggested that FR901228 enhanced adenovirus transduction by increasing CAR and v integrin RNA levels, thereby enhancing viral entry [7]. However, their study did not demonstrate that these increased RNA levels were associated with increased protein levels or kinetics. In our study, a kinetic analysis of the effect on the FR901228-assisted AAV-mediated transduction of U251MG cells showed that the transduction efficiency peaked when cells were treated with FR901228 at the time of transduction. This is in sharp contrast to the case of the effect of FR901228 on the enhanced adenovirus-mediated transduction. Since enhanced viral entry into the cell is a primary function of FR901228 regarding improved adenovirus transduction, transduction efficiency of the adenovirus was preferentially enhanced when the cells were pretreated with FR901228 before transduction [10].

Interestingly, we observed that type 2 and type 5 rAAV differed from each other in the efficiency of their transduction of the U251MG and 9L cells. The differences in the transduction efficiency of the AAV vectors derived from distinct serotypes may be due to the fact that each AAV serotype recognizes a different receptor and that different cell types may express different levels of these receptors. Type 2 AAV uses the cell surface heparan sulfate proteoglycan (HSPG) as a receptor [11]. However, cell surface expression of HSPG alone is insufficient for type 2 AAV infection and FGF-R1 is also required as a coreceptor for successful viral entry into the host cell [12]. Type 5 AAV transduction requires 2,3-linked sialic acid [13] as well as PDGF-R [14] for efficient binding and transduction. These observations indicate that optimized expression of a transgene borne by rAAV will require the careful selection of the appropriate vector serotype with respect to the target cell.

Our data also suggest that the use of FR901228 in combination with AAV vector infection may improve viral entry into the cells, but also requires additional mechanisms to benefit the target cells for the efficient transduction. Association of the acetylated histone H3 in the episomal AAV vector genome was characterized by using the chromatin immunoprecipitation assay. Characterization of the chromatin modification in the rAAV genome with FR901228 suggested that improved expression of the transgene depends on the chromatin state of the AAV genome in the infected cells rather than viral entry. These results suggest that the superior transduction induced by HDAC inhibitor treatment is actually due to an enhancement of transgene expression associated with chromatin modification rather than to increased viral entry. Thus, epigenetic regulatory mechanisms may be involved in the HDAC inhibitor-mediated improvement of the transduction of cancer cells with rAAV. The rAAV concatemer may need to be present in a histone-associated chromatin form in the cells before efficient transgene expression can occur.

Our study suggests that the improved rAAV-mediated transduction induced by HDAC inhibitor was due to an enhancement of transgene expression rather than increased viral entry. This phenomenon may be related to the proposed histone-associated chromatin form of the rAAV concatemer in transduced cells. The depsipeptide fermentation product FR901228 is currently being tested in clinical trials as an anti-cancer drug. Therefore, to utilize such a compound to assist rAAV-mediated cancer gene therapy is theoretically and practically reasonable. The use of HDAC inhibitors may enhance the utility of rAAV-mediated transduction strategies for future clinical investigation.

MATERIALS AND METHODS

Recombinant AAV production. The EGFP expression cassette driven by the CMV promoter was ligated into pAAVLacZ [15] and pAAV5-RNL [16] to form the proviral plasmids pAAV2EGFP and pAAV5EGFP. rAAV types 2 and 5 that express the EGFP gene (AAV2EGFP and AAV5EGFP) were generated using the proviral plasmids. The luciferase expression cassette driven by the CMV promoter in pLNCL [17] was cloned into pAAVLacZ to create pAAV2Luc. A rAAV type 2 that expresses the luciferase gene (AAV2Luc) was generated using pAAV2Luc. Likewise, the HSV-*tk* cDNA contained in the pAVS6TK [18] was subcloned into pAAV5-RNL to create pAAV5TK. A rAAV type 5 that expresses the HSV-*tk* gene driven by the CMV promoter (AAV5TK) was generated using pAAV5TK. Transfection of 293 cells with the proviral plasmid, AAV helper plasmid pAAV2H [15] or pAAV5H [16], and adenoviral helper plasmid pAdeno was performed according to the previously described protocol [19] associated with an

active gassing [20]. The physical titer of the viral stock was determined by dot-blot hybridization with plasmid standards.

HDAC inhibitors. The HDAC inhibitor FR901228 (obtained from Fujisawa Pharmaceutical Co., Ltd.) is a depsipeptide fermentation product from *Chromobacterium violaceum* [21]. FR901228 strongly inhibits the proliferation of tumor cells by arresting cell cycle transition and is now being tested in clinical trials [22]. FR901464 (obtained from Fujisawa Pharmaceutical Co., Ltd.) and TSA (Sigma–Aldrich Corp., St. Louis, MO, USA) are also prepared as HDAC inhibitors [21].

