

High-Efficiency Transduction of Fibroblasts and Mesenchymal Stem Cells by Tyrosine-Mutant AAV2 Vectors for Their Potential Use in Cellular Therapy

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Abstract

Adeno-associated virus 2 (AAV2) vectors transduce fibroblasts and mesenchymal stem cells (MSCs) inefficiently, which limits their potential widespread applicability in combinatorial gene and cell therapy. We have reported that AAV2 vectors fail to traffic efficiently to the nucleus in murine fibroblasts. We have also reported that site-directed mutagenesis of surface-exposed tyrosine residues on viral capsids leads to improved intracellular trafficking of the mutant vectors, and the transduction efficiency of the single tyrosine-mutant vectors is ~10-fold higher in human cells. In the current studies, we evaluated the transduction efficiency of single as well as multiple tyrosine-mutant AAV2 vectors in murine fibroblasts. Our results indicate that the Y444F mutant vectors transduce these cells most efficiently among the seven single-mutant vectors, with >30-fold increase in transgene expression compared with the wild-type vectors. When the Y444F mutation is combined with additional mutations (Y500F and Y730F), the transduction efficiency of the triple-mutant vectors is increased by ~130-fold and the viral intracellular trafficking is also significantly improved. Similarly, the triple-mutant vectors are capable of transducing up to 80–90% of bone marrow-derived primary murine as well as human MSCs. Thus, high-efficiency transduction of fibroblasts with reprogramming genes to generate induced pluripotent stem cells, and the MSCs for delivering therapeutic genes, should now be feasible with the tyrosine-mutant AAV vectors.

Overview Summary

Fibroblasts and mesenchymal stem cells (MSCs) are promising targets for gene and cell therapy. Recombinant adeno-associated virus 2 (AAV2) vectors are currently in use in several Phase I/II clinical trials for gene therapy. However, the existing data show that AAV2 vector-mediated transduction of fibroblasts and MSCs is inefficient. We observed that AAV2 vectors containing mutations in three surface-exposed tyrosine residues significantly increase transduction efficiency in fibroblasts, as well as in both human and murine primary MSCs. The increased transduction efficiency of these triple-mutant AAV2 vectors correlates well with improved viral intracellular trafficking in fibroblasts. These data provide strong evidence that high-efficiency gene delivery and transgene expression by AAV vectors are indeed feasible, which should facilitate the generation of induced pluripotent stem cells, as well as the use of MSCs in combinatorial gene and cell therapy.

Introduction

MESENCHYMAL STEM CELLS (MSCs) and fibroblasts are attractive targets for gene and cell therapies of in-

herited and acquired disorders. MSCs are multipotent, non-hematopoietic stem cells capable of self-renewal and can be used as a means of delivering genes to repair or regenerate damaged or diseased tissues and organs (Reiser *et al.*, 2005).

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Thus, the use of viral vectors for MSC gene transfer has gained prominence, with retroviral and lentiviral vectors being the most rigorously pursued (Chuah *et al.*, 2000; Zhang *et al.*, 2002; Shakhbazou *et al.*, 2008; Barzilay *et al.*, 2009; Uchibori *et al.*, 2009). Second, mouse fibroblasts have been used to develop induced pluripotent stem (iPS) cells by Yamanaka and colleagues (Takahashi and Yamanaka, 2006). In subsequent studies, both mouse and human fibroblasts have been reprogrammed *in vitro* into iPS cells through viral or nonviral transduction of combinations of several transcription factors, including Oct4, Sox2, Myc, Klf4, Nanog, Lin28, H-TERT, and SV40 L-T (Takahashi *et al.*, 2007; Wernig *et al.*, 2007; Yu *et al.*, 2007; Park *et al.*, 2008). More recently, successful gene replacement in the iPS cells derived from autologous skin was reported and the corrected iPS cells were differentiated into hematopoietic progenitors, which led to correction of sickle cell anemia in a murine model *in vivo* (Hanna *et al.*, 2007). These studies indicate that an effective patient-specific cell therapy using autologous MSCs or fibroblasts can be developed in the near future. However, it is of note that retroviruses and lentivirus are either the etiologic agents of, or are intimately associated with, malignant disorders (Weiss *et al.*, 1984). Recombinant retro- and lentiviral vectors can also undergo random integration into host chromosome, thereby elevating the risk of insertional mutagenesis (Cockrell and Kafri, 2007; Nair, 2008). Thus, development of alternative vector systems, such as adeno-associated virus (AAV), needs to be pursued, given the proven safety of AAV vectors in several Phase I/II clinical trials (Bainbridge *et al.*, 2008; Cideciyan *et al.*, 2008; Hauswirth *et al.*, 2008; Maguire *et al.*, 2008).

The adeno-associated virus 2 (AAV2) is a small, single-stranded DNA-containing nonpathogenic human parvovirus with the ability to transduce a wide variety of cells and tissues both *in vitro* and *in vivo* (Muzyczka, 1992; Flotte *et al.*, 1993; Xiao *et al.*, 1996; Snyder *et al.*, 1997). However, a number of nonpermissive cell types have also been identified (Ponnazhagan *et al.*, 1996; Bartlett *et al.*, 1999; Girod *et al.*, 1999). Even in cells that allow infection, the level of infection or transgene expression is variable due to well-documented impediments in virus-host cell interactions during the life cycle of AAV2 (Qing *et al.*, 1999, 2001; Hansen *et al.*, 2000, 2001a; Zhong *et al.*, 2006). In murine fibroblasts, the rate-limiting step for AAV-mediated transgene expression appears to be the intracellular trafficking and capsid uncoating steps, as we have demonstrated earlier (Hansen *et al.*, 2000). And, in MSCs, the efficiency of AAV2 vector-mediated transduction has so far been not optimal (Ju *et al.*, 2004; Kumar *et al.*, 2004; McMahan *et al.*, 2006; Stender *et al.*, 2007). Thus, it is critical to develop novel AAV vectors that can target and transduce both of these cell types at high-efficiency, for their widespread applicability in combinatorial gene and cell transfer applications.

Our recent studies have yielded insights into key steps in the intracellular trafficking of AAV and led the development of novel AAV vectors that are capable of high-efficiency transduction at lower doses (Zhong *et al.*, 2007, 2008a). Then we found that mutations of critical surface-exposed tyrosine residues on AAV2 capsids circumvent the ubiquitination step, thereby avoiding proteasome-mediated degradation and resulting in high-efficiency transduction by these vectors in human cells *in vitro* and murine hepatocytes *in vivo* (Zhong *et al.*, 2008b). As described by Markusic *et al.* (2010), we gen-

erated multiple combinations of tyrosine mutants of AAV2 capsids, including a triple-mutant (Y730F+ Y500F+ Y444F), consisting of the three best performing single tyrosine-mutants, and evaluated whether the transduction efficiency could be augmented further. Of all the combinations tested, the triple-mutant vector had the highest level of gene transfer, ~4-fold more efficient than the single mutants. Both the Y730F and triple-mutant vectors provided long-term therapeutic expression of human factor IX in a hemophilia B mouse model. Based on these data, the current studies were designed to first test if the introduction of tyrosine mutations into the AAV2 viral capsid could significantly improve murine fibroblast and MSC gene transfer and also deduce the underlying mechanism of enhanced transduction, if any, by the tyrosine-mutant vectors in these therapeutically important cell types.

Materials and Methods

Recombinant AAV2 vectors, plasmids, antibodies, and chemicals

Highly purified stocks of self-complementary AAV2 (scAAV2) wild-type (WT) vectors containing the enhanced green fluorescence protein (EGFP) gene driven by the chicken β -actin (CBA) promoter (scAAV2-EGFP-WT) were generated as described previously and quantified by slot-blot hybridization as described (Kube and Srivastava, 1997; Liu *et al.*, 2003). An AAV2-helper plasmid, pACG-2, containing the AAV2 rep gene with an ACG start codon, was generously provided by Dr. R. Jude Samulski (University of North Carolina at Chapel Hill, Chapel Hill, NC). An scAAV2 cloning vector, pdsCBA-EGFP, was a kind gift from Dr. Xiao Xiao (University of North Carolina at Chapel Hill). All primary antibodies were purchased from BD Biosciences Pharmingen (San Jose, CA), and Dynabeads sheep anti-rat IgG was obtained from Invitrogen (Carlsbad, CA). All chemicals and reagents used in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Construction of surface-exposed tyrosine-mutant AAV2 capsid plasmid

A two-stage procedure, based on QuikChange II site-directed mutagenesis (Stratagene, La Jolla, CA), was performed using plasmid pACG-2 as described previously (Wang and Malcolm, 1999). In brief, in stage one, two PCR extension reactions were performed in separate tubes for each mutant. One tube contained the forward PCR primer and the other contained the reverse primer (Zhong *et al.*, 2008b). In stage two, the two reactions were mixed and a standard PCR mutagenesis assay was carried out as per the manufacturer's instructions. PCR primers were designed to introduce changes from tyrosine to phenylalanine residues, as well as a silent change to create a new restriction endonuclease site for screening purposes (Zhong *et al.*, 2008b). All multiple mutant AAV2 capsid plasmids were constructed using standard subcloning strategies. All mutants were screened with the appropriate restriction enzyme and were sequenced prior to use.

Cells

Murine fibroblasts. The murine fibroblast cell line NIH 3T3 was obtained from the American Type Culture Collection (Manassas, VA). Murine embryo fibroblast (MEF)

cultures were generated from the WT (WT-MEF) or FKBP52-knockout (FKBP52-KO MEF) mice as described previously (Zhao *et al.*, 2006). Cells were cultured and maintained in DMEM/F12 media (GIBCO, Carlsbad, CA) containing 10% fetal bovine serum (FBS) and 1% (v/v) concentrations of penicillin and streptomycin and supplemented with 2 mM L-glutamine.

