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SHORT COMMUNICATION

Strategies for improving the transduction efficiency of single-stranded adeno-associated virus vectors in vitro *and* in vivo

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Recombinant vectors based on adeno-associated virus type 2 (AAV) target the liver efficiently, but the transgene expression is limited to ~5% of murine hepatocytes. Viral second-strand DNA synthesis continues to be a rate-limiting step for efficient transduction by the single-stranded AAV (ssAAV) vectors. This is due, in part, to the presence of a cellular chaperone (FK506-binding) protein, FKBP52, phosphorylated forms of which interact with the D-sequence in the inverted terminal repeats of AAV2 genome and inhibit the viral second-strand DNA synthesis. Our previous studies have documented that dephosphorylation of FKBP52 at tyrosine residues by the cellular T-cell protein tyrosine phosphatase (TC-PTP), and at serine/threonine residues by protein phosphatase 5 (PP5) enhances viral second-strand DNA synthesis and consequently, the transgene expression. We have also reported that coinfection with a self-complementary AAV (scAAV)-TC-PTP vector results in up to sixfold increase in the transduction efficiency of conventional ssAAV2 vectors in primary murine hepatocytes in vivo*. We reasoned that coinfection with scAAV-TC-PTP and scAAV-PP5 vectors may lead to a further increase in the transduction efficiency of ssAAV2 vectors. We demonstrate here that this strategy does indeed lead to ~16-fold increase in the transduction efficiency of conventional ssAAV vectors in primary murine hepatocytes* in vivo *following tail-vein injections. Neither scAAV2-TC-PTP nor scAAV2-PP5 vectors alone or together had any adverse effect on the hepatocytes. Thus, this coinfection strategy may be useful for achieving expression from recombinant ssAAV2 vectors containing larger genes, such as coagulation factor VIII, which exceed the packaging capacity of scAAV vectors, for the potential gene therapy of hemophilia A.*
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Adeno-associated virus 2 (AAV), a nonpathogenic human parvovirus, has gained attention as a potentially safe vector for gene transfer and gene therapy.[1,2] The AAV genome is a single-stranded DNA, which is transcriptionally inactive.[3,4] Thus, failure to undergo viral second-strand synthesis remains a predominant rate-limiting step in the observed low efficiency of single-stranded AAV (ssAAV) vector-mediated transgene expression both *in vitro* and *in vivo*.[5–14] The use of self-complementary AAV (scAAV) vectors that bypass the requirement for viral second-strand DNA synthesis can circumvent this problem.[13] However, their widespread use is limited by their limited packaging capacity (~3.3 kb),[15] which is significantly less than that of conventional ssAAV vectors (~6 kb).[16] It is therefore important to design strategies to improve the transduction efficiency of conventional ssAAV vectors. In our previous studies, we have identified a 52-kDa cellular protein, FKBP52, that interacts specifically with the D-sequence within the inverted terminal repeat of the AAV genome.[10,17] FKBP52 is phosphorylated at both tyrosine (*Tyr*) and serine or threonine (*Ser/Thr*) residues. *Ser/Thr-or Tyr*-phosphorylated forms of FKBP52 inhibit the viral second-strand DNA synthesis by ~40 and ~90%, respectively, leading to inefficient transgene expression.[8,9,11,17–19] We have also documented that FKBP52 is dephosphorylated at *Tyr* residues by the cellular T-cell protein tyrosine phosphatase (TC-PTP), and at *Ser/Thr* residues by protein phosphatase 5 (PP5).[9,12] Dephosphorylated FKBP52 can no longer bind to the D-sequence, which in turn, facilitates the viral second-strand DNA synthesis, and augments the transgene expression from ssAAV2 vectors. We have demonstrated augmentation in ssAAV vector-mediated transgene expression in transgenic mice overexpressing TC-PTP,[9] and in mice deficient in FKBP52.[18] Similarly, delivery of

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TC-PTP via a scAAV vector, or overexpression of PP5 following stable transfection, also led to an increase in transgene expression from ssAAV vectors. However, the observed increases were only modest, approximately 5- to 6-fold.[11,12]