Cells and culture. The malignant human glioma cell line U251MG, the malignant rat glioma cell line 9L, the laryngeal epidermoid carcinoma cell line HEp-2, and the human maxillary sinus cancer cell line NKO-1 were used in this study. Cells were cultured in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2. Human embryonic kidney 293 cells were cultured with D-MEM:F12 (1:1 mixture) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2. Luciferase assay was performed on the luminometer (Fluoroskan Ascent FL, Thermo Labsystems, Beverly, MA, USA) using the Bright-Glo Reagent kit (Promega, Madison, WI, USA).

FACS analysis. Approximately 5×10^4 cells were analyzed on the FACScan (Becton–Dickinson, San Jose, CA, USA) with CellQuest software (Becton–Dickinson). Cells were incubated with a PE-labeled monoclonal antibody (13C2) specific for human integrin v chain (CD51; Cymbus Biotechnology Ltd., Chandlers Ford, UK) for 30 min on ice. The 7-aminoactinomycin-D (Via-Probe; Pharmingen, San Diego, CA, USA)-negative cell fraction, which contains the viable cells, was used to detect EGFP- and/or PE-positive cells.

Western blot analysis. Detection of histone acetylation by FR901228 in U251MG cells was performed as described [7]. Western blot analysis of the cells incubated in the presence or absence of FR901228 for 24 h was performed using either a rabbit polyclonal antibody against histone H3 or one against acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:2000 in 5% milk. The probed membrane was incubated with an anti-rabbit immunoglobulin horseradish peroxidase-linked antibody and developed by ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Determination of transgene copy number. Tumor cells were infected with 1×10^4 genome copies/cell of rAAV in the presence of FR901228. The high-molecular-weight DNA was extracted from the cells (DNA Extraction Kit; Qiagen, Inc., Hilden, Germany) 0, 2, 4, 12, and 24 h later. The copy numbers were determined by quantitative PCR analysis of 100 ng of the DNA by using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) as described in the supplementary information.

mRNA analysis of coreceptors for the AAV. U251MG cells were incubated with recombinant AAV either alone (1×10^4 genome copies/cell) or together with FR901228 (0.3 or 3 ng/ml) for 24 h. mRNA was isolated from the cell culture using an RNeasy mini kit (Qiagen) and reverse-transcribed into a single-stranded cDNA using the SuperScript Preamplification System (Invitrogen, Carlsbad, CA, USA). FGF-R1 or PDGF-R mRNA was quantitated by real-time PCR as described in the supplementary information.

PCR analysis of immunoprecipitated DNA. Chromatin immunoprecipitation was performed following the Upstate Biotechnology ChIP kit protocol. U251MG cells were transduced with AAV vector at 1×10^4 genome copies/cell, pCMV-EGFP, or pAAV2EGFP in the presence or absence of the 1 ng/ml FR901228. Twenty-four hours after the transduction, chromatin proteins of interest were cross-linked to DNA. After preclearing, isotype-antibody control or anti-acetylated histone H3 or anti-histone H3 antibody (Upstate Biotechnology) was added to the sonicated chromatin solution and incubated overnight at 4°C with agitation. Resulting immune complexes were collected by the salmon sperm DNA–protein A agarose slurry. The eluted samples were treated with proteinase K and purified by phenol/chloroform extraction. Precipitated DNAs were analyzed for the vector-derived promoter by quantitative PCR with an ABI Prism 7700 sequence detection system as described in the supplementary information.

In vivo analysis of enhanced transgene expression. U251MG cells were treated with PBS ($n = 3$) or transduced with a recombinant AAV2 expressing luciferase (AAV2Luc) at 1×10^4 genome copies/cell for 1 h ($n = 5$), and then 3×10^6 of the transduced cells in 100 μl PBS containing 25% (v/v) basement membrane matrix (Matrigel; BD Biosciences, Franklin Lakes, NJ, USA) were inoculated subcutaneously into male BALB/c *nu/nu* mice (Clea Japan, Tokyo, Japan) along with intraperitoneal injection of FR901228 at 1 mg/kg or the same volume of vehicle. Twenty-four hours after the administration of FR901228, optical bioluminescence imaging was performed using the CCD camera (Xenogen Corp., Alameda, CA, USA). After intraperitoneal injection of reporter substrate D-luciferin (375 mg/kg body wt), mice were imaged for scans.

To analyze the effect of FR901228 on the enhanced tumor elimination *in vivo*, 9L tumor cells were transduced with an AAV5TK at 1×10^4 genome copies/cell for 1 h, and then 3×10^6 of the transduced cells in 100 μl PBS containing 25% (v/v) Matrigel were inoculated subcutaneously into BALB/c mice. The tumor-bearing animals received an intraperitoneal injection of FR901228 at 3 mg/kg (group 1, $n = 6$; group 3, $n = 10$) or PBS (group 2, $n = 6$). The animals were also exposed to ganciclovir at 100 mg/kg per day (groups 2 and 3) or PBS (group 1) for 14 consecutive days by intraperitoneal placement of the miniosmotic pumps (Alzet, Palo Alto, CA, USA) according to the manufacturer's instructions. Tumor growth was monitored two to three times a week by measuring two perpendicular tumor diameters using calipers and the volumes were calculated as $a \times b^2 \times 0.5$, where a is the length and b is the width of the tumor in millimeters. Animals with tumors larger than 2 cm in diameter were euthanized.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymthe.2005.11.010.

