Adeno-Associated Virus 5 Transduces Adipose-Derived Stem Cells with Greater Efficacy Than Other Adeno-Associated Viral Serotypes

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Adipose-derived stem cells (ASCs) have shown potential in the treatment of a myriad of diseases; however, infusion of cells alone is unlikely to provide the full range of potential therapeutic applications. Transient genetic manipulation of ASCs could increase their repair and regeneration characteristics in a disease-specific context, essentially transforming them into drug-eluting depots. The goal of this study was to determine the optimal parameters necessary to transduce ASCs with recombinant adeno-associated virus (rAAV), an approved gene therapy vector that has never been associated with disease. Transduction and duration of gene expression of the most common recombinant AAV vectors were tested in this study. Among all tested serotypes, rAAV5 resulted in both the highest and longest term expression. Furthermore, we determined the glycosylation profile of ASCs before and after neuraminidase treatment and demonstrate that rAAV5 transduction requires plasma membrane-associated sialic acid. Future studies will focus on the optimization of gene delivery to ASCs, using rAAV5 as the vector of choice, to drive biological drug delivery, engraftment, and disease correction.

Keywords: adipose-derived stem cells, adeno-associated virus, stem cell therapy, gene therapy

INTRODUCTION

FROM CHRONIC WOUNDS to Crohn's disease, stem cell therapy is taking center stage as a viable therapeutic approach for numerous genetic and acquired diseases.¹⁻³ Investigation of the clinical relevance of adipose-derived stem cells (ASCs) in disease treatment has received particular attention because of the relative ease of obtaining ASCs and their robust nature *in vitro* and *in vivo*. ASCs are capable of surviving autotransplantation even in relatively ischemic environments, and are often amply abundant in modern Americans via simple outpatient (or even office-based) lipoaspiration or excisional lipectomy. In addition to low morbidity at harvest, when compared with bone marrow biopsies for bone marrow-derived stem cells (BMCs), the yield per gram of ASCs in lipoaspirate is severalfold higher than that of BMCs from bone marrow.⁴ As mesenchymal stem cells, ASCs are multipotent for a

variety of tissues.^{3,5} Furthermore, their antiinflammatory properties could hold promise in combating the inflammatory milieu associated with many conditions.^{3,6-8} These preliminary results, along with several *in vitro* and animal studies, suggest that ASCs hold much clinical promise.

The potential clinical benefits of ASCs, when acting alone, are unlikely to be enough to effectively combat complex disease conditions. The ability to modify autotransplanted ASCs to be drug-eluting cells holds the potential to further enhance their healing effects by providing a personalized cocktail of molecular products in the direct vicinity of the damaged tissue being treated. Genetic modification of ASCs has been attempted using several gene-based technologies including nonviral and virus-mediated gene transfer.⁹⁻¹¹ In contrast to many gene delivery systems, which demonstrate toxicity and safety concerns, recombinant adeno-associated virus (rAAV) is a safe and effective means of

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transducing nondividing primary tissue with sustained gene expression.^{12,13} Moreover, an AAV-based vector for the treatment of lipoprotein lipase deficiency, Glybera, just recently became the first approved gene therapy in the Western world.¹⁴

Because each AAV serotype demonstrates distinct tissue tropism, we asked which AAV serotype is most effective at transducing human-derived ASCs. Because many AAV serotypes use glycosylated molecules as receptors, we determined the glycosylation profile of ASCs and investigated how well this profile correlates with AAV transduction. Last, we asked whether AAV transduction would alter the stem cell surface marker characteristics of ASCs. Here we report that rAAV5 is the most efficient rAAV serotype for ASC transduction and that cell surface sialic acid is a requirement for transduction. Overall, these data indicate that ASC transduction by AAV5 will provide an excellent and versatile system for creating ASC drug-eluting therapeutics.

MATERIALS AND METHODS

Ethics statement

All human tissue samples were obtained after written informed consent had been provided, with approval from the Wright State University and Miami Valley Hospital IRB committees (WSU IRB#4772). All data were analyzed anonymously and according to the principles expressed in the Declaration of Helsinki.