In an attempt to further augment the transduction efficiency of ssAAV vectors, we reasoned that coinfection with AAV-TC-PTP and AAV-PP5 vectors together may exert, at the very least, an additive effect. As both TC-PTP and PP5 cDNA sizes (~2.5 kb) are within the packaging capacity of scAAV vectors[15] and AAV2 remains the best characterized serotype for gene transfer protocols,[20,21] we examined whether coinfection with scAAV2-TC-PTP+scAAV2-PP5 vectors might augment the transduction efficiency of conventional ssAAV2 vectors. We reasoned that rapid and simultaneous expression of TC-PTP and PP5 from scAAV vectors would lead to complete dephosphorylation of FKBP52 at *Tyr* and *Ser/Thr* residues, resulting in a more efficient viral second-strand DNA synthesis and augmentation of the transduction efficiency of conventional ssAAV2 vectors. This strategy is schematically represented in Figure 1. Tyrosine- and serine/threonine-phosphorylated forms of FKBP52 block viral second-strand DNA synthesis resulting in poor transgene expression from an ssAAV vector (panel a). However, if scAAV-TC-PTP and scAAV-PP5 vectors are admixed with a conventional ssAAV vector prior to transduction, expression of TC-PTP and PP5 from the scAAV vectors, which would not require viral second-strand DNA synthesis, would cause dephosphorylation of FKBP52 at both tyrosine and serine/threonine residues. This would lead to a more efficient second-strand DNA synthesis of the ssAAV vector resulting in high-efficiency transgene expression (panel b).

We first tested this hypothesis in transient transfection assays using lipofectamine (Lipofectamine2000, Invitrogen, Carlsbad, CA, USA) and TC-PTP and/or PP5 cDNA expression plasmids in three different established human cell lines known to contain varied levels of *Tyr* or *Ser/Thr* forms of cellular FKBP52[8] *in vitro*. HeLa (human cervical carcinoma), 293 (adenovirus-transformed human embryonic kidney) and KB (human nasopharyngeal carcinoma) cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained as monolayer cultures in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% newborn calf serum (NCS) and 1% (by volume) of 100% stock solution of antibiotics (10 000 U penicillin+10 000 μg streptomycin). Highly purified stocks of a recombinant ssAAV2 vector containing the enhanced green fluorescent protein reporter gene driven by the cytomegalovirus promoter (CMVp-EGFP) were purchased from Virapur LLC (San Diego, CA, USA). Recombinant expression plasmids containing the Rous sarcoma virus (RSV) promoter-driven murine TC-PTP, and human PP5 cDNAs were generously provided by Dr Michel Tremblay (McGill University, Montreal, QC, Canada) and Dr David J Chen and Dr Benjamin PC Chen (UT Southwestern Medical Center at Dallas, TX, USA). Approximately 5×10^5 HeLa, 293 or KB cells were plated in each well in six-well plates and incubated at 37 °C for 12 h. Cells were transfected with 4 μg of recombinant TC-PTP and/or PP5 expression plasmids. After 24 h, cells were washed once with IMDM and then either mock infected or infected at 37 °C for 2 h with 1500 particles per cell of a recombinant AAV2-EGFP vector as described previously.[17] Cells were incubated in complete IMDM containing 10% NCS and 1% antibiotics for 48 h. The transduction efficiency of ssAAV2 was measured by EGFP imaging using an Axiovert 25

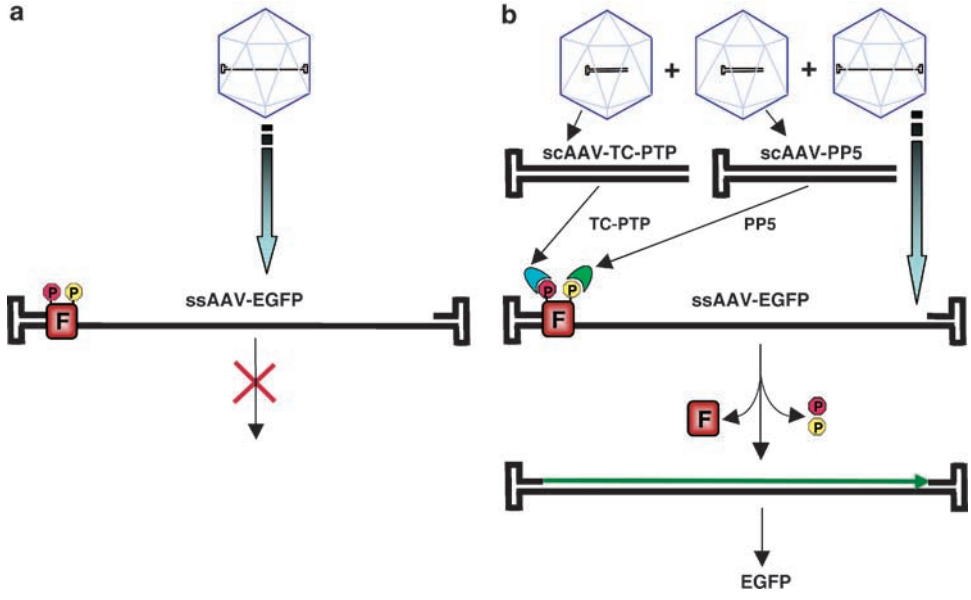

Figure 1 Schematic representation of failure of single-stranded adeno-associated virus (ssAAV) vectors to undergo viral second-strand DNA synthesis due to the presence of tyrosine- and serine/threonine-phosphorylated forms of FKBP52 (**a**). Strategy to augment the second-strand DNA synthesis of a conventional ssAAV vector by coinfection with scAAV-TC-PTP and scAAV-PP5 vectors (**b**). FKBP52 (F), phosphorylated at tyrosine residues (red symbol) and serine/threonine (yellow symbol), which strongly inhibits the second-strand DNA synthesis of a conventional ssAAV vector, is dephosphorylated at tyrosine residues by T-cell protein tyrosine phosphatase (TC-PTP; blue semi-oval), and at serine/threonine residues by protein phosphatase 5 (PP5; green semi-oval) expressed from scAAV-TC-PTP and scAAV-PP5 vectors, which allows more efficient viral second-strand DNA synthesis of conventional ssAAV vector, and consequently, leads to more efficient transgene expression.

fluorescence microscope (Carl Zeiss Inc., Thornwood, NY, USA). Images from five visual fields of mock-infected and vector-infected cells were analyzed quantitatively by ImageJ analysis software (NIH, Bethesda, MD, USA). Transgene expression (mean value) was assessed as total area of green fluorescence (pixel^2) per visual field. These results are shown in Figure 2a. Compared with control cells infected with ssAAV2-EGFP vector alone, the transduction efficiency in three different cell types was augmented by approximately 1.7- to 3.6-fold when TC-PTP alone was overexpressed, and by approximately 2- to 3.5-fold when PP5 alone was overexpressed. These findings were comparable with our previous studies, where we observed approximately fivefold increase in transduction efficiency of ssAAV2 vectors in 293 cells stably transfected with a human PP5 expression plasmid.[12] Consistent with our hypothesis, we observed an additive effect in the increase in EGFP expression in HeLa, KB and 293 cells when both TC-PTP and PP5 expression plasmids were cotransfected (Figure 2b).

In anticipation of extending these studies to an *in vivo* animal model, we next wished to deliver TC-PTP and PP5 genes via scAAV vectors. We envisaged that rapid expression of TC-PTP and PP5 from these vectors would lead to complete dephosphorylation of FKBP52 resulting in efficient second-strand synthesis of a conventional ssAAV vector. The scAAV2 plasmids containing the RSV promoter-driven murine TC-PTP (scAAV2-TC-PTP) and the RSV promoter-driven human PP5 (scAAV2-PP5) were constructed by standard cloning methods as described previously.[11,12] Highly purified stocks of recombinant scAAV2-TC-PTP and scAAV2-PP5 vectors were generated by the triple-plasmid transfection protocol as described previously.[22] The physical particle titers of recombinant vector stocks were determined by quantitative DNA slot-blot analyses.[23] Approximately 5×10^5 HeLa, 293 or KB cells were plated in each well in six-well plates and incubated at 37 °C for 12 h. Cells were washed once with IMDM and then either mock infected or infected at 37 °C for 1 h with 2500 particles per cell of recombinant scAAV2-TC-PTP and/or scAAV2-PP5 vectors followed by infection with 5000 particles per cell of a recombinant ssAAV2-EGFP vector. Cells were incubated in complete IMDM containing 10% NCS and 1%

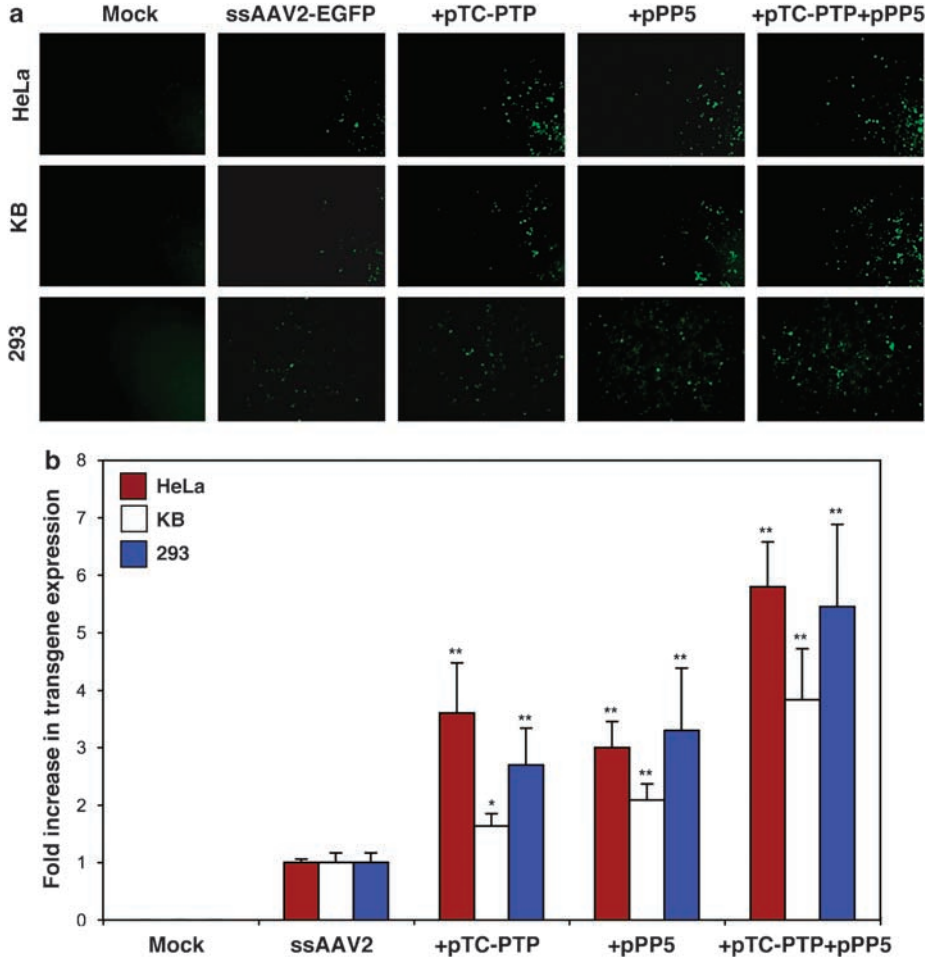

Figure 2 Comparative analysis of single-stranded adeno-associated virus (ssAAV) vector-mediated transgene expression in HeLa, KB and 293 cells following lipofectamine-mediated transient transfection with T-cell protein tyrosine phosphatase (TC-PTP) and/or protein phosphatase 5 (PP5) expression plasmids. (**a**) Transgene expression was detected by fluorescence microscopy 48 h post-infection. Representative images are shown. Original magnification, ×50. (**b**) Quantitative analyses of the data from (**a**). Images from five visual fields were analyzed quantitatively by ImageJ analysis software. Transgene expression (mean value) was assessed as total area of green fluorescence (pixel^2) per visual field. Analysis of variance (ANOVA) was used to compare test results with the control enhanced green fluorescent protein (EGFP) expression. *$P<0.05$, **$P<0.01$.

antibiotics. Forty-eight hours post-infection, cells were visualized under a fluorescence microscope. Whereas mock-infected HeLa, KB and 293 cells showed no EGFP expression, only a few cells transduced with ssAAV2-EGFP vector had green fluorescence (Figure 3a). When cells were coinfected with scAAV2-TC-PTP or scAAV2-PP5 vectors, up to fourfold increase in the transduction efficiency was observed, which is similar to approximately sixfold increase observed in the transgene expression from ssAAV2 in our previous study.[11] Interestingly, HeLa cells showed the highest fold (fourfold) increase in transgene expression when coinfected with scAAV2-TC-PTP; in 293 cells this increase (3.6-fold) was highest when scAAV2-PP5 was coinfected (Figure 3b). These data correlate well with our previous studies that showed that FKBP52 is phosphorylated predominantly at *Tyr* residues in HeLa cells, whereas it is phosphorylated predominantly at *Ser/Thr* residues in 293 cells.[7,8,11,12] Coinfection of both scAAV2-TC-PTP and scAAV2-PP5 vectors, further augmented the transduction efficiency by approximately 5- to 7-fold in all three cell types (Figure 3b). These results demonstrate that recombinant scAAV2-TC-PTP and scAAV2-PP5 vectors together as helper viruses can additively augment the transduction efficiency of ssAAV2-EGFP vectors.

We also wished to evaluate the potential efficacy of scAAV2-TC-PTP and scAAV2-PP5 vectors as helper viruses, alone or in combination, in murine hepatocytes *in vivo*. Approximately 5×10^{10} physical particles of ssAAV2-EGFP vectors alone, or those admixed with scAAV2-TC-PTP and/or scAAV2-PP5 vectors at a 1:1 ratio, were injected into C57BL/6 mice intravenously via the tail vein. Phosphate-buffered saline (PBS)-injected mice were used as an appropriate control. Liver sections from three hepatic lobes of the PBS-injected and vector-injected mice 2 weeks after injection were examined for the transduction efficiency of the ssAAV2-EGFP vector. Consistent with previously published studies,[11,24,25–29] little green fluorescence occurred in hepatocytes 2 weeks after injection of conventional ssAAV2-EGFP alone (Figure 4a); however, coinjection with scAAV2-TC-PTP or scAAV2-PP5 vectors led to approximately 6- and 9-fold increase in the transduction of ssAAV2-EGFP vector, respectively (Figure 4b). This difference in helper function between TC-PTP and PP5 may be due to the fact that in hepatocytes, FKBP52 is present predominantly

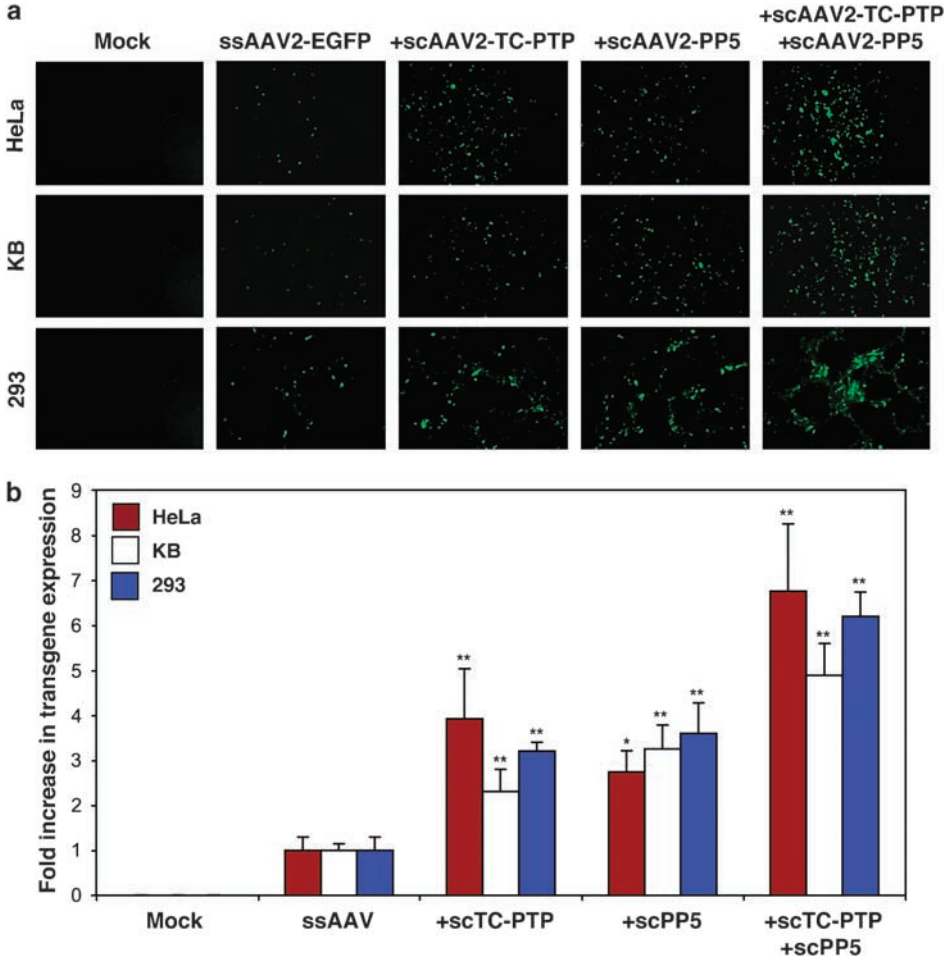

Figure 3 Comparative analysis of single-stranded adeno-associated virus (ssAAV) vector-mediated transgene expression in HeLa, KB and 293 cells following transduction with ssAAV2-EGFP vector alone, or with coinfection with scAAV2-TC-PTP and/or scAAV2-PP5 vectors. (**a**) Transgene expression was detected by fluorescence microscopy 48 h post-infection. Representative images are shown. Original magnification, $\times 50$. (**b**) Quantitative analyses of the data from (**a**). Transgene expression was assessed as described in the legend to Figure 1. $^{*}P < 0.05$, $^{**}P < 0.01$.

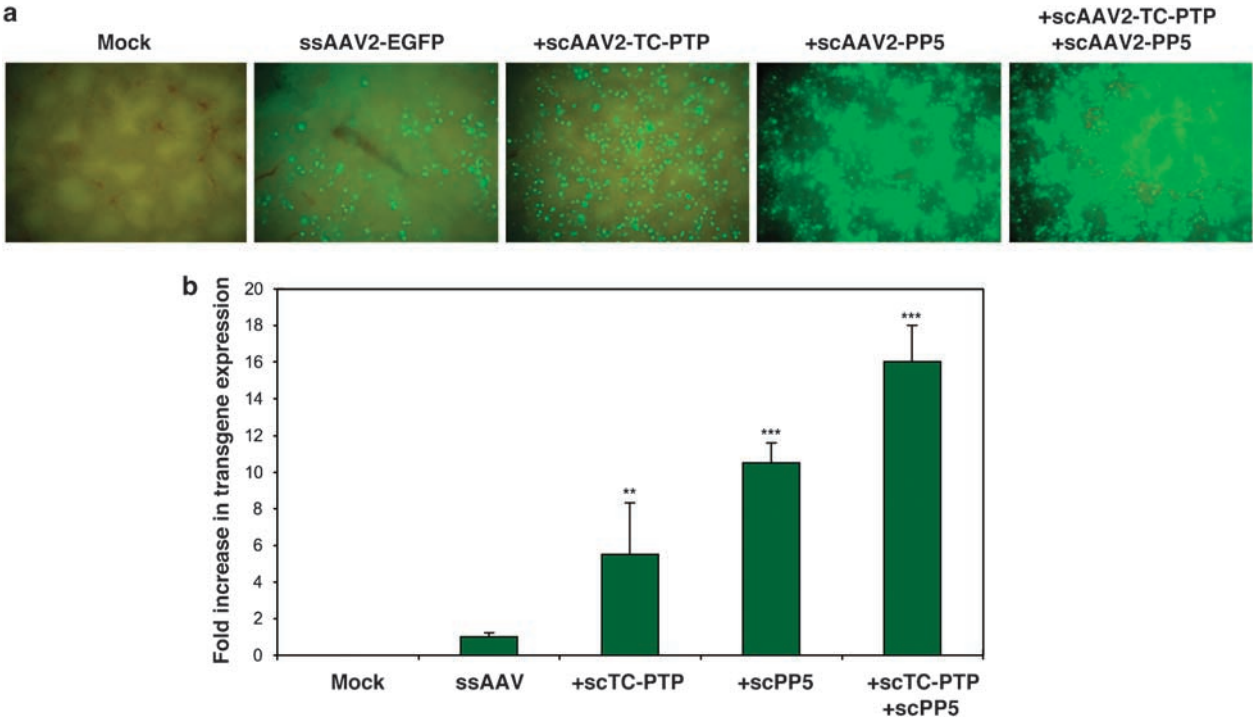

Figure 4 Single-stranded adeno-associated virus (ssAAV) vector-mediated transduction of hepatocytes in normal C57BL/6 mice injected with ssAAV2-EGFP vector alone, or coinjected with either scAAV2-TC-PTP and/or scAAV2-PP5 vectors. (**a**) Transgene expression was detected by fluorescence microscopy 2 weeks post-injection of 5×10^{10} ssAAV2-EGFP vector particles/animal and coinjection of 5×10^{10} particles each of scAAV2-TC-PTP and/or scAAV2-PP5 vectors/animal via the tail vein. Representative images are shown. (**b**) Quantitative analyses of the data from (**a**). Transgene expression was assessed as described in the legend to Figure 1. $^{**}P < 0.01$, $^{***}P < 0.001$.

phosphorylated at *Ser/Thr* residues. Coinjection of scAAV2-TC-PTP and scAAV2-PP5 vectors together resulted in an ~16-fold increase in the ssAAV2-EGFP vector transduction efficiency, further corroborating our hypothesis that coadministration of scAAV2-TC-PTP and scAAV-PP5 vectors can substantially increase the transduction efficiency of an ssAAV vector. The injection of scAAV2-TC-PTP and/or scAAV2-PP5 vectors did not lead to major histological abnormalities in the liver. Both PBS- and the helper virus-injected groups were grossly normal. The liver tissue from all PBS- or helper virus-injected animals had no evidence of any toxicity or any pathological lesions, as examined by an experienced pathologist certified by the American College of Veterinary Pathologists. A set of representative data shown in Figure 5 corroborates that TC-PTP and PP5 over expression was nontoxic in murine hepatocytes.

Although it is conceivable that complete dephosphorylation of FKBP52 might be achievable by biochemical means, and to our knowledge, specific inhibitors of epidermal growth factor receptor protein tyrosine kinase, such as Tyrphostin 23 or gefitinib are available.[30,31] However Ser/Thr kinase inhibitors are rare.[32,33] As these drugs are either in the developmental phase or are less efficacious,[34] and might even be toxic, the vector coadministration strategy presented here is an attractive alternative for gene therapy. However, it remains to be seen whether this strategy would be useful for an ssAAV vector carrying a therapeutic gene in a human trial. It is, nonetheless, tempting to speculate that at the very least, the therapeutic vector dosage could be reduced by a log to overcome problems associated with vector load and

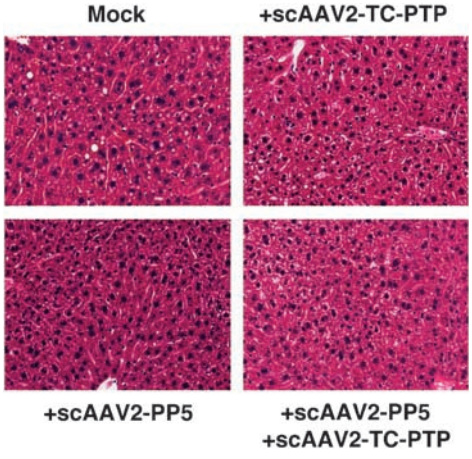

Figure 5 Morphological analysis of murine hepatocytes 2 weeks post-injection of either phosphate-buffered saline (PBS) or 5×10^{10} ssAAV2-EGFP vector particles/animal with coinjection of 5×10^{10} particles each of scAAV2-TC-PTP and/or scAAV2-PP5 vectors/animal. Liver sections were stained with hematoxylin eosin (HE), and visualized under a light microscope. Original magnification, $\times 400$.

immune response seen in clinical trials.[35] Furthermore, given that AAV2 remains the sole serotype vector currently in use in human gene therapy,[20,21,36–40] coupled with the fact that it is also the best characterized in terms of vector toxicology, it is conceivable that this strategy could be employed to augment transgene expression

from single-stranded vectors containing large genes such as coagulation factor VIII in the liver in clinical trials in patients with hemophilia A. In our current studies, the TC-PTP and the PP5 genes were under the control of the RSV promoter. It is likely that by using a hepatocyte-specific promoter, such as the transthyretin promoter,[41] efficacious helper functions could be achieved at reduced helper virus doses. Similarly, the use of serotypes other than AAV2 might also allow a further reduction in the required doses of scAAV-TC-PTP and scAAV-PP5 helper viruses. The apparent lack of toxicity of scAAV2-TC-PTP and scAAV2-PP5 vectors, at least in experimental mice, suggests that this approach might also be safe. This is further supported by the fact that toxicological studies performed previously in normal C57BL/6 mice transduced with a scAAV2-TC-PTP vector showed no evidence of toxicity up to 13 weeks;[11] deliberate overexpression of TC-PTP was not deleterious in transgenic mice for up to more than 1.5 years of age;[9,18] and overexpression of PP5 did not affect cellular growth.[12] The development of PP5-transgenic, and TC-PTP/PP5-bigenic mice, currently underway, should yield information on the safety profile as well as on the underlying mechanism of enhanced transduction of ssAAV2 vectors by scAAV-TC-PTP and scAAV-PP5 helper viruses.

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