

Efficient gene expression by self-complementary adeno-associated virus serotype 2 and 5 in various human cancer cells

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Abstract. The feasibility of various self-complementary AAV (scAAV) serotypes as efficient gene delivery vehicles in human cancer cells was evaluated. To dissect the transduction characteristics, we infected a variety of human cancer cells with scAAV1-6 or scAAV8 expressing GFP. scAAV2 led to the best transduction efficiency with nearly complete transgene expression at 1000 MOI in most cancer cells, regardless of cell/tissue origins. scAAV5 could also induce effective gene expression, even though gene transfer potency by scAAV5 was poorer than that by scAAV2. Substantial portion of transgene expression lasted over a month following gene delivery by both scAAV 2 and scAAV5, indicating that long-term gene expression can occur. Moreover, co-infection of scAAV2 and scAAV5 can induce simultaneous transgene expressions introduced via each vector. Thus, the current study provide evidence that scAAV2 and scAAV5 vectors are excellent gene transfer tools in a wide variety of human cancer cells, independently driving persistent transgene expression.

Introduction

The promising potential of recombinant adeno-associated virus (rAAV) as a gene delivery tool has been recently documented in cancer gene therapy (1,2). Adeno-associated virus, a member of the non-pathogenic parvovirus family, consists of a single-stranded DNA as a genome (3). The AAV genome is able to integrate itself into the human chromosome at various sites but most commonly on chromosome 19q (4,5), resulting in persistent transgene expression by rAAV (3). In fact, rAAV2, the most widely studied rAAV serotype, can mediate long-term gene expression in both *in vitro* and *in vivo* (6,7).

For transgene expression, the conventional single-stranded rAAV2 (ssAAV) genome has to become double-stranded via complementary-strand synthesis, once the rAAV2 gets into a target cell (3). Therefore, the conversion of single-stranded rAAV2 genome into double-stranded form has been a major limiting step in efficient transduction by rAAV2. To overcome this rate-limiting step, several research groups have generated preformed double-stranded rAAV and demonstrated its usefulness in gene transduction (8-10). Transduction efficiency (TE) could be dramatically improved by hiring this self-complementary rAAV (scAAV) of 5- to 140-fold increase, compared to that by ssAAV (8,9).

A wide variety of AAV serotypes, other than AAV2, have been additionally isolated (11,12). Moreover, rAAVs originated from various AAV serotypes have been generated by replacing the rAAV2 capsid with other capsids and the potential of these pseudotyped-rAAVs have been intensely investigated. Several studies have shown that other rAAV serotypes have advantages in effectively transducing various cell/tissue types due to their distinct tropisms (13). For example, rAAV1, rAAV6 and rAAV8 vectors show better transduction efficiencies (TEs) in skeletal muscle (14,15) or heart (16,17). In addition, rAAV1 and rAAV5 can transduce the vascular endothelial cells of airways more efficiently (18).

Therefore, it would be very interesting to examine the characteristics of transduction by different rAAV serotypes based on scAAV on human cancer cells from distinct cell/tissue origins. In the present study, we transduced a wide variety of human cancer cells of different origins with scAAV serotypes 1-6 and 8. We then asked whether there was any difference in the TE depending on tissue origin. We also determined whether long-term transgene expression can occur by scAAV in human cancer cells. Finally, we examined transduction pattern by co-treatment of different scAAVs. The results suggest that scAAV2 and scAAV5 can efficiently transduce various cancer cells, regardless of tissue origin. The data also indicate that persistent and simultaneous transgene expression can occur by scAAV 2 and scAAV5.

Materials and methods

Cell culture. 293T and human glial cancer U251 cells were kindly provided by Dr J. Jung and Dr Y. Lee at Harvard