MSC isolation and purification. Murine bone marrow-derived MSCs were isolated from C57BL/6 mice by negative selection as described previously (Baddoo *et al.*, 2003). In brief, bone marrow obtained from the long bones of 4-week-old C57BL/6 mice were suspended in α -MEM containing L-lutamine, but no ribonucleosides or deoxyribonucleosides (Invitrogen) and supplemented with 10% FBS (Sigma-Aldrich), 100 U/ml penicillin, and 100 U/ml streptomycin. Whole bone marrow cells were cultured in the same medium in 100-mm culture dishes (1.45×10^6 cells/cm²) at 37°C with 5% CO₂ for 72 hr, and nonadherent cells were removed by aspiration. Cells were cultured for an additional 5–7 days with a single medium change and harvested by gentle scraping after incubation in 0.25% trypsin with 1 mM EDTA for 5 min. To remove cell clumps, the cell pellet was dispersed by gentle agitation, resuspended in 20 ml of Hanks' balanced salt solution (HBBS), and filtered through a 70-mm filter (Falcon, Franklin Lakes, NJ). Cells were resuspended in HBSS at a maximum density of 4×10^7 cells/ml and incubated on a rotator for 1 hr at 4°C prior to immunodepletion. For immunodepletion, anti-CD11b, anti-CD34, and anti-CD45 antibodies (rat anti-mouse IgG) ($1 \mu\text{g}/10^6$ cells) (BD Biosciences Pharmingen) were added and cells were incubated on a rotator for 40 min at 4°C. Cells were spun down at 4°C and supernatant was removed. The cells pellets were resuspended in 0.2 ml of washed anti-rat IgG-conjugated Dynabeads and 1 ml of HBSS with 10% FBS, and incubated on a rotator for 20 min at 4°C. CD11b⁻, CD34⁻, and CD45⁻ positive cells were separated from negative cells by Dynabeads sheep anti-rat IgG (Invitrogen). The immunodepleted cells were plated at approximately 1×10^6 cells per T75 flask and cultured in complete medium at 37°C with 5% CO₂ in a humidified chamber with medium changes two or three times weekly. The expression profiles of surface antigens on the immunodepleted MSCs were evaluated by flow cytometry using rat anti-mouse antibodies (BD Biosciences Pharmingen) against CD9, CD29 and CD81, which are uniformly expressed in murine MSCs.

Human MSCs were purchased from ScienCell Research Laboratories (Carlsbad, CA) and maintained in the complete MSC culture medium obtained from ScienCell Research Laboratories.

Recombinant AAV2 vector transduction assays in vitro

Approximately 1×10^5 murine NIH 3T3, WT-MEF, or FKBP52-KO MEF cells were plated in 12-well plates and incubated at 37°C for 12 hr. Cells were washed once with DMEM/F12 media and then infected at 37°C for 2 hr either with phosphate-buffered saline (PBS, mock) or with the WT or the single or multiple tyrosine-mutant scAAV2-EGFP vectors at a multiplicity of infection (MOI) of 500, 1,000, or 2,000 vgs/cell in various experiments. To compare the efficiency of the WT and triple-mutant vectors, in the uncoating

process and transgene expression under DNA replication stress, VP16 (etoposide, Sigma-Aldrich) was added at a final concentration of 10 μM to murine WT-MEF cells or FKBP52-KO MEF cells. Forty-eight hours after infection, transgene expression was detected by fluorescence microscopy using an Axiovert 25 fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Images from five visual fields were analyzed quantitatively by ImageJ analysis software (National Institutes of Health, Bethesda, MD). Transgene expression was assessed as total area of green fluorescence (pixel²) per visual field (mean \pm SD).

Approximately 1×10^5 murine or human MSCs (within five passages) were used for transductions with recombinant AAV2 vectors in 12-well plates as described previously (Zhong *et al.*, 2004, 2007). Transgene expression was evaluated 5 days after transduction by EGFP imaging using fluorescence microscopy. Analysis of variance (ANOVA) was used to compare test results with the control, and they were determined to be statistically significant.

Isolation of nuclear and cytoplasmic fractions from MEFs and Southern blot analysis for AAV2 intracellular trafficking

Nuclear and cytoplasmic fractions from murine WT-MEF cells or FKBP52-KO MEF cells were isolated as described previously (Hansen *et al.*, 2000; Zhong *et al.*, 2004). In brief, mock-infected or the WT or the triple-mutant scAAV2-EGFP vector-infected cells were washed twice with PBS at 18 hr postinfection, treated with 0.01% trypsin, and washed extensively with PBS to remove any adsorbed and unadsorbed virus particles. Cell pellets were gently resuspended in 200 μl of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 5 min, after which 10 μl of 10% NP-40 was added, and observed under a light microscope. Samples were mixed gently and centrifuged for 5 min at 500 rpm at 4°C. Supernatants (cytoplasmic fractions) were decanted and stored on ice. Pellets (nuclear fractions) were washed twice with 1 ml of hypotonic buffer and stored on ice. The purity of each fraction was determined to be >95%, as measured by the absence of acid phosphatase activity (nuclear fractions) and absence of histone H3 (cytoplasmic fractions) as described previously (Hansen *et al.*, 2000; Zhong *et al.*, 2004). Low-M_r DNA samples from nuclear and cytoplasmic fractions were isolated and electrophoresed on 1% agarose gels followed by Southern blot hybridization using a ³²P-labeled EGFP-specific DNA probe as described previously (Zhong *et al.*, 2004). Densitometric scanning of autoradiographs for the quantitation was performed using ImageJ analysis software.

Separation of intact AAV2 virions from MEFs and Southern blot analysis to determine rate of AAV capsid uncoating

Equivalent numbers ($\sim 2 \times 10^6$) of WT-MEF cells or FKBP52-KO MEF cells were infected with the WT or the triple-mutant scAAV2-EGFP vectors at 1×10^6 vgs/cell at 37°C for 2 hr. The nuclear and cytoplasmic fractions were isolated 18 or 48 hr postinfection as described above. The supernatants (cytoplasmic fraction) were decanted. After washing twice with 1 ml of hypotonic buffer, the pellets

(nuclear fraction) were resuspended in 400 μ l of PBS containing 2 mM MgCl₂. Nuclear fractions (200 μ l each) were either mock-treated, or digested with Benzoylase nuclease (Novagen Inc., Madison, MI) (100 U/ml) at 37°C for 1 hr. EDTA was added to a final concentration of 100 mM, followed by heat inactivation of Benzoylase nuclease at 75°C for 15 min. Low-M_r DNA was isolated by the method described previously (Hansen *et al.*, 2000, 2001a; Zhong *et al.*, 2004), and AAV genomes were electrophoresed on 1% agarose gels and analyzed by Southern blot hybridization using a ³²P-labeled EGFP-specific DNA probe as described above. Densitometric scanning of autoradiographs, for the quantitation of relative amounts of viral genomes, was evaluated with ImageJ analysis software (National Institutes of Health).

Results

Single mutations in surface-exposed tyrosine residues improve the transduction efficiency of AAV2 vectors in murine NIH 3T3 cells

To develop the optimal AAV vectors for fibroblast and MSC gene transfer, we first tested the transduction efficiency of each of the seven (Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, and Y730F) surface-exposed tyrosine-mutant scAAV2-EGFP vectors in comparison with that of the WT vectors in murine NIH 3T3 cells *in vitro* under identical conditions. From the results shown in Fig. 1, it is evident that whereas mock-infected NIH 3T3 cells showed no green fluorescence, the transduction efficiency of each of the tyrosine-mutant vectors, 48 hr postinfection, was significantly higher

compared with that of the WT scAAV2-EGFP vector at 2,000 viral particles per cell. Specifically, the transduction efficiency of Y444F vectors was maximal, ~193-fold higher than that of the WT vector (Fig. 1B). Similarly, the Y730F and the Y500F mutants also had significant enhancement in EGFP expression (~57- and 44-fold, respectively). It is interesting to note that the tyrosine-mutant efficacy data from NIH 3T3 cells are different from our previously published data with HeLa cells (Zhong *et al.*, 2008b), where the Y730F mutant was the most efficacious (~11-fold higher transduction efficiency vs. the WT vector) (Fig. 2). These data suggest that tyrosine mutants have different levels of efficacy in different cell/tissue types, possibly due to the differences in the intracellular milieu in different species and/or conformation-dependent binding and accessibility to host cellular factors.

Multiple mutations in the surface-exposed tyrosine residues of AAV2 vectors further improve the transduction efficiency in primary WT murine fibroblasts but not in FKBP52-KO murine fibroblasts

To examine whether various combinations of the three best performing single tyrosine mutants (Y444F > Y730F > Y500F) could lead to further enhancement in the transduction efficiency, we first compared the efficacy of these single mutants with double (Y444+730F) and triple (Y444+500+730F) tyrosine-mutant vectors in WT- MEFs. As expected, the transduction efficiency of each of the single and multiple tyrosine-mutant vectors was significantly higher compared with that of the WT scAAV2-EGFP vector at an MOI of 1,000

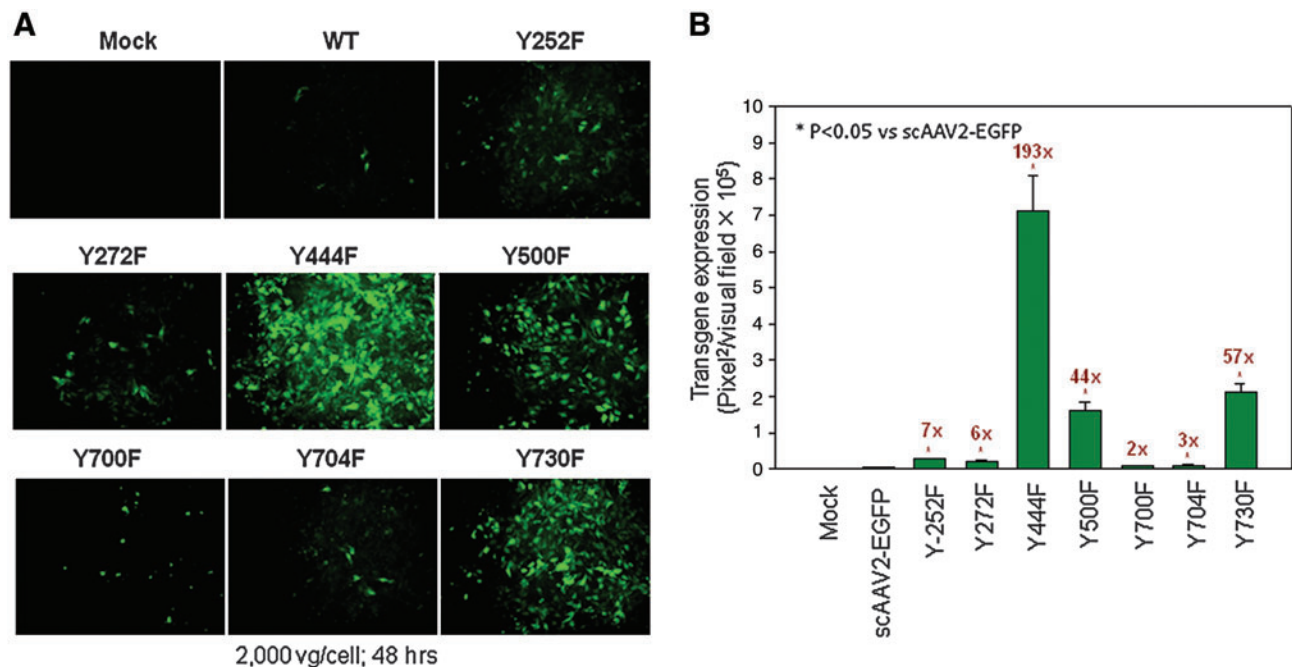


FIG. 1. Comparative analyses of AAV2-mediated transduction of murine NIH 3T3 cells with tyrosine-mutant capsid scAAV2-EGFP vectors. **(A)** Cells were either mock-infected, or infected with the WT or the singly tyrosine-mutant scAAV2-EGFP vectors at an MOI of 2,000 vgs/cell. Transgene expression was detected by fluorescence microscopy at 48 hr postinfection. Original magnification $\times 100$. **(B)** Quantitative analyses of AAV2 transduction efficiency in NIH 3T3 cells. Images from five visual fields were analyzed quantitatively by ImageJ analysis software. Transgene expression was assessed as total area of green fluorescence (pixel²) per visual field (mean \pm SD). ANOVA was used to compare test results with the control, and they were determined to be statistically significant. * $p < 0.05$ vs. WT scAAV2-EGFP. Color images available online at www.liebertonline.com/hum.

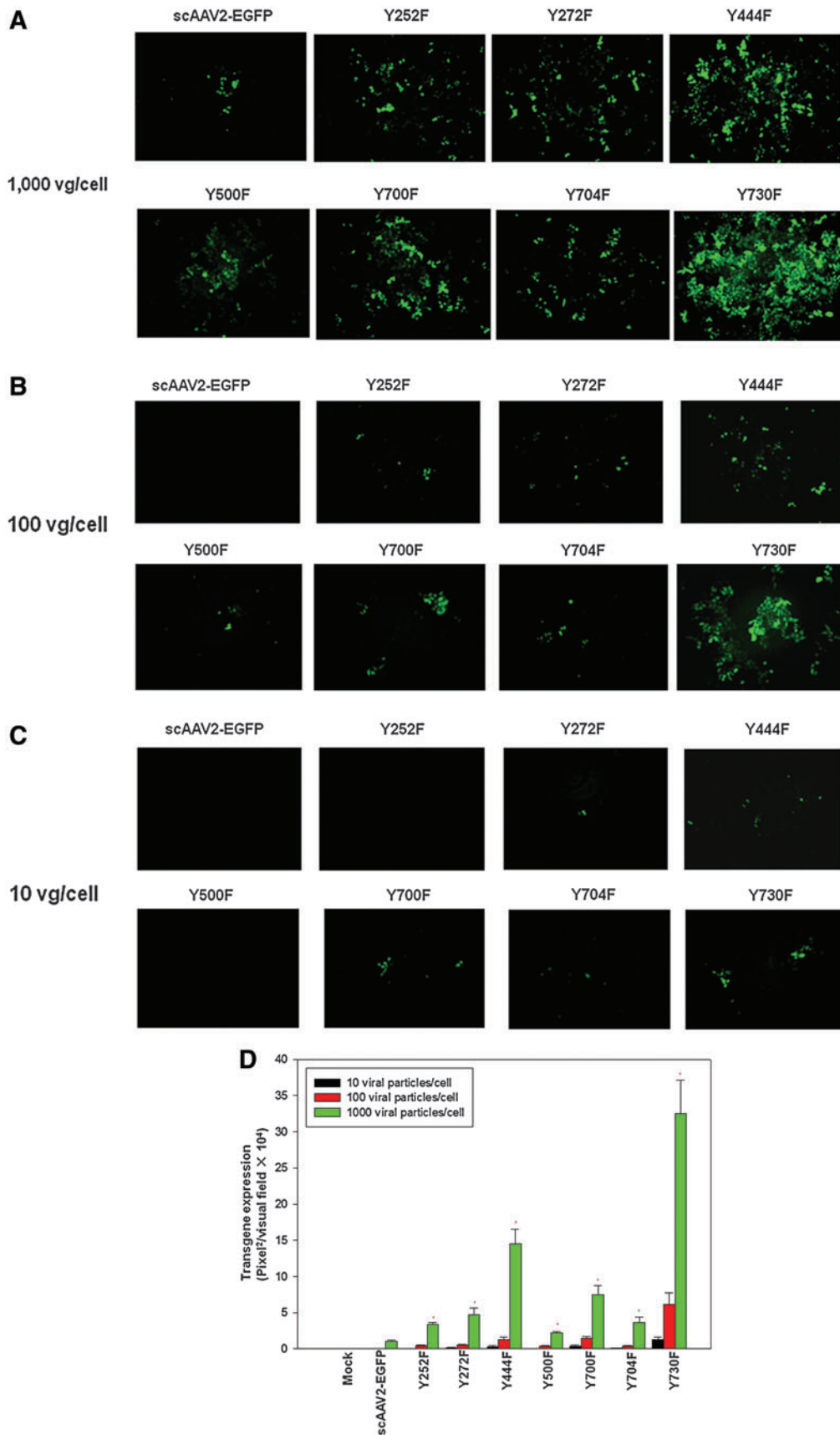


FIG. 2. Comparative analyses of AAV2-mediated transduction of human HeLa cells with tyrosine-mutant scAAV2-EGFP vectors at different MOIs. (A–C) Cells were either mock-infected, or infected with the WT or single-mutant scAAV2-EGFP vectors at the various indicated MOIs per cell. Transgene expression was detected by fluorescence microscopy at 48 hr post-infection. Original magnification $\times 100$. (D) Quantitative analyses of AAV2 transduction efficiency were performed as described in the legend to Fig. 1. * $p < 0.05$ vs. WT scAAV2-EGFP. Color images available online at www.liebertonline.com/hum.

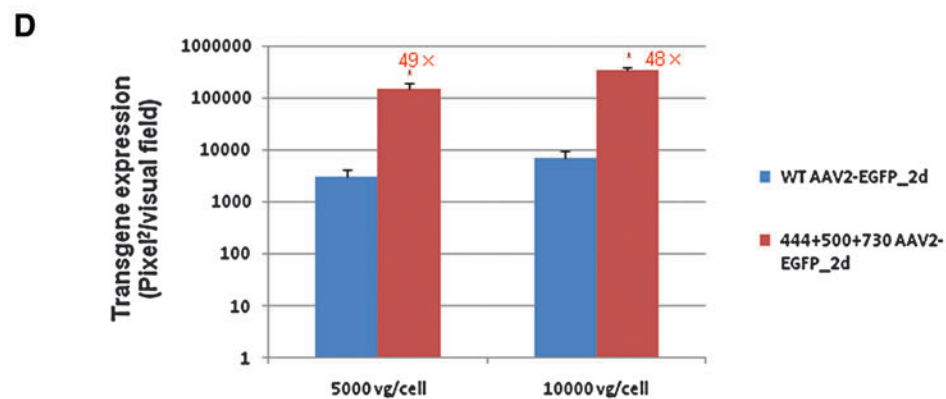
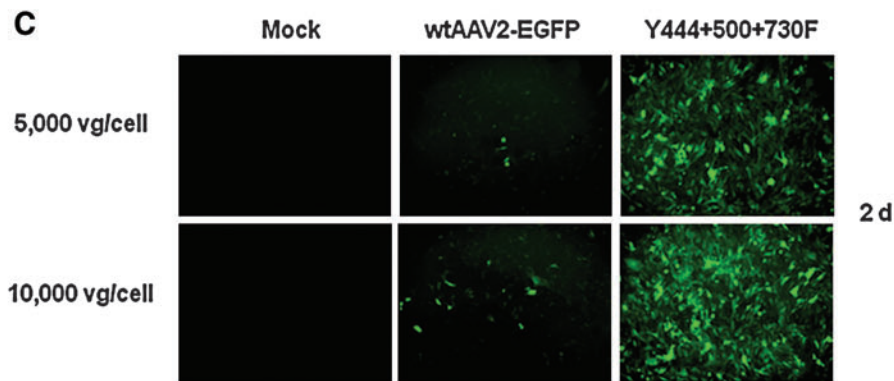
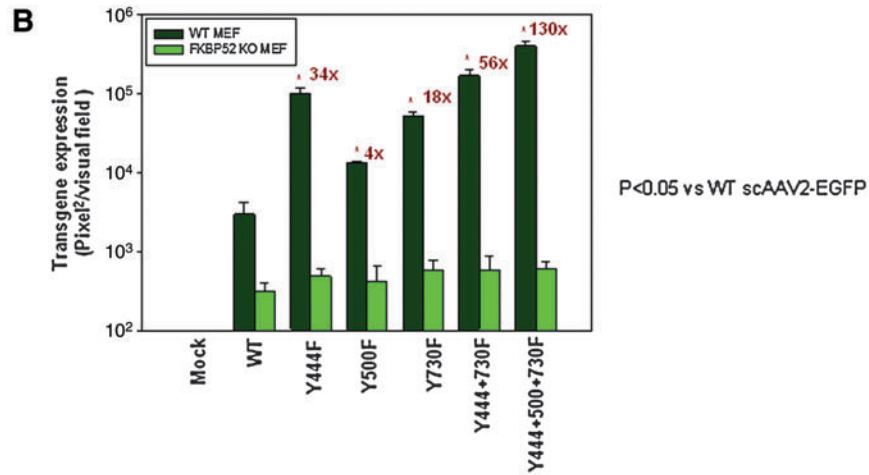
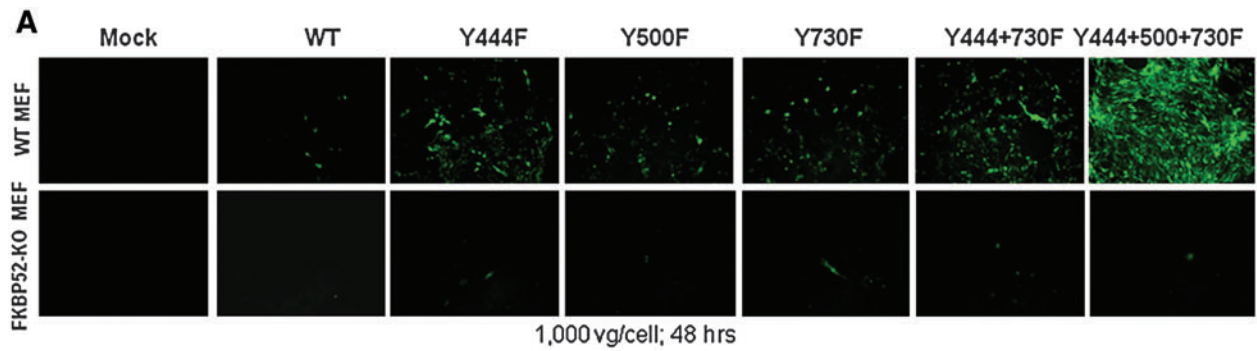


FIG. 3. Comparative analyses of AAV2-mediated transduction of normal (A–E) and FKBP52-KO (A) MEFs with tyrosine-mutant scAAV2-EGFP vectors. Cells were either mock-infected, or infected with the WT or single-, double-, or triple-mutant scAAV2-EGFP vectors at various indicated MOIs per cell. Transgene expression was detected by fluorescence microscopy at various time points postinfection. (E) Bright-field images demonstrate similar cell confluence. Original magnification $\times 100$. (B and D) Quantitative analyses of AAV2 transduction efficiency for data in A and C were performed as described in the legend to Fig. 1. * $p < 0.05$ vs. WT scAAV2-EGFP. Color images available online at www.liebertonline.com/hum.

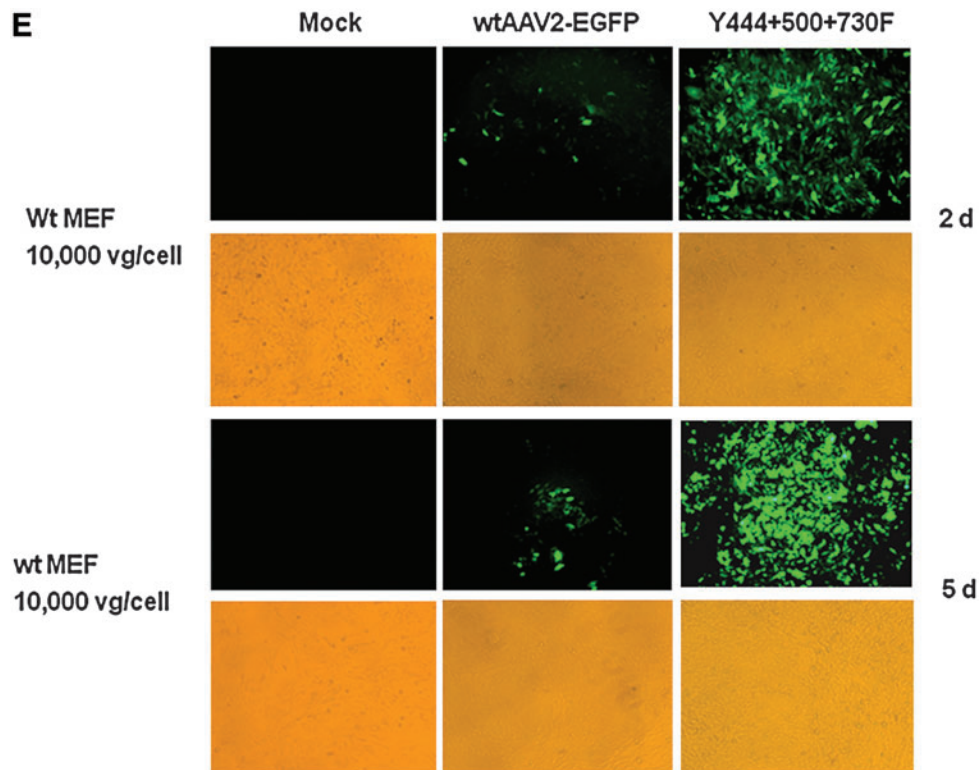


FIG. 3. (Continued).

vg/cell, 48 hr postinfection (Fig. 3A and B). Interestingly, the triple-mutant (Y444+500+730F) led to an additional ~4-fold increase in transgene expression, followed by a ~1.5-fold increase with the Y444+730F double-mutant, compared with the single-mutant vectors. The efficacy of the triple-mutant vector in WT-MEFs is in agreement with our recent studies, where we have documented that the triple-mutant vector had the highest level of gene transfer, ~3–5-fold more efficient than the single mutants in the HeLa cells *in vitro* and in murine hepatocytes *in vivo* (Markusic *et al.*, 2010). The transduction efficiency of the triple-mutant vector was ~130-fold higher than that of the WT AAV vector. These vectors were also tested in NIH 3T3 and 20,000 and WT-MEFs at an MOI of 5,000 and 10,000 for 2 days or 5 days (Figs. 3C–E and 4). As can be seen, whereas WT AAV2 vectors transduced either NIH 3T3 or WT-MEF cells very poorly, even at high MOIs, the triple-mutant AAV2 led to high efficiency transduction under identical conditions (~50–350-fold increase).

As we have previously shown that the tyrosine-mutant vectors can traffic efficiently into the nucleus bypassing the cellular proteasome-mediated degradation (Zhong *et al.*, 2008a,b) and that a host-cell chaperone protein, FKBP52, is required for intracellular trafficking of WT AAV (Zhao *et al.*, 2006), we wished to examine if FKBP52 could mediate enhanced transduction of the tyrosine-mutant vectors. To test this hypothesis, we compared the transduction efficacy of the WT and triple-mutant scAAV vectors in FKBP52-KO MEFs. As can be seen in Fig. 3A, little or no transgene expression was seen in FKBP52-KO MEFs infected with either the WT or the triple-mutant vectors, compared with the WT-MEFs (Fig. 3A). These data suggest that FKBP52, being a cellular chap-

erone protein, plays an important role in the intracellular trafficking and/or uncoating process of the triple-mutant vectors as we have described previously for the WT vectors (Zhao *et al.*, 2006).

FKBP52 is required for intracellular trafficking of the WT but not for the triple-mutant AAV2 vectors

As we only detected very low transgene expression in FKBP52-KO MEFs infected with either the WT or the triple-mutant vectors, we next examined the fate of the input viral DNA in these cells in comparison with the WT-MEFs. Low- M_r DNA samples from nuclear or cytoplasmic fractions of mock- or vector-transduced cells were isolated 18 hr post-transduction and analyzed on Southern blots. As can be seen in Fig. 5A (lane 5), and the densitometric scanning in Fig. 5B, ~45% of the input AAV2 DNA was present in the nuclear fraction in the WT-MEF cells infected with the WT vector, consistent with our previously published studies (Zhong *et al.*, 2007). Interestingly, the amount of the input triple-mutant AAV2 vector DNA in the nuclear fraction was increased to ~65% (Fig. 5A, lane 6, and B). These results further confirm that, as with single-mutant tyrosine vectors (Zhong *et al.*, 2008b), multiple mutations in the surface-exposed tyrosine residues also facilitate enhanced nuclear transport of AAV2 vectors. However, in FKBP52-KO MEFs, in which ~30% input DNA of the WT vectors was detected in the nuclear fraction (Fig. 5A, lane 11, and B), nearly 75% of the input DNA was seen in the nuclear fraction from cells transduced with the triple-mutant vectors. These data suggest a diminished role for FKBP52 in the intracellular trafficking of the triple-mutant vectors compared with the WT

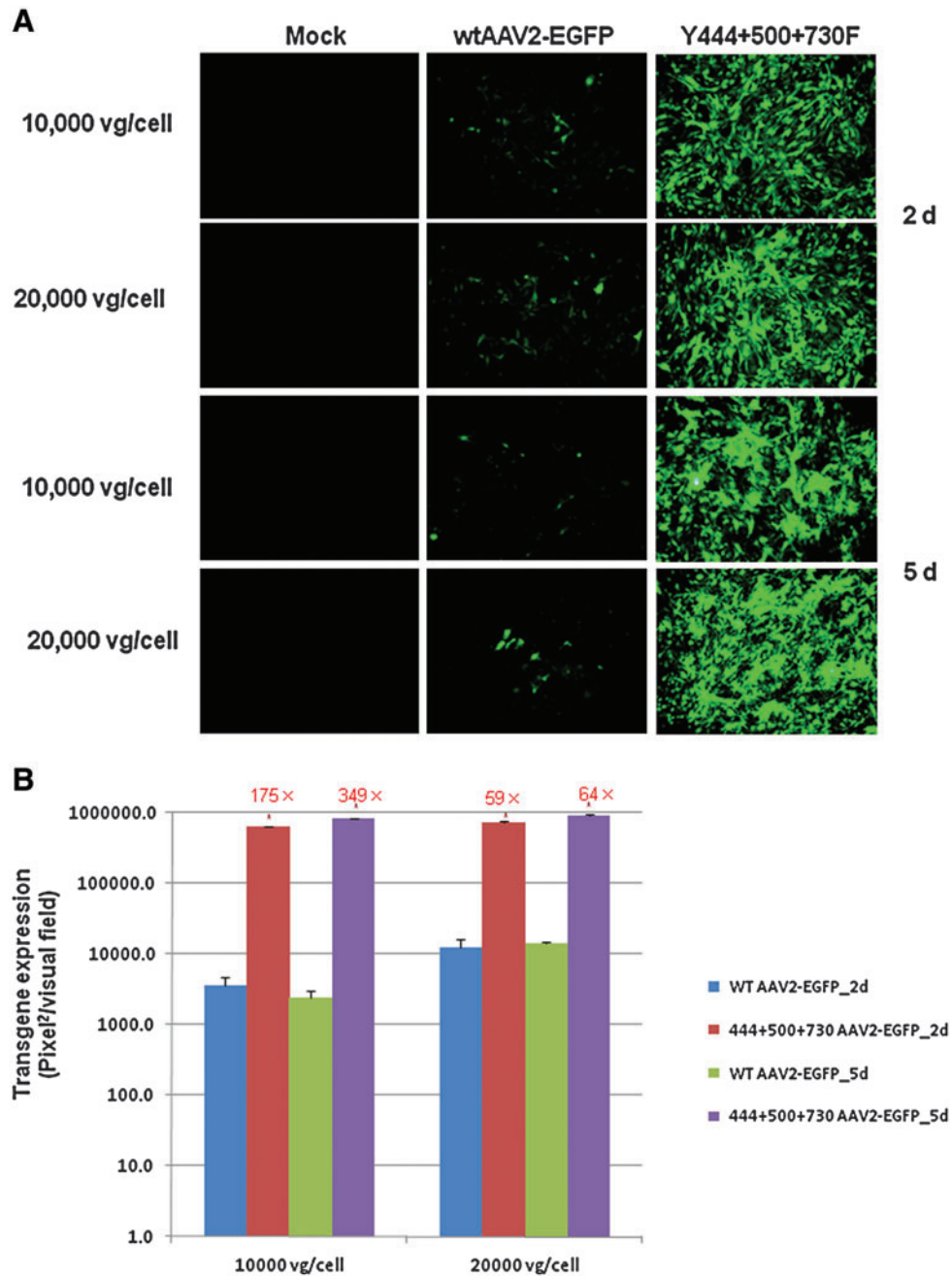


FIG. 4. Comparative analyses of AAV2-mediated transduction of murine NIH 3T3 cells with tyrosine-mutant scAAV2-EGFP vectors at different MOIs and time points. **(A)** Cells were either mock-infected, or infected with the WT or triple-mutant scAAV2-EGFP vectors at the various indicated MOIs per cell. Transgene expression was detected by fluorescence microscopy at various time points after infection. Original magnification $\times 100$. **(B)** Quantitative analyses of AAV2 transduction efficiency were performed as described in the legend to Fig. 1. $*p < 0.05$ vs. WT scAAV2-EGFP. Color images available online at www.liebertonline.com/hum.

vectors, possibly due to the absence of the tyrosine residues at positions 730, 500, and 444 that may provide binding sites for the dephosphorylated forms of FKBP52 in the cytosol, and then traffic the vectors into the nucleus (Zhong *et al.*, 2007). The absence of these critical residues on AAV capsids in tyrosine-mutant vectors may prevent binding with FKBP52, and thus promote nuclear translocation by avoiding the ubiquitin/proteasomal pathway, because other tetrapeptide-containing immunophilins such as FKBP38 are

known to anchor with proteasomes in the cytosol (Nakagawa *et al.*, 2007).

FKBP52 is involved in the uncoating of AAV2 vectors

To investigate the extent of AAV vector uncoating in the WT-MEFs and FKBP52-KO MEFs, cells were infected with the WT or the triple-mutant scAAV vectors, and nuclear fractions were isolated 18 and 48 hr postinfection. Samples

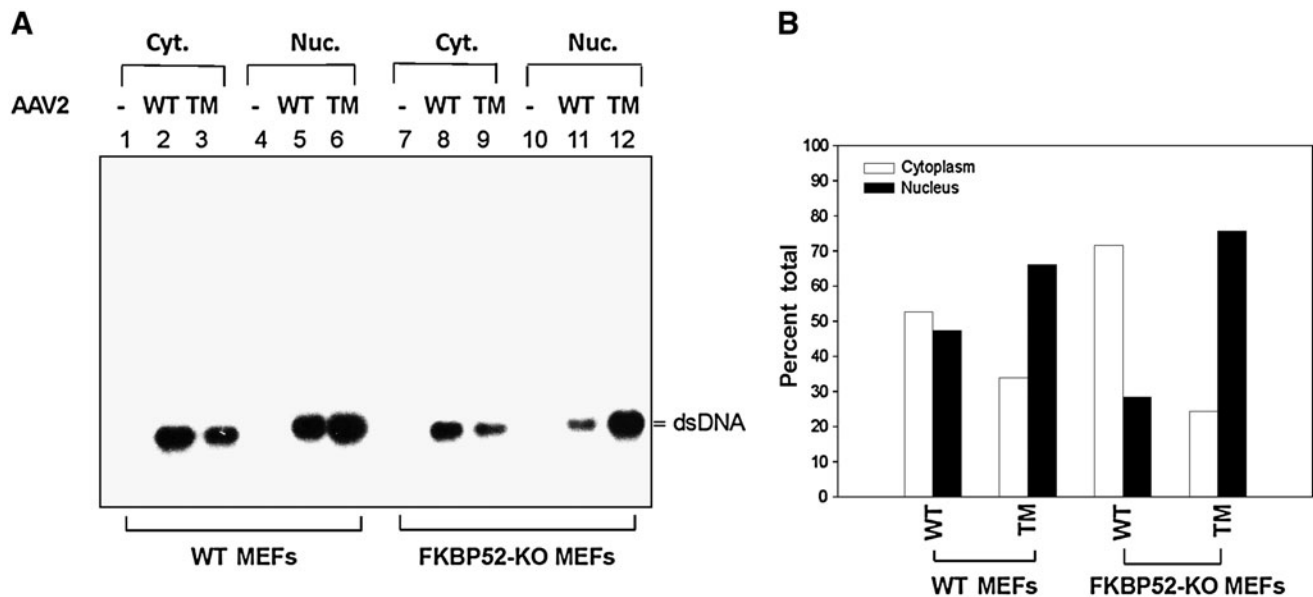


FIG. 5. Southern blot analyses of intracellular trafficking of WT and the triple-mutant AAV vector genomes in normal and FKBP52-KO MEFs. **(A)** Low- M_r DNA samples isolated from mock-transduced or vector-transduced cells were analyzed on Southern blots using a ^{32}P -labeled EGFP-specific DNA probe. **(B)** Densitometric quantitation of the data in A.

were either mock-treated or treated with Benzonase nuclease, which would be expected to digest the virus DNA after uncoating of the capsid structure. It is important to note that others and we have shown that DNA in the intact nuclei can be completely digested with the DNase protocol used here (Zhao *et al.*, 2006). Low- M_r DNA isolated from equivalent numbers of cells was then analyzed on Southern blots. These results are shown in Fig. 6. As can be seen in Fig. 6A and its quantitative analysis in Fig. 6C, 18 hr posttransduction in WT-MEFs, the triple-mutant vector had a better uncoating profile due to its enhanced trafficking ability, as only ~45% of DNA for the triple-mutant vector remained resistant to Benzonase nuclease digestion compared with ~80% of the input DNA of the WT AAV vector. A similar trend of uncoating extended up to 48 hr in WT-MEFs for both vectors. However, in the absence of FKBP52, as in FKBP52-KO MEFs (Fig. 6B), almost equal amounts (~100%) of input WT and triple-mutant vectors remained intact. These data strongly suggest that FKBP52 is required for the capsid uncoating of both the WT and the triple-mutant vectors. The rapid uncoating of the triple-mutant vectors at 48 hr in WT-MEFs (~48% to 2%), but not in FKBP52-KO MEFs (100% to 90%), or 18 hr postinfection, also suggests that nucleus-localized FKBP52 can facilitate uncoating of the triple-mutant vectors in the absence of cytoplasmic FKBP52 bound to AAV capsids, as is the case with the WT vectors. In this context, it is interesting to note that ~80% of cellular FKBP52 is localized to the nucleus (Czar *et al.*, 1994a,b).

A direct correlation exists between the rate of capsid uncoating and the extent of transgene expression

Treatment with topoisomerase inhibitors, such as etoposide (VP16), has previously been shown to improve transgene expression from AAV vectors (Koeberl *et al.*, 1997). To examine whether enhanced transgene expression could be

achieved from the fraction of uncoated DNA in both WT and FKBP52-KO MEFs, cells were treated with VP16 following infections with the WT and the triple-mutant scAAV vectors. As can be seen in Fig. 7, a significantly higher level of transgene expression was observed in WT-MEFs treated with VP16 for both the WT and the triple-mutant AAV vectors compared with that in the FKBP52-KO MEF cells. This increase in transgene expression corroborated well with the levels of vector genomes released following FKBP52-mediated uncoating of AAV capsids to serve as templates for VP16 activity.

Tyrosine mutations improve the transduction efficiency of AAV2 vectors in primary murine MSCs

To establish the feasibility of the use of tyrosine-mutant vectors for MSC gene transfer, the transduction efficiency of each of the seven surface-exposed tyrosine-mutant scAAV2-EGFP vectors (Y252F, Y272F, Y444F, Y500F, Y700F, Y704F, and Y730F) was analyzed in murine MSCs. Primary murine bone marrow-derived MSCs were isolated from C57BL/6 mice as described previously (Baddoo *et al.*, 2003). Transgene expression was detected by fluorescence microscopy 5 days postinfection. As shown in Fig. 8A, the transduction efficiency of the Y500F and Y730F vectors was ~3–10-fold higher than that of the WT vector, consistent with our previous studies (Zhong *et al.*, 2008b), as well as those described by Markusic *et al.* (2010). The transduction efficiency of the Y444F vectors was ~13-fold higher than that of the WT vectors (Fig. 8A). In addition, the following combinations of the mutants were also evaluated in murine MSCs at 2,000 vgs/cell: three double: Y444+500F, Y444+730F, Y500+730F; one triple: Y444+500+730F; and one septuple: Y252+272+444+500+700+704+730F. The combination of mutant Y444F with other mutants, such as two double-mutants (Y444+500F and Y444+730F), the triple-mutant (Y444+500+730F), and the

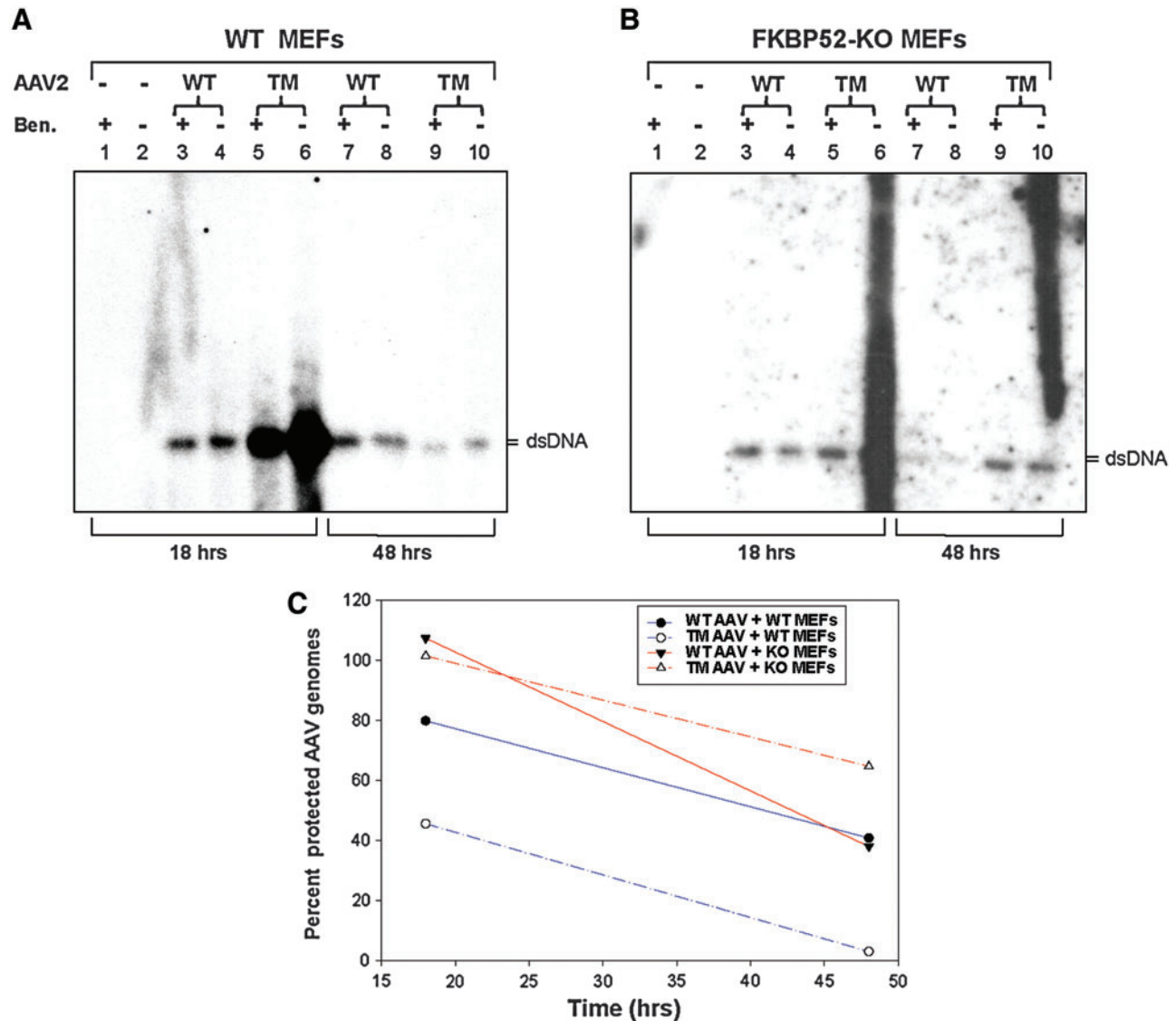


FIG. 6. Southern blot analyses for uncoating of WT and the triple-mutant AAV vectors in normal (**A**) and FKBP52-KO (**B**) MEFs. Equivalent amounts of nuclear samples isolated from mock-transduced or vector-transduced cells at 18 hr or 48 hr were either not treated, or treated exhaustively with Benzonase nuclease. Low M_r DNA isolated from these samples was analyzed on Southern blots using a ^{32}P -labeled EGFP-specific DNA probe as described above. (**C**) Densitometric quantitation of the data in A and B. Color images available online at www.liebertonline.com/hum.

septuple-mutant, led to ~21–31-fold and ~45-fold increases in EGFP transgene expression in murine MSCs compared with the WT vectors (Fig. 8B). These data indicate that the triple-mutant is the optimal tyrosine-mutant AAV2 vector for efficient transduction of primary murine MSCs. The enhanced transduction efficiency of the triple-mutant vector offers a potentially useful approach for generating iPS cells.

Tyrosine mutations also improve the transduction efficiency of AAV2 vectors in primary human MSCs

We next examined whether single or multiple tyrosine mutations could also improve the transduction efficiency of AAV2 vectors in primary human MSCs. The efficacy of WT as well as single and multiple tyrosine-mutant vectors was

evaluated in primary human bone marrow-derived MSCs *in vitro*. The efficiency of single tyrosine-mutant vectors was significantly higher, and ranged between 5- and 6-fold, compared with that of the WT vector (Fig. 9) at 5,000 vgs/cell. The multiple-mutant vectors led to a further increase in EGFP expression (Fig. 9A), in which two double mutants (Y444+500F and Y444+730F) led to ~7–8-fold increase. The triple-mutant (Y444+500+730F) vectors led to ~11-fold increase, and the septuple-mutant resulted in ~9-fold increase in EGFP transgene expression compared with the WT vectors (Fig. 9B). As determined by fluorescence microscopy, ~80–90% of these cells were transduced by the triple-mutant vectors 5 days posttransduction. This enhanced transduction potential of human MSCs mediated by the triple-mutant vector offers a potentially promising platform for therapeutic applications.

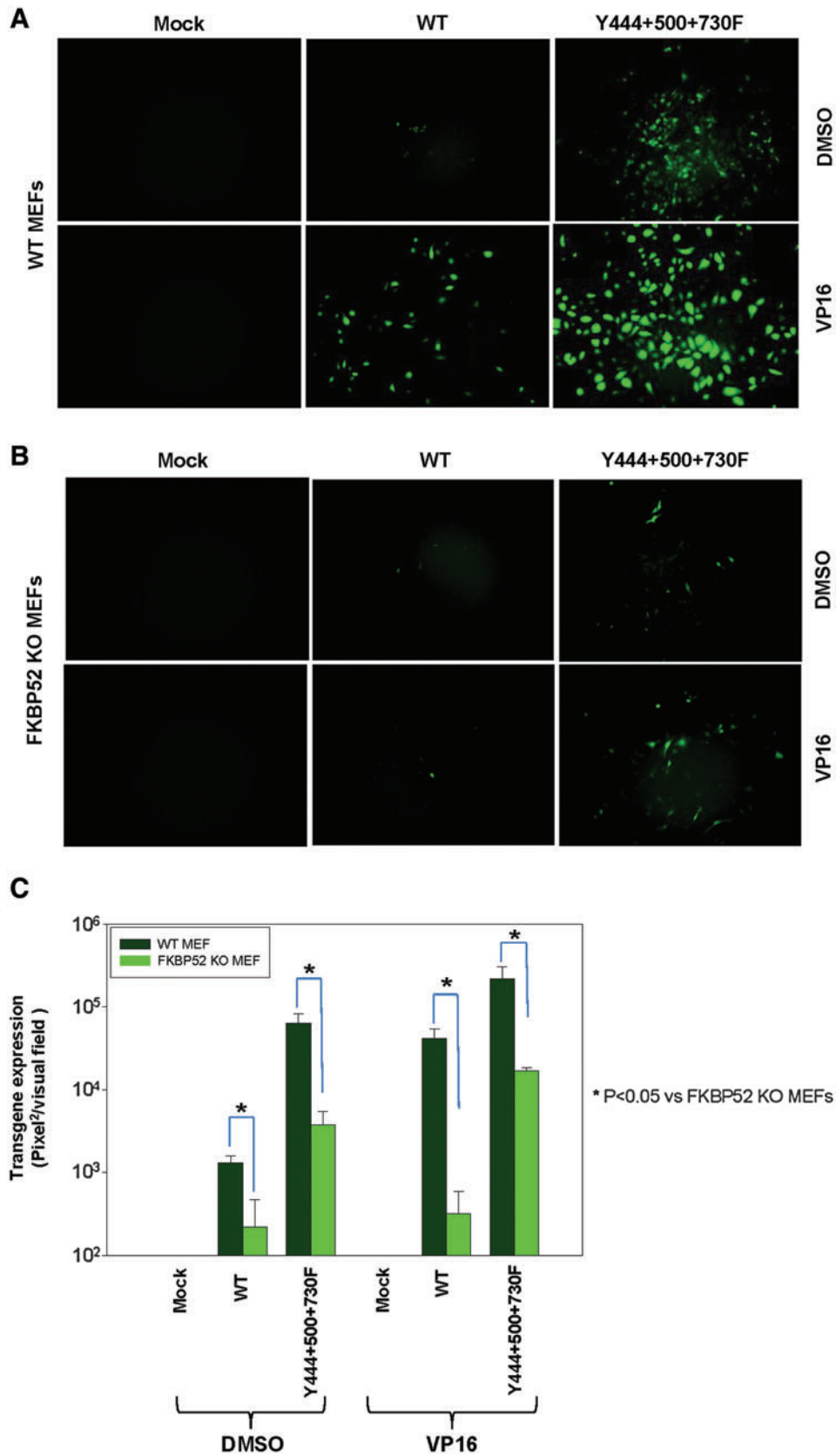


FIG. 7. Comparative analyses of AAV2-mediated transduction of normal (**A**) and FKBP52-KO (**B**) MEFs with WT and triple-mutant scAAV2-EGFP vectors with and without treatment with VP16. Cells were infected either mock-infected, or infected with the WT or triple-mutant scAAV2-EGFP vectors at an MOI of 500 vgs/cell. Transgene expression was detected by fluorescence microscopy 48 hr postinfection. Original magnification $\times 100$ x. (**C**) Quantitative analyses of AAV2 transduction efficiency was performed as described in the legend to Fig. 1. * $p < 0.05$ vs. WT scAAV2-EGFP. Color images available online at www.liebertonline.com/hum.

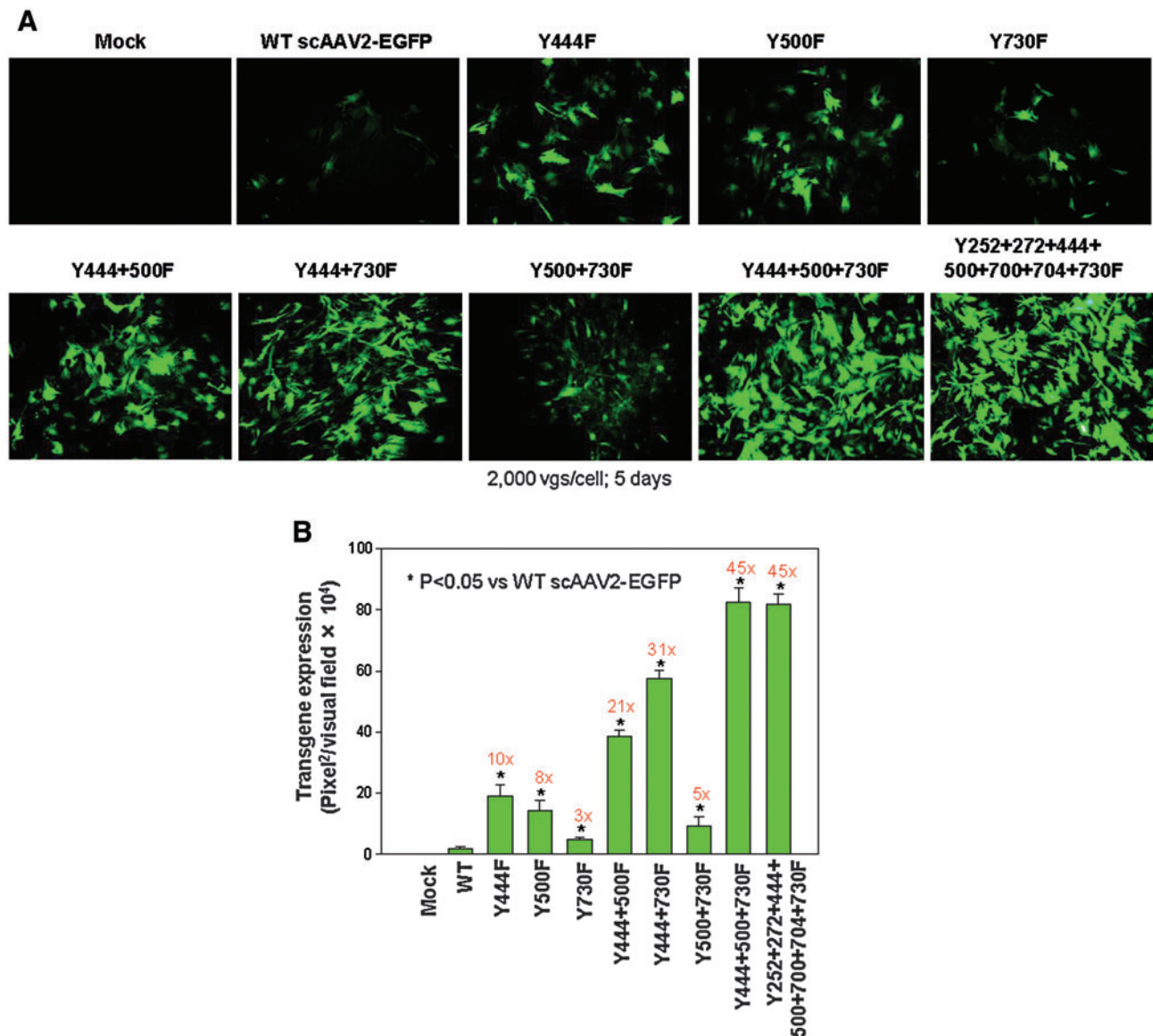


FIG. 8. Comparative analyses of AAV2-mediated transduction of murine MSCs with tyrosine-mutant capsid scAAV2-EGFP vectors. **(A)** Murine bone marrow-derived MSCs were infected by surface-exposed tyrosine-mutant capsid scAAV2-EGFP vector at an MOI of 2,000 vgs/cell. Transgene expression was detected by fluorescence microscopy at 5 days postinfection. Original magnification $\times 100$. **(B)** Quantitative analyses of AAV2 transduction efficiency in murine MSCs. * $p < 0.05$ vs. WT scAAV2-EGFP. Color images available online at www.liebertonline.com/hum.

Discussion

Correction of genetic defects is one of the most elegant and fundamental approaches to the treatment of human diseases, including cancer, AIDS, metabolic and inherited diseases (Anderson, 1998). However, despite continuous improvement in vector technology, efficient and specific delivery of a therapeutic gene to the target cells still remains a major block in clinical gene transfer studies. Alternatively, stem cells, including the hematopoietic stem cells (HSCs), MSCs, and more recently iPS cells, are considered attractive targets for *ex vivo* modification with gene therapy vectors for the potential cellular and gene therapy of inherited and acquired disorders (Takahashi and Yamanaka, 2006; Zhong *et al.*, 2006; Stender *et al.*, 2007). These cells possess the properties

of self-renewal, are capable of differentiating into multiple lineages, have the ability to home to sites of injury, and, most importantly, are hypoimmunogenic when *ex vivo* transduced, making allogeneic transplantation of these cells a possibility. Of these, the use of MSCs and iPS cells is considered widely feasible over that of HSCs due to their ease of isolation and a high capacity for *in vitro* expansion and further genetic modifications with viral vectors.

Remarkable progress has been made in the recent past in using retroviral or lentiviral vectors to deliver therapeutic genes into MSCs (Zhang *et al.*, 2002; Lee *et al.*, 2004; Reiser *et al.*, 2005; Barzilay *et al.*, 2009; Jacome *et al.*, 2009) or in deriving iPS cells from adult somatic cells (Welstead *et al.*, 2008; Sommer *et al.*, 2009) and the subsequent use of gene-modified iPS cells for therapeutic globin gene transfer

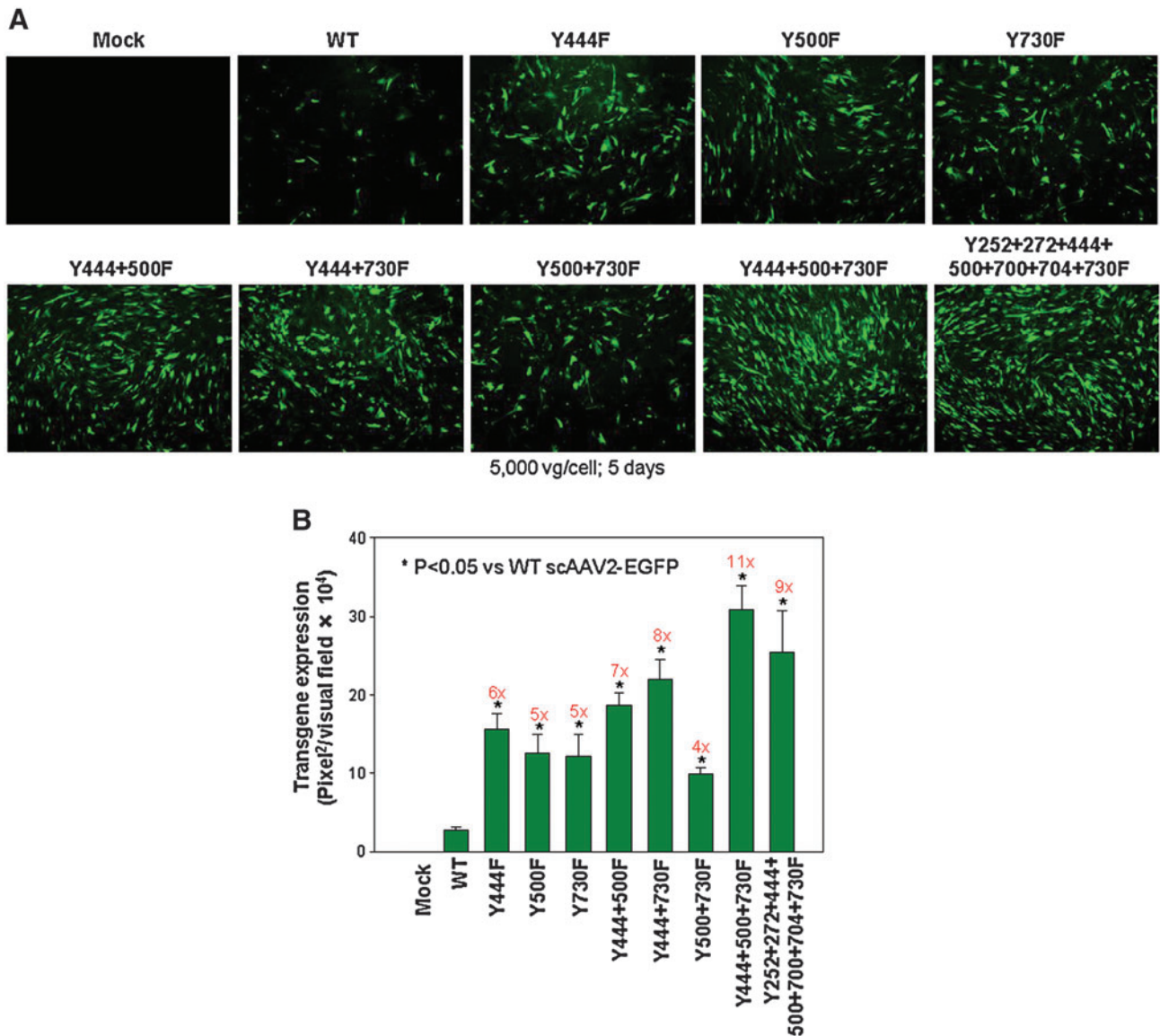


FIG. 9. Comparative analyses of AAV2-mediated transduction of human MSCs with multiple tyrosine-mutant capsid scAAV2-EGFP vectors. **(A)** Murine bone marrow-derived MSCs were infected by single, double, triple, and septuple Y-F mutant scAAV2-EGFP vectors at an MOI of 5,000 vgs/cell. Transgene expression was detected by fluorescence microscopy 5 days postinfection. Original magnification ×100. **(B)** Quantitative analyses of AAV2 transduction efficiency in murine MSCs. **p* < 0.05 vs. WT scAAV2-EGFP. Color images available online at www.liebertonline.com/hum.

(Townes, 2008). However, both retroviral and lentiviral vectors undergo random integration into host chromosomal DNA, and there is persistent risk for insertional mutagenesis (Cockrell and Kafri, 2007; Nair, 2008). The development of leukemia in four treated children in an X-linked severe combined immunodeficiency clinical trial using retroviral vectors has re-emphasized the risks related to gene therapy (Hacein-Bey-Abina *et al.*, 2003; Kohn *et al.*, 2003; Nair, 2008). More recently, nonintegrating adenoviral vectors (Stadtfeld *et al.*, 2008) and plasmid DNAs (Okita *et al.*, 2008) have been used to deliver Klf4, Oct3/4, and Sox2 genes to fibroblasts, but the efficiency of reprogramming is low, ranging between 0.01 and 0.1%. Thus, we believe that further development of alternative vector systems, such as AAV, needs to be pursued for their potential ability to infect MSCs and fibroblasts, as well as to

generate iPS cells at high efficiency, given the proven safety of AAV vectors in several clinical trials (Bainbridge *et al.*, 2008; Hauswirth *et al.*, 2008; Maguire *et al.*, 2008).

Despite the impressive safety record of AAV vectors, the efficacy of gene transfer in MSCs and in fibroblasts is suboptimal (Reiser *et al.*, 2005). A comparative study of viral and nonviral vectors showed that AAV serotypes 1, 2, 4, 5, and 6 were least effective in rat and rabbit MSCs compared with other means of gene transfer (McMahon *et al.*, 2006). In contrast, a vector dose-dependent enhancement in transduction efficiency of 1–65% has been reported in human MSCs, but those authors concluded that host cellular barriers may contribute to the relatively restricted transgene expression observed in MSCs (Stender *et al.*, 2007). Importantly, the AAV2 vector-transduced MSCs in this study retained their

multipotential activity compared with the untransduced controls (Stender *et al.*, 2007). Similarly, in fibroblasts, we and others have reported ~30-fold lower levels of AAV2 DNA replication when compared with 293 or HeLa cells (Hansen *et al.*, 2000; Bhargava and Trempe, 2009). Therefore, it becomes important to elucidate the fundamental steps in the life cycle of AAV and its host cell interactions to overcome barriers leading to a decreased transduction efficiency of AAV in both MSCs and fibroblasts.

We have previously undertaken systematic studies in fibroblasts, including cell-surface receptor- and coreceptor-mediated viral binding and entry (Hansen *et al.*, 2000), intracellular trafficking (Hansen *et al.*, 2000, 2001a; Zhao *et al.*, 2006), nuclear transport (Hansen *et al.*, 2001b), uncoating and viral second-strand DNA synthesis (Zhao *et al.*, 2006), and identified intracellular trafficking as the major impediment for efficient transgene expression from WT AAV vectors. Subsequently, we documented that, during the process of navigation through the late endosome, EGFR-PTK-induced tyrosine phosphorylation of AAV2 capsid proteins promotes ubiquitination and degradation of AAV2, thus leading to impairment of viral nuclear transport and decrease in transduction efficiency in intact cells (Zhong *et al.*, 2007, 2008a). Based on these studies, we mutated each of the seven surface-exposed tyrosine residues on viral capsids that are phosphorylated by EGFR-PTK, to yield AAV2 vectors with significantly increased transduction efficiency in HeLa cells *in vitro* as well as in C57BL/6 mice *in vivo*, compared with their WT counterpart (Zhong *et al.*, 2008b). In the present study, we have further improved the transgene expression in fibroblasts by >130-fold, using a combination of either single or multiple tyrosine-mutants and demonstrated that the triple-mutant AAV2 is by far the optimal vector to transduce fibroblasts.

As the level of transgene expression from this triple-mutant vector was compromised in FKBP52-KO fibroblasts, we further examined the role of FKBP52 in both intracellular trafficking and uncoating of the triple-mutant vector. Our detailed subcellular studies suggest that FKBP52 plays a major role in intracellular trafficking of the WT AAV vector, but not the triple-mutant vector. However, FKBP52 is required for uncoating of both the WT and the triple-mutant AAV vectors. It is possible that FKBP52 binds to different regions of the AAV capsid, depending on whether they are in the cytosol or in the nucleus, and surface-exposed tyrosine residues in the WT vector may provide docking sites for cytosolic FKBP52, but not nuclear FKBP52. This could explain why the triple-mutant vectors can still traffic efficiently into the nucleus in FKBP52-KO cells, by an additive effect of FKBP52's ability to escape from the proteasomal machinery, but it gets trapped in the nucleus of these cells during the uncoating process. Alternatively, it is possible for FKBP52 to have dual roles in intracellular trafficking and capsid uncoating by a molecular switch involving its phosphorylation and dephosphorylation status. Cytoplasmic FKBP52 may bind to phosphorylated-tyrosine residues present in the WT AAV capsid and facilitate its intracellular trafficking, but while inside the nucleus, FKBP52 may become phosphorylated to initiate uncoating. In the absence of AAV capsid-bound cytoplasmic FKBP52 within the nucleus, as with the triple-mutant vectors, the phosphorylated forms of nuclear FKBP52 may substitute for uncoating activity. Then, in this

proverbial vicious cycle of AAV–host cell protein interactions, phosphorylated FKBP52 blocks the viral second-strand DNA synthesis, as we have described previously (Mah *et al.*, 1998; Qing *et al.*, 1998). Further studies are needed to confirm this hypothesis and the trafficking and uncoating advantage or disadvantage, if any, using the septuple tyrosine-mutant vectors.

The availability of a highly efficacious triple-mutant AAV vector that can transduce fibroblasts ~130-fold more efficiently, and exhibits transgene expression in ~90% of infected cells, can now be exploited to generate iPS cells at a higher frequency. Similarly, the efficacy of AAV2 tyrosine-mutant vectors in transducing both murine and human MSCs was also documented. Given that AAV2 remains the predominant serotype vector currently in use in human gene therapy (Flotte *et al.*, 1996, 2004; Kay *et al.*, 2000; Aitken *et al.*, 2001; Wagner *et al.*, 2002; Manno *et al.*, 2003; Snyder and Francis, 2005), coupled with the fact it is also the best characterized in terms of vector toxicology, we achieved >90% transduction efficiency in MSCs at MOIs of ≤5,000 of the tyrosine-mutant vectors, levels that will be capable of achieving physiological correction with most therapeutic proteins. This is critical, because the requirement of relatively high vector doses (>10,000–100,000) has been reported to achieve only suboptimal transduction of MSCs with AAV vectors (McMahon *et al.*, 2006; Stender *et al.*, 2007). The availability of a large repertoire of multiple tyrosine-mutant AAV2 vectors and other AAV serotypes (AAV1–AAV12) (Daya and Berns, 2008) offers hope for achieving 100% transduction at reduced vector doses in the therapeutically important fibroblasts and MSCs.

In summary, this study clearly demonstrates that a thorough understanding of the fundamental steps in AAV–host cell interactions can lead to development of efficacious vectors that can optimally transduce semipermissive cells, which have significant implications in the use of AAV vectors in both gene and cell therapy.

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Author Disclosure Statement

No competing financial interests exist.

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