Isolation and culture of human-derived ASCs

After informed consent had been obtained, patient-derived ASCs were isolated from surgical lipectomy or lipoaspirate specimens; specimens were deidentified and kept separate. Excised lipectomy specimens were minced sharply into small pieces. Specimens were washed at least three times and then digested in type I collagenase (2 mg/ml) for 15–30 min, while incubated at 37°C and manually shaken every 5 min to isolate the stromovascular fraction (SVF) known to contain ASCs. After sample centrifugation at 1200×*g* for 5 min, the supernatant was discarded and the cellular pellet was resuspended in medium and cells were counted. Cells were seeded onto standard tissue culture plates at an initial seeding density of 20,000–100,000 cells/cm². The medium (Dulbecco's modified Eagle's medium [DMEM], 10% fetal calf serum, 1% antibiotic–antimycotic solution; A5955; Sigma-Aldrich, St. Louis, MO) was changed the next day to remove nonadherent cells. The medium was changed every other day until 90% confluence was

reached. ASCs were detached with TrypLE (Life Technologies, Carlsbad, CA) and used for experiments at passage 3 or 4. The viability of the cells was monitored via cell counts, trypan blue exclusion assays, and visual morphology as the cells were passaged.

ASC characterization

Identification and characterization of the ASC population were performed by an immunofluorescence multicolor flow cytometry strategy (Accuri C4; BD Biosciences, San Jose, CA) derived from the recommendations of the 2013 joint position statement from the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT).¹⁵ Immunofluorescently linked antibodies for flow cytometry were obtained from e-Bioscience (San Diego, CA). Antibodies against surface markers CD45 (leukocyte common antigen) and CD31 (PECAM-1) were used to exclude hematopoietic/lymphocytic and endothelial lineages, respectively. The SVF was identified by the stem cell markers CD105 (endoglin), CD90 (Thy-1), CD44 (hyaluronic acid receptor), CD73 (L-VAP-2), and CD13 (APN). Within the SVF, ASCs were distinguished from bone marrow-derived mesenchymal stem cells (MSCs) via the absence of CD106 (VCAM-1) expression and positive expression of CD36 (GPIIb) assessed by flow cytometry.

Transduction of human-derived ASCs by rAAV

Triple-passaged human-derived ASCs were seeded at a density of 2×10⁴ cells per 24-well plate. rAAV serotypes 1, 2, 4, 5, 8, and 9, encoding the enhanced green fluorescent protein (eGFP) gene under the control of the cytomegalovirus (CMV) promoter, were supplied by the University of Iowa Vector Core (Iowa City, IA). The vectors were based on a backbone with the structural Rep proteins from AAV2 followed by the capsid serotype (rAAV2/1, 2/2, 2/4, 2/5, 2/8, or 2/9). rAAV viral genome titer was determined by qPCR, transduced titer by fluorescence-activated cell sorting for eGFP, and silver staining was done to further assess titer and viral purity. Viral genome titer was used to calculate the multiplicity of infection (MOI). Each rAAV serotype was used to transduce triple-passaged ASCs. rAAV serotypes were diluted with DMEM to the MOI indicated in text. Growth medium was aspirated 24 hr after seeding, and the cells were rinsed with phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS^{−/−}). Each diluted rAAV serotype was added to each well in a

24-well plate at the MOI indicated in text in a total volume of 250 μ l. The cells were then incubated for 4 hr at 37°C and 5% CO₂. Mock-transduced (medium only) ASCs were used as the negative control. The inoculum was then removed, cells were rinsed with culture medium, and fresh culture medium was added. All cells were incubated at 37°C and 5% CO₂ until further analysis.

Fluorescence microscopy

AAV transduction and lectin staining were evaluated by laser scanning confocal microscopy (Olympus FV1000) with a $\times 20$ or $\times 60$ oil immersion lens and also by fluorescence microscopy (Nikon Eclipse TE2000-S) at magnifications of $\times 4$ and $\times 10$. Three to five images from three separate experiments on ASCs from different donors were quantified by ImageJ analysis of mean fluorescence intensity. Error bars represent the standard error.