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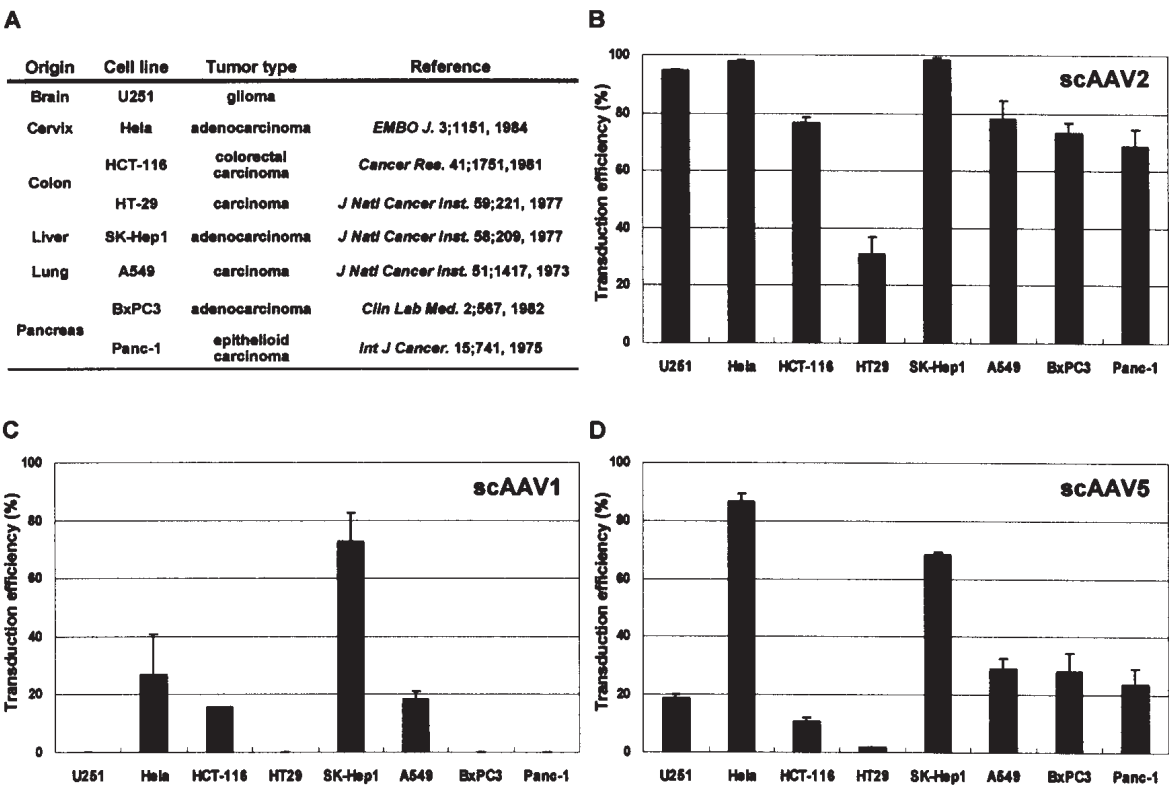


Figure 1. Transduction of various human cancer cells by distinct scAAV serotypes. Table (A) represents the information about the human cancer cells used in the present study. Cells were infected with scAAV2 (B), scAAV1 (C) or scAAV5 serotypes (D) encoding GFP gene at MOI 1000. To estimate transduction efficiency (TE), GFP expression in the cells was analyzed on 48 h post-infection (p.i.) by flow cytometry (n>3, mean values \pm SD). scAAV2 led to the highest TE, regardless of cancer cell types.

Medical School and University of Pittsburgh, respectively. The 293 cells and other human cancer cells (hepatocellular SK-Hep1, cervical HeLa, colon HCT116, HT-29, lung A549, pancreatic Bx-PC3 and Panc-1) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All the cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (50 μ g/ml) in a 5% CO₂ incubator at 37°C.

Recombinant adeno-associated virus (rAAV) preparation and titration. For the generation of self-complementary rAAV serotypes, pHpa-trs-SK was obtained from Dr J. Samulski (University of North Carolina) as pscAAV plasmid (8). A set of pRepCap plasmids carrying capsid genes for serotypes 1-6 or 8 were also provided by Dr C. High from University of Pennsylvania. All the rAAVs were synthesized based on triple-transfection using calcium phosphate method mixing pHpa-trs-SK encoding GFP or RFP, pRepCap and pXX6 (Stratagene, La Jolla, CA) encoding adenoviral helper genes (19,20). Briefly, about 1x10⁷ cells in a 15-cm culture dish were transfected with 50 μ g of the plasmids for 2 days. From cell lysates that included the supernatant after removing cell debris, pure population of rAAVs were prepared by sequential two-steps of CsCl gradients. After dialysis using 50 mM Tris-buffer (pH 7.8) containing 1 mM MgCl and 10% sorbitol, the purified rAAVs were aliquoted and stored at -80°C. The total number of rAAV particles was calculated by TaqMan-based real-

time PCR analysis (TaqMan universal master Mix, Applied Biosystems, CA, USA) quantifying the number of viral genome encoding a cytomegalovirus (CMV) promoter. The primers and TaqMan probe were designed to target the CMV promoter. Their sequences were 5'-CGTTACATAACTTACGGTAA ATG-3' (forward primer), 5'-ATACGTCATTATTGACGTC AATG-3' (reverse primer), 5'-FAM CCTGGCTGACCGCCC AACGACTAMRA-3' (TaqMan probe). The reaction was carried out as described previously by using DNA engine OPTICON 2 system (MJ Research, NV, USA) (21).