REFERENCES

1. Carter, B. J. (2004). Adeno-associated virus and the development of adeno-associated virus vectors: a historical perspective. *Mol. Ther.* **10:** 981–989.
2. Okada, T., *et al.* (2002). Adeno-associated virus vectors for gene transfer to the brain. *Methods* **28:** 237–247.
3. Zaiss, A. K., Liu, Q., Bowen, G. P., Wong, N. C., Bartlett, J. S., and Muruve, D. A. (2002). Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. *J. Virol.* **76:** 4580–4590.
4. Kanazawa, T., *et al.* (2001). Gamma-rays enhance rAAV-mediated transgene expression and cytocidal effect of AAV-HSVtk/ganciclovir on cancer cells. *Cancer Gene Ther.* **8:** 99–106.
5. Kanazawa, T., *et al.* (2003). Suicide gene therapy using AAV-HSVtk/ganciclovir in

doi:10.1016/j.ymthe.2005.11.010

combination with irradiation results in regression of human head and neck cancer xenografts in nude mice. *Gene Ther.* **10:** 51–58.
6. Kanazawa, T., *et al.* (2004). Topoisomerase inhibitors enhance the cytocidal effect of AAV-HSVtk/ganciclovir on head and neck cancer cells. *Int. J. Oncol.* **25:** 729–735.
7. Kitazono, M., Goldsmith, M. E., Aikou, T., Bates, S., and Fojo, T. (2001). Enhanced adenovirus transgene expression in malignant cells treated with the histone deacetylase inhibitor FR901228. *Cancer Res.* **61:** 6328–6330.
8. Chen, W. Y., Bailey, E. C., McCune, S. L., Dong, J. Y., and Townes, T. M. (1997). Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. *Proc. Natl. Acad. Sci. USA* **94:** 5798–5803.
9. Nakai, H., Yant, S. R., Storm, T. A., Fuess, S., Meuse, L., and Kay, M. A. (2001). Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. *J. Virol.* **75:** 6969–6976.
10. Vanoosten, R. L., Moore, J. M., Ludwig, A. T., and Griffith, T. S. (2005). Depsipeptide (FR901228) enhances the cytotoxic activity of TRAIL by redistributing TRAIL receptor to membrane lipid rafts. *Mol. Ther.* **11:** 542–552.
11. Summerford, C., and Samulski, R. J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.* **72:** 1438–1445.
12. Qing, K., Mah, C., Hansen, J., Zhou, S., Dwarki, V., and Srivastava, A. (1999). Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat. Med.* **5:** 71–77.
13. Walters, R. W., *et al.* (2001). Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. *J. Biol. Chem.* **276:** 20610–20616.
14. Di Pasquale, G., *et al.* (2003). Identification of PDGFR as a receptor for AAV-5 transduction. *Nat. Med.* **9:** 1306–1312.
15. Okada, T., *et al.* (2001). Development and characterization of an antisense-mediated prepackaging cell line for adeno-associated virus vector production. *Biochem. Biophys. Res. Commun.* **288:** 62–68.
16. Chiorini, J. A., Kim, F., Yang, L., and Kotin, R. M. (1999). Cloning and characterization of adeno-associated virus type 5. *J. Virol.* **73:** 1309–1319.
17. Okada, T., *et al.* (1997). Inhibition of gene expression from the human c-erbB gene promoter by a retroviral vector expressing anti-gene RNA. *Biochem. Biophys. Res. Commun.* **240:** 203–207.
18. Okada, T., *et al.* (2001). AV.TK-mediated killing of subcutaneous tumors in situ results in effective immunization against established secondary intracranial tumor deposits. *Gene Ther.* **8:** 1315–1322.
19. Okada, T., *et al.* (2002). Adeno-associated viral vector-mediated gene therapy of ischemia-induced neuronal death. *Methods Enzymol.* **346:** 378–393.
20. Okada, T., *et al.* (2005). Large-scale production of recombinant viruses using a large culture vessel with active gassing. *Hum. Gene Ther.* **16:** 1212–1218.
21. Nakajima, H., Kim, Y. B., Terano, H., Yoshida, M., and Horinouchi, S. (1998). FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. *Exp. Cell Res.* **241:** 126–133.
22. Sandor, V., *et al.* (2002). Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. *Clin. Cancer Res.* **8:** 718–728.