Lectin staining

Human-derived triple-passaged ASCs were stained with fluorescein isothiocyanate (FITC)-conjugated lectins according to the manufacturer's protocol (see Table 1; Vector Laboratories, Burlingame, CA). Mock-treated unstained ASCs were used as negative controls and included in each experiment. Cells were seeded at a density of 2×10^4 on collagen-coated 8-well chamber slides. Twenty-four hours after seeding, cells were chilled on ice and washed with ice-cold PBS^{−/−} and 1% bovine serum albumin (BSA). Cells were treated with 2.5 μ l of FITC-conjugated lectins diluted in ice-cold PBS^{−/−} and 1% BSA buffer (250 μ l), and incubated for 1 hr at 4°C in a dark environment to prevent endocytosis and bleaching of the fluorophores. After the incubation period, ASCs were washed three times in ice-cold PBS^{−/−} and 1% BSA buffer and fixed with freshly prepared 4% paraformaldehyde in PBS^{−/−} for 15 min. The cells were then washed twice with 500 μ l of ice-cold PBS^{−/−} with 1% BSA. The cells were mounted with Vectashield mounting medium with DAPI (Vector Laboratories) and images were taken, using a confocal microscope (Olympus FV1000) with a $\times 60$ oil immersion lens.

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Neuraminidase treatment

Human-derived ASCs were pretreated with a 20-mU/ml concentration of neuraminidase (250 μ l; *Vibrio cholerae*; Sigma-Aldrich) for 2 hr at 37°C. After neuraminidase treatment of ASCs, cells were washed and infected with rAAV, as described previously, or stained with FITC-labeled lectin. rAAV transduction was assessed 48 hr posttransduction by fluorescence microscopy.

Statistical analysis

Data were expressed as mean and standard error of at least three replicates and analyzed with one-way analysis of variance (ANOVA) using GraphPad software (GraphPad Software, San Diego, CA). A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Human-derived ASC isolation and stem cell surface marker characterization

Between 60 and 120 ml of human adipose tissue was processed from four different donors, and the SVF was obtained as described previously. SVF cells were prepared for flow cytometry to determine the expression of stem cell, endothelial, and leukocyte cell surface markers. To delineate the ASC subpopulation from the total SVF, cells negative for CD31 (endothelial), CD45 (hematopoietic), and CD106 (MSC) markers and positive for cell surface markers CD105, CD90, CD44, CD73, CD13, and CD36,

Table 1. Lectins used to determine the glycoconjugate profile of adipose-derived stem cells and the corresponding rAAV serotypes that bind to the glycosylation motif

Lectin	Ligand motifs	AAV binding
N-Acetylglucosamine (sialic acid)-binding lectins		
<i>Maackia amurensis</i> leukoagglutinin (MAL) from <i>Maackia amurensis</i>	Neu5Ac/Gcα2,3Galβ1,4Glc(NAc)	AAV1, AAV4, AAV5
Wheat germ agglutinin (WGA) from <i>Triticum vulgaris</i>	GlcNAcβ1-4GlcNAcβ1-4GlcNAc, Neu5Ac (sialic acid)	AAV4, AAV5
N-Acetylgalactosamine (galactose)-binding lectins		
Peanut agglutinin (PNA) from <i>Arachis hypogaea</i>	Galβ1-3GalNAcα1-Ser/Thr	AAV9
<i>Ricinus communis</i> agglutinin (RCA) from <i>Ricinus communis</i>	Galβ1-4GalNAcβ1-R	AAV9
Soybean agglutinin (SBA) from soybean	N-Acetylgalactosamine (GalNAc) residues	
Mannose-binding lectins		
Concanavalin A (ConA) from <i>Canavalia ensiformis</i>	α-D-Mannosyl and α-D-glucosyl residues	AAV9
Fucose-binding lectins		
<i>Ulex europaeus</i> agglutinin (UEA) from <i>Ulex europaeus</i>	Fucα1-2Gal-R	

rAAV, recombinant adeno-associated virus.

were considered ASCs (Supplementary Fig. S1; supplementary data are available online at <http://online.liebertpub.com/hgtb>). On the basis of these markers, our ASC yields were routinely from 80% to more than 95% of the cells isolated. Similar to previous descriptions, ASCs exhibited a spindle-like shape (80–100 μ m in diameter and 200 μ m in length) and viability was routinely more than 85% by trypan blue exclusion.^{16,17} Given the high percentage of ASCs, no further purification or selection was performed for these studies.