Transduction of cells. Cells in 48-well plates were treated with rAAVs at various multiplicities of infection (MOIs) at ~50% cellular confluence. Mock-treated cells used as a control were the cells with no rAAV treatment. The cells were further maintained until designated times with or without subculturing the cells. Transgene products, such as GFP and RFP were observed under an inverted fluorescent microscope (Leica DMIRB, Leica, Germany). The pictures were taken when necessary at the original magnification of either x100 or x200. The fluorescent pictures were merged by using MetaVue software (Molecular Devices, PA, USA).

Flow cytometric analysis. Both transduction efficiency (TE) and the mean fluorescence intensity (MFI) was determined using flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA, USA). Transduced cells were harvested by trypsinizing the cells with 0.05% trypsin-EDTA. The cells

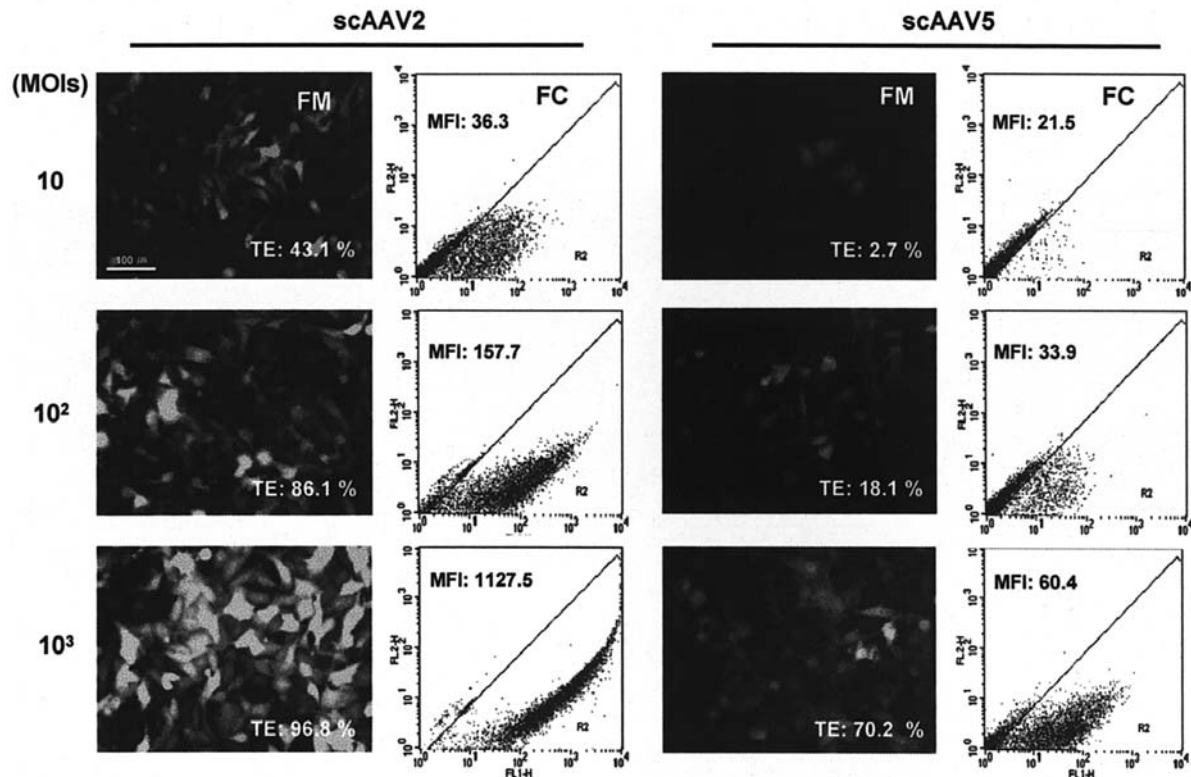


Figure 2. Characterization of GFP expression by scAAV2 or scAAV5 at different MOIs. SK-Hep1 cells were treated with scAAV2 or scAAV5-GFP at MOIs of 10, 100 or 1000. Forty-eight hours p.i., TE and mean fluorescence intensity (MFI) were measured using a flow cytometer. The pictures were taken under fluorescent microscope, original magnification x200. When the cells were infected with higher MOI, the more the cells were transduced or expressed the higher the MFI. FM; fluorescent microscopy, FC; flow cytometry.

were then washed in PBS by centrifugation. After the removal of PBS, the cells were fixed in ice-cold PBS containing 4% paraformaldehyde. Data were analyzed using CellQuest program following flow cytometry. The percentage of GFP positive cells was defined as the fraction beyond the region of 99.9% of uninfected control cells.

Results

Both scAAV serotypes 2 and 5 can efficiently transduce a variety of human cancer cells. To analyze the transduction efficiency (TE) by scAAVs based on various AAV serotypes, we infected different human cancer cells with scAAV1-6 or 8 expressing green fluorescent protein (GFP). As described in Fig. 1A, the origins of the cancer cells were diverse. Nevertheless, the data indicate that the cells were most efficiently transduced by scAAV2 serotype (Fig. 1B). TE by scAAV2 at multiplicity of infection (MOI) 1000 was marked as high as 98.4 ± 0.9 , 97.9 ± 0.5 or $94.7 \pm 0.4\%$ in SK-Hep-1, HeLa or U251 cells, respectively. The lowest gene transfer by scAAV2 was noticed in HT-29 cells at $31.0 \pm 5.7\%$. The second effective gene transfer was acquired using scAAV5 serotype (Fig. 1D). Similar pattern of TE among cancer cells was observed in the case of scAAV5, except U251 with a substantial decrease in TE by scAAV5. Serotype scAAV1 could only transduce a significant portion of certain cancer cell types, such as SK-Hep1 (Fig. 1C). Other rAAV serotypes showed extremely low TE in all eight human cancer cells (data not shown).

These results suggest that scAAV2 and scAAV5 would be the most effective rAAV gene transfer vectors in various human cancer cells.

Higher transduction efficiency can be achieved by treating cancer cells with higher MOIs of scAAVs. To verify if effective gene delivery could be obtained by treatment of more particles of scAAVs, SK-Hep1 cells were incubated with scAAV2-GFP or scAAV5-GFP at various MOIs. Flow cytometric analysis for both TE and the mean fluorescent intensity (MFI) suggested that higher transgene expression occurred with increasing MOIs (Fig. 2). In the presence of scAAV2, TEs at MOI=10 and 100 were 43.1 and 86.1%, implying that nearly entire cells were transduced at MOI=100 (Fig. 2). Additionally, MFIs by scAAV2 continuously increased from 36.3 at MOI=10 to 157.7 (MOI=100) and 1127.5 (MOI=1000). In case of scAAV5, TE at MOI=10 was relatively low at the level of 2.7%. However, TEs dramatically increased to 18.1% at MOI=100 and 70.2% at MOI=1000. However, MFIs by scAAV5 stay at the similar degree regardless of MOIs. Another type of cancer cells, HeLa cells, showed similar characteristics of transgene expression at identical experimental conditions (data not shown). Together with Fig. 1, the data confirm that scAAV2 can transduce the cells more efficiently than scAAV5. Additionally, the results imply that transgene expression per cell by scAAV can be further intensified by treating more viruses into cells experiencing near 100% TE.

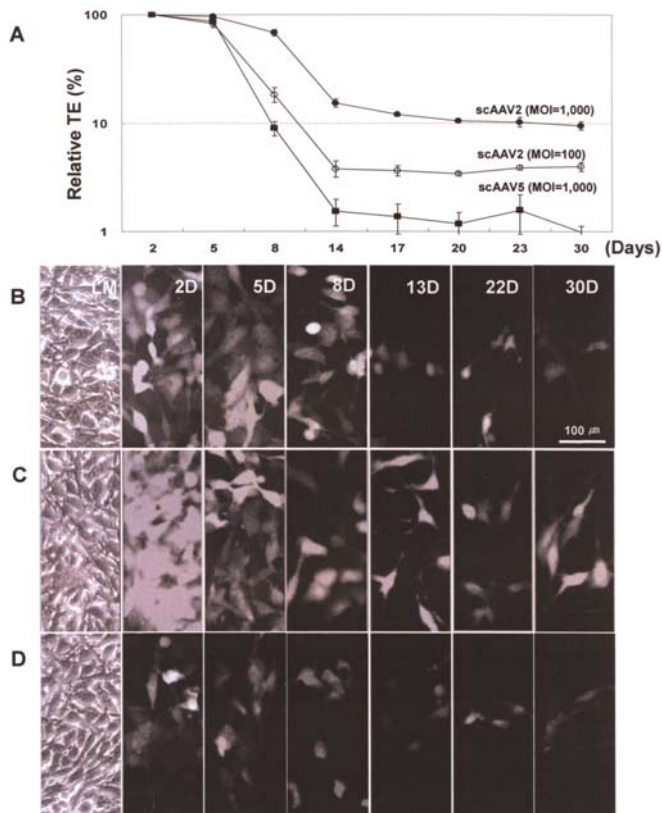


Figure 3. Long-term gene expression by scAAVs in SK-Hep1 cells. Cells were treated with scAAV2-GFP or scAAV5-GFP at MOI=100 or 1000, respectively. The cells were cultivated and GFP signal was analyzed by using both a flow cytometer (A) and a fluorescent microscope at designated times [(B), scAAV2 at MOI 100; (C), scAAV2 at MOI 1000; (D), scAAV5 at MOI 1000; original magnification x200]. The relative TE in A was calculated as follows; TE at n days p.i./TE at 2 days p.i. x 100 (%). The results show that transgene GFP signal persists over time. The experiments were performed three times independently.