Transduction of ASCs with rAAV vectors

rAAV is a highly promising gene therapy vector. Because each AAV serotype demonstrates distinct tissue tropism, we asked which AAV serotype is most effective at transducing human-derived ASCs. Triple-passaged ASCs were infected with six different rAAV serotypes (1, 2, 4, 5, 8, and 9) encoding the eGFP gene under the control of the CMV promoter. The MOI ranged from 10 to 10^6 viral genomes (VG)/cell. The cells were examined under a fluorescence microscope or confocal microscope, and nontransduced ASCs were used as the negative control to eliminate background autofluorescence. We found that rAAV5-eGFP had the greatest trans-

duction efficiency as compared with other serotypes in ASCs from four different donors (>70% GFP positive at an MOI of 10^6 VG/cell; Fig. 1). rAAV serotypes 1 and 2 were also able to transduce ASCs at an MOI of 10^6 VG/cell whereas rAAV serotypes 4, 8, and 9 were not able to transduce ASCs. At an MOI of 10^6 VG/cell, rAAV5 produced sustained eGFP expression, with eGFP-positive cells detectable 8 days postinfection (Fig. 2). By contrast, rAAV1 and rAAV2 lost gene expression by day 4 (data not shown). Moreover, rAAV5-eGFP expression was observed down to an MOI of 100 (Fig. 3). Overall, rAAV5-eGFP expression was high and the most sustained over the duration of examination as indicated in text.

ASCs remain viable and retain their stem cell surface marker profile after rAAV transduction

We next asked whether rAAV transduction changed the viability or expression of stem cell surface markers on the ASCs. ASCs were transduced with rAAV serotypes 2, 5, and 8, or mock transduced and cell counts, trypan blue exclusion, and flow cytometry assays were performed. No difference in cell number or viability was observed, indicating that there is no overt cellular toxicity after rAAV

Figure 1. Adipose-derived stem cells (ASCs) are transduced by recombinant adeno-associated virus serotype 5 (rAAV5) to a greater extent than by rAAV serotypes 1, 2, 4, 8, or 9. (A) rAAV1; (B) rAAV2; (C) rAAV4; (D) rAAV5; (E) rAAV8; (F) rAAV9; (G) no virus. Oil immersion confocal microscopy; original magnification, $\times 60$. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bars: 10 μ m. (H) Mean fluorescence intensity of ASCs transduced by various AAV serotypes. Color images available online at www.liebertpub.com/hgtb

Figure 2. rAAV5 transduction persists for more than 1 week in ASCs. Multiplicity of infection (MOI), 10⁶ viral genomes/cell. (A) rAAV5, day 2; (B) rAAV5, day 4; (C) rAAV5, day 6; (D) rAAV5, day 8. Fluorescence microscopy; original magnification, $\times 10$. Scale bars: 40 μ m. (E) Mean fluorescence intensity of ASCs transduced with rAAV5, measured on various days. Color images available online at www.liebertpub.com/hgtb

transduction of ASCs (data not shown). No change in the cell surface stem cell marker expression profile was observed after transducing the ASCs with the various rAAV serotypes, suggesting that ASCs maintain their stemlike cell surface characteristics after rAAV transduction (Supplementary Fig. S1).

Lectin binding profile of ASCs

Previous studies have demonstrated that rAAVs generally bind specific sugar motifs (e.g., sialic acid, mannose, or heparan sulfate) on the cell surface as primary binding receptors¹² (Table 1). However, none of these studies have been performed on ASCs. Lectins have been widely used to identify the extracellular glycosylation pattern of cells because of their high binding affinity and carbohydrate specificity. The lectin binding profile on ASCs showed that they are extensively glycosylated but not fucosylated (Fig. 4). The human-derived ASCs express abundant *N*-acetylgalactosamine, sialic acid, and mannose molecules, which correlates strongly with the transduction profiles for AAV serotypes 1, 2, and 5 in ASCs.

Figure 3. rAAV5 is able to transduce ASCs over a wide range of MOIs. (A) 10⁸; (B) 10⁶; (C) 10⁴; (D) 10² viral genomes/cell. Fluorescence microscopy; original magnification, $\times 4$. Scale bars: 50 μ m. (E) Mean fluorescence intensity of ASCs transduced at various MOIs with rAAV5-GFP. GFP, green fluorescent protein. Color images available online at www.liebertpub.com/hgtb

AAV5 requires sialic acid for internalization into ASCs

To further confirm that the sialylation of cultured human ASCs was responsible for rAAV5 transduction, cells were treated with neuraminidase from *Vibrio cholerae*, which removes 2,3-, 2,6-, and 2,8-sialic acid from cell surface glycoproteins. Consistent with sialic acid removal, no *Maackia amurensis* lectin (MAL) lectin binding was observed after the neuraminidase treatment of ASCs (Fig. 5A and B). Consistent with this, rAAV5 transduction at an MOI of 10⁶ VG/cell was completely inhibited by sialic acid removal (Fig. 5C and D), whereas rAAV2 transduction was not affected (Fig. 5E and F). These data indicate that, similar to other cell types,^{18–20} rAAV5 requires sialic acid for binding and internalization in human-derived ASCs.

DISCUSSION

In this study we assessed the transduction profiles of the most commonly used and clinically relevant rAAV vectors on human ASCs *in vitro*. We found that rAAV serotypes 1, 2, and 5 demonstrate efficient gene transfer in ASCs. Interestingly, only

Figure 4. ASCs are extensively glycosylated (green), but are not fucosylated. (A) *Maackia amurensis* lectin (MAL), 2,3-N- and O-linked sialic acid; (B) wheat germ agglutinin (WGA), 2,3-N- and O-linked sialic acid; (C) concanavalin A (ConA), α -mannose and α -glucose; (D) peanut agglutinin (PNA), N-acetylgalactosamine and D-galactose; (E) *Ricinus communis* agglutinin (RCA), terminal galactose residues; (F) soybean agglutinin (SBA), N-acetylgalactosamine; (G) *Ulex europaeus* agglutinin (UEA 1), L-fucose. Oil immersion confocal microscopy; original magnification, $\times 60$. Nuclei are stained with DAPI (blue). Scale bars: 10 μ m. (H) Percentage of ASCs positive for lectin staining by flow cytometry without (white columns) or with (green columns) neuraminidase pretreatment. Color images available online at www.liebertpub.com/hgtb

rAAV5 showed relatively long-term transduction. To our knowledge, we identify for the first time the glycosylation profile of ASCs. We also show that, consistent with the literature, 2,3-N-linked sialic acid glycosylation correlates with efficient rAAV5 transduction.²¹ Together these data indicate that AAV5 is the most suitable rAAV serotype for studies that seek to enhance the therapeutic efficacy of ASC autotransplantation by expression or secretion of beneficial therapeutic molecules.

Although rAAV is not the most common vector system in gene therapy trials today, it is perhaps the most promising vector system. AAV is a non-integrating helper-dependent virus that has never been associated with disease in humans. Moreover, Glybera, the first human gene therapy approved in the Western world, is based on rAAV1. With

promising clinical trial results for many more genetic diseases, such as hemophilia B, Leber's congenital amaurosis, and muscular dystrophy, it is likely that more rAAV serotypes will enter the marketplace within the next few years.^{22–24} This will pave the way for other applications that use rAAV to treat acquired diseases and combination therapies with stem cells. Moreover, there is also the potential for rAAV-based therapies to correct defective genes and inherited genetic diseases through homologous recombination in stem cells.²⁵ To this end, we asked whether one rAAV serotype was best for ASC transduction. In agreement with previous studies, we show that AAV serotypes 1, 2, and 5 efficiently transduce ASCs.^{26–28} Interestingly, our results are in contrast to mouse adipose tissues, which are more effectively transduced by

Figure 5. rAAV5 transduction of ASCs is inhibited by removal of cell surface sialic acid by neuraminidase treatment. ASCs stained with *Maackia amurensis* lectin (MAL) in green, 2,3 N- and O-linked sialic acid before and after neuraminidase treatment. Oil immersion confocal microscopy; original magnification, $\times 60$. Nuclei are stained with DAPI (blue). Scale bars: $10\ \mu\text{m}$. rAAV5 and rAAV2 transduction of ASCs after neuraminidase treatment. Fluorescence microscopy; original magnification, $\times 10$. Scale bars: $40\ \mu\text{m}$. Color images available online at www.liebertpub.com/hgtb

rAAV8 and rAAV9.²⁹ The difference between human and mouse highlights the importance of comparative studies between human and murine ASCs and tailoring the experimental questions being asked and the organismal system being tested.

For the first time we show that rAAV5 shows the most prolonged gene expression of the rAAV vectors tested. This is important because the duration of gene expression is likely to correlate with therapeutic efficacy. Although gene expression was reduced by day 8, which would limit therapeutic gene expression, this could possibly be a benefit. ASCs are expected to engraft and differentiate based on