A significant portion of transgene expression lasts over time following gene transfer by scAAVs in human cancer cells. The duration of transgene expression by scAAV in human cancer cells was examined by infecting SK-Hep1 cells with either scAAV2- or scAAV5-GFP at 100 or 1000 MOI, respectively. The cells were maintained over 30 days by sub-culturing at the ratio of 1:6 or 1:10 every 2 or 3 days (Fig. 3). GFP signal began to diminish around 5 days post scAAV introduction and sharply reduced within day 14 to $3.8 \pm 0.6\%$ with scAAV2 (MOI=100), $15.3 \pm 1.4\%$ with scAAV2 (MOI=1000), and $1.5 \pm 0.4\%$ with scAAV5 (MOI=1000). Then, the transgene expression remained at similar levels during experimental period. In other human cancer cells including BxPC3 and HeLa cells, the transgene expression retained over time a similar pattern (data not shown). Therefore, the results indicate that scAAV2 and scAAV5 can induce a certain degree of long-term gene expression in human cancer cells.

Gene transfer by scAAV2 and scAAV5 can occur concomitantly in cancer cells. To evaluate the possibility of simultaneous gene expression by different scAAV vectors, SK-Hep1 cells were co-infected with scAAV2-RFP and scAAV5-GFP at MOI=10000 and 5000, respectively. The

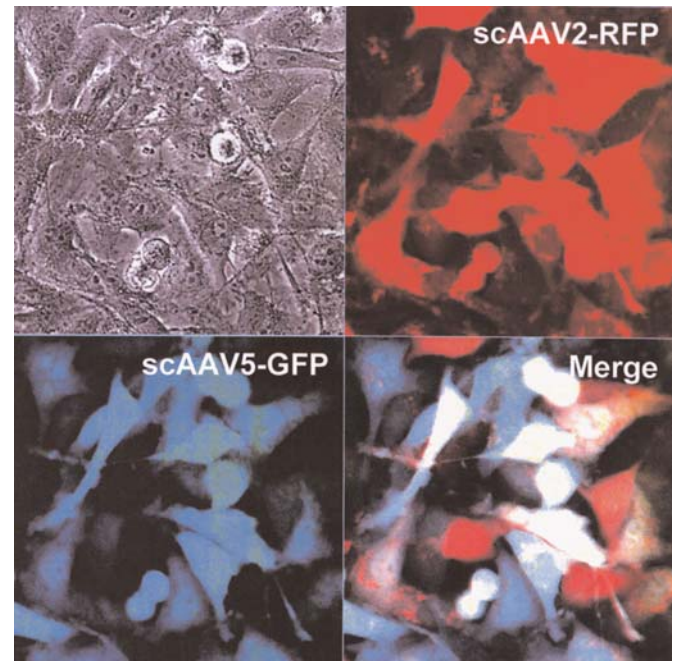


Figure 4. Co-treatment of SK-Hep1 cells with scAAV2-RFP and scAAV5-GFP. SK-Hep1 cells were simultaneously incubated for 72 h with scAAV2-RFP at MOI 10000 and scAAV5-GFP at MOI 5000. GFP and RFP expression was examined under fluorescent microscope. LM, inverted light microscopy. Merge, merged signal of GFP (green) and RFP (red).

scAAV2-RFP is the scAAV2 vector encoding red fluorescent protein (RFP) as a reporter gene. We used 2 times higher MOI of scAAV2-RFP than that of scAAV5-GFP due to less fluorescent intensity of RFP, compared to that of GFP. As shown in Fig. 4, many cells efficiently synthesized both transgene products, which were readily detected under fluorescent microscope. Moreover, GFP and RFP transgene products were co-localized in many cells (Merge; indicated by yellow or orange colours). The data thus demonstrates that scAAV2 and scAAV5 can simultaneously deliver and express different transgenes in the same cell.

Discussion

The present study provides evidence that scAAV2 and scAAV5 among various scAAV serotypes can effectively transduce different human cancer cells regardless of their cell/tissue origins. The significant portions of cells were effectively transduced by scAAV2 or scAAV5 at MOI=1000, even though scAAV2 generally showed the higher TE. In SK-Hep1 and HeLa cells, originated from hepatocellular and cervical carcinoma, TE was found to be the highest. The lowest TE was observed in HT29 colon cancers. Hacker *et al* have also demonstrated that scAAV2 can most effectively transduce several human cancer cells including primary melanoma cells (2). Unlike our data, however, scAAV1 and scAAV3, respectively, induced a superior TE to scAAV5. This discrepancy might be simply caused by the difference of cell lines examined. Nevertheless, more study has to be done to explain the difference, which is crucial to utilizing scAAVs for gene delivery in cancer cells.

Recently, we showed that TE by single-stranded rAAV serotype 2 (ssAAV2) was not high, even in SK-Hep1 or HeLa cells (7). We then demonstrated that TE can be dramatically enhanced by treating the cells with hydroxyurea and tyrphostin-1. Both reagents can confer the conversion of single-stranded rAAV genome to double-stranded form, the essential process for transgene expression by ssAAV2 (2,22-24). Moreover, we observed that the TE by the preformed double-stranded scAAV2 was >10 times higher than that by ssAAV2 with no chemical treatment in HeLa cells (data not shown). Thus, scAAV is more effective at transducing human cancer cells in comparison to ssAAV.

Several *in vivo* studies have indicated that the long-term transgene expression can occur by rAAV vectors in various cells or tissues (6,25,26). Similar to gene transfer by ssAAV, significant portion of infected cancer cells could maintain transgene expression ability by both scAAV2 and scAAV5 (Fig. 3) (7). Previous studies have suggested that long-term gene expression by ssAAV is due to either the integration of rAAV genome to chromosome (27,28) or genomic persistence in its episomal conformation (29-31). Even though ssAAV vectors preferentially integrate into actively transcribed regions (32), the terminal repeats in each end of the rAAV vector have minimal promoter activity, unlike that of retroviruses characterized by strong promoter activity (33). More importantly, there is no evidence indicating that the rAAV vector can cause cancers *in vivo* (34,35). Finally, rAAV vectors have been proven as the safe gene transfer vehicle by several clinical trials, such as for cystic fibrosis or Canavan disease treatment (36,37). Collectively, this suggests that scAAV vectors could effectively and safely express a gene of interest at a long-term level in human cancer cells.

The present study also demonstrates that independent co-transduction can be readily achieved utilizing scAAV2 and scAAV5 at the same time. The expression profile of rAAV receptor on cell surface is deeply involved in TE by rAAV, similar to other recombinant virus-based vectors. scAAV2 binds to heparan sulfate proteoglycans (38) and other co-receptors (39-42), while scAAV5 binds to 2,3-N-linked sialic acid of the cell surface (43,44) and platelet-derived growth factor receptor (45,46). Thus, the co-expression of different cancer therapeutic genes by concomitant infection of scAAV2 and scAAV5 might be easily obtained for combined gene therapy. One of the major limitation using rAAV2 is the presence of pre-existing neutralizing antibodies against AAV2 in the general human population (47-50). By contrast, the prevalence of neutralizing antibody against AAV5 has been observed at low levels, particularly in cystic fibrosis patients (51-53). Furthermore, Kuck *et al* showed that pre-existing immune response against rAAV2 may have no influence on the transduction of rAAV5 vectors in mouse studies (54). Taken together, scAAV5 serotype holds a promising potential of avoiding the adverse immune responses against rAAV2-based gene therapy. Thus, scAAV5 vectors can be used simultaneously with scAAV2 vector for combined treatment or independently to avoid unwanted host immune response.

A body of recent studies have showed anti-tumor efficacy by rAAV2 encoding various anti-cancer transgenes, such as tissue inhibitor of metalloproteinase-1 (55), mutant endo-

statin (26), and nm23H1 (56) or VEGFR neutralizing antibody (57). In consideration for current rAAV-based cancer gene therapies, the present study provides the rationale that scAAV2 vectors in combination with scAAV5 vectors may improve anti-tumor efficacy for successful cancer gene therapy.

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References

- Li C, Bowles DE, van Dyke T and Samulski RJ: Adeno-associated virus vectors: Potential applications for cancer gene therapy. *Cancer Gene Ther* 12: 913-925, 2005.
- Hacker UT, Wingenfeld L, Kofler DM, *et al*: Adeno-associated virus serotypes 1-5 mediated tumor cell directed gene transfer and improvement of transduction efficiency. *J Gene Med* 7: 1429-1438, 2005.
- Berns KI, Muzyczka N, Knipe DM and Howley PM: *Field's virology*. Vol. 2, 4th edition. Lippincott-Raven, Philadelphia, PA, pp2327-2359, 2001.
- Kotin RM, Siniscalco M, Samulski RJ, *et al*: Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA* 87: 2211-2215, 1990.
- Rivadeneira ED, Popescu NC, Zimonjic DB, *et al*: Sites of recombinant adeno-associated virus integration. *Int J Oncol* 12: 805-810, 1998.
- Watanabe M, Nasu Y, Kashiwakura Y, *et al*: Adeno-associated virus 2-mediated intratumoral prostate cancer gene therapy: long-term maspin expression efficiently suppresses tumor growth. *Hum Gene Ther* 16: 699-710, 2005.
- Kim SJ, Nam YR, Shin O, *et al*: Treatment with hydroxyurea and tyrphostin-1 significantly improves the transduction efficiency of recombinant adeno-associated viruses in human cancer cells. *Oncol Rep* 14: 1475-1479, 2005.
- McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P and Samulski RJ: Adeno-associated virus terminal repeat (tr) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo*. *Gene Ther* 10: 2112-2118, 2003.
- McCarty DM, Monahan PE and Samulski RJ: Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther* 8: 1248-1254, 2001.
- Wang Z, Ma HI, Li J, Sun L, Zhang J and Xiao X: Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors *in vitro* and *in vivo*. *Gene Ther* 10: 2105-2111, 2003.
- Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J and Wilson JM: Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA* 99: 11854-11859, 2002.
- Gao G, Vandenberghe LH and Wilson JM: New recombinant serotypes of AAV vectors. *Curr Gene Ther* 5: 285-297, 2005.
- Grimm D and Kay MA: From virus evolution to vector revolution: use of naturally occurring serotypes of adeno-associated virus (AAV) as novel vectors for human gene therapy. *Curr Gene Ther* 3: 281-304, 2003.
- Blankinship MJ, Gregorevic P, Allen JM, *et al*: Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6. *Mol Ther* 10: 671-678, 2004.
- Louboutin JP, Wang L and Wilson JM: Gene transfer into skeletal muscle using novel AAV serotypes. *J Gene Med* 7: 442-451, 2005.
- Su H, Huang Y, Takagawa J, *et al*: Aav serotype-1 mediates early onset of gene expression in mouse hearts and results in better therapeutic effect. *Gene Ther* 13: 1495-1502, 2006.
- Wang Z, Zhu T, Qiao C, *et al*: Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat Biotechnol* 23: 321-328, 2005.
- Chen S, Kapturczak M, Loiler SA, *et al*: Efficient transduction of vascular endothelial cells with recombinant adeno-associated virus serotype 1 and 5 vectors. *Hum Gene Ther* 16: 235-247, 2005.

19. Song JJ, Kim JH, Lee H, *et al*: Enhancement of gene transfer efficiency into human cancer cells by modification of retroviral vectors and addition of chemicals. *Oncol Rep* 7: 119-124, 2000.
20. Nam YR, Kim SJ, Lee B, *et al*: Analysis of the production efficiency and titration of various recombinant adeno-associated viruses. *Oncol Rep* 12: 761-766, 2004.
21. Moon MS, Joo CH, Hwang IS, *et al*: Distribution of viral rna in mouse tissues during acute phase of coxsackievirus b5 infection. *Intervirology* 48: 153-160, 2005.
22. Russell DW, Alexander IE and Miller AD: DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. *Proc Natl Acad Sci USA* 92: 5719-5723, 1995.
23. Smith AD, Collaco RF and Trempe JP: Enhancement of recombinant adeno-associated virus type 2-mediated transgene expression in a lung epithelial cell line by inhibition of the epidermal growth factor receptor. *J Virol* 77: 6394-6404, 2003.
24. Douar AM, Poulard K, Stockholm D and Danos O: Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. *J Virol* 75: 1824-1833, 2001.
25. Mochizuki S, Mizukami H, Ogura T, *et al*: Long-term correction of hyperphenylalaninemia by AAV-mediated gene transfer leads to behavioral recovery in phenylketonuria mice. *Gene Ther* 11: 1081-1086, 2004.
26. Subramanian IV, Bui Nguyen TM, Truskinovsky AM, Tolar J, Blazar BR and Ramakrishnan S: Adeno-associated virus-mediated delivery of a mutant endostatin in combination with carboplatin treatment inhibits orthotopic growth of ovarian cancer and improves long-term survival. *Cancer Res* 66: 4319-4328, 2006.
27. Duan D, Fisher KJ, Burda JF and Engelhardt JF: Structural and functional heterogeneity of integrated recombinant AAV genomes. *Virus Res* 48: 41-56, 1997.
28. Miller DG, Rutledge EA and Russell DW: Chromosomal effects of adeno-associated virus vector integration. *Nat Genet* 30: 147-148, 2002.
29. Snyder RO, Spratt SK, Lagarde C, *et al*: Efficient and stable adeno-associated virus-mediated transduction in the skeletal muscle of adult immunocompetent mice. *Hum Gene Ther* 8: 1891-1900, 1997.
30. Fisher KJ, Jooss K, Alston J, *et al*: Recombinant adeno-associated virus for muscle directed gene therapy. *Nat Med* 3: 306-312, 1997.
31. Schnepf BC, Jensen RL, Chen CL, Johnson PR and Clark KR: Characterization of adeno-associated virus genomes isolated from human tissues. *J Virol* 79: 14793-14803, 2005.
32. Russell DW: AAV loves an active genome. *Nat Genet* 34: 241-242, 2003.
33. Haberman RP, McCown TJ and Samulski RJ: Novel transcriptional regulatory signals in the adeno-associated virus terminal repeat A/D junction element. *J Virol* 74: 8732-8739, 2000.
34. Bell P, Wang L, Lebherz C, *et al*: No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. *Mol Ther* 12: 299-306, 2005.
35. Kay MA and Nakai H: Looking into the safety of AAV vectors. *Nature* 424: 251, 2003.
36. Wagner JA, Nepomuceno IB, Messner AH, *et al*: A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrastomies. *Hum Gene Ther* 13: 1349-1359, 2002.
37. McPhee SW, Janson CG, Li C, *et al*: Immune responses to AAV in a phase I study for Canavan disease. *J Gene Med* 8: 577-588, 2006.
38. Summerford C and Samulski RJ: Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 72: 1438-1445, 1998.
39. Qing K, Mah C, Hansen J, Zhou S, Dwarki V and Srivastava A: Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat Med* 5: 71-77, 1999.
40. Summerford C, Bartlett JS and Samulski RJ: Alphavbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med* 5: 78-82, 1999.
41. Kashiwakura Y, Tamayose K, Iwabuchi K, *et al*: Hepatocyte growth factor receptor is a coreceptor for adeno-associated virus type 2 infection. *J Virol* 79: 609-614, 2005.
42. Akache B, Grimm D, Pandey K, Yant SR, Xu H and Kay MA: The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3 and 9. *J Virol* 80: 9831-9836, 2006.
43. Kaludov N, Brown KE, Walters RW, Zabner J and Chiorini JA: Adeno-associated virus serotype 4 (aav4) and aav5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *J Virol* 75: 6884-6893, 2001.
44. Walters RW, Yi SM, Keshavjee S, *et al*: Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. *J Biol Chem* 276: 20610-20616, 2001.
45. Di Pasquale G, Davidson BL, Stein CS, *et al*: Identification of PDGFR as a receptor for AAV-5 transduction. *Nat Med* 9: 1306-1312, 2003.
46. Ding W, Zhang L, Yan Z and Engelhardt JF: Intracellular trafficking of adeno-associated viral vectors. *Gene Ther* 12: 873-880, 2005.
47. Blacklow NR, Hoggan MD, Sereno MS, *et al*: A seroepidemiologic study of adenovirus-associated virus infection in infants and children. *Am J Epidemiol* 94: 359-366, 1971.
48. Blacklow NR, Hoggan MD and Rowe WP: Serologic evidence for human infection with adenovirus-associated viruses. *J Natl Cancer Inst* 40: 319-327, 1968.
49. Erles K, Sebkova P and Schlehofer JR: Update on the prevalence of serum antibodies (igg and igm) to adeno-associated virus (aav). *J Med Virol* 59: 406-411, 1999.
50. Chirmule N, Probert K, Magosin S, Qian Y, Qian R and Wilson J: Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther* 6: 1574-1583, 1999.
51. Tobiasch E, Burguete T, Klein-Bauernschmitt P, Heilbronn R and Schlehofer JR: Discrimination between different types of human adeno-associated viruses in clinical samples by pcr. *J Virol Methods* 71: 17-25, 1998.
52. Halbert CL, Miller AD, McNamara S, *et al*: Prevalence of neutralizing antibodies against adeno-associated virus (AAV) types 2, 5 and 6 in cystic fibrosis and normal populations: implications for gene therapy using aav vectors. *Hum Gene Ther* 17: 440-447, 2006.
53. Hildinger M, Auricchio A, Gao G, Wang L, Chirmule N and Wilson JM: Hybrid vectors based on adeno-associated virus serotypes 2 and 5 for muscle-directed gene transfer. *J Virol* 75: 6199-6203, 2001.
54. Kuck D, Lau T, Leuchs B, *et al*: Intranasal vaccination with recombinant adeno-associated virus type 5 against human papillomavirus type 16 11. *J Virol* 80: 2621-2630, 2006.
55. Zacchigna S, Zentilin L, Morini M, *et al*: Aav-mediated gene transfer of tissue inhibitor of metalloproteinases-1 inhibits vascular tumor growth and angiogenesis *in vivo*. *Cancer Gene Ther* 11: 73-80, 2004.
56. Li J, Zhou J, Chen G, *et al*: Inhibition of ovarian cancer metastasis by adeno-associated virus-mediated gene transfer of nm23h1 in an orthotopic implantation model. *Cancer Gene Ther* 13: 266-272, 2006.
57. Fang J, Qian JJ, Yi S, *et al*: Stable antibody expression at therapeutic levels using the 2A peptide. *Nat Biotechnol* 23: 584-590, 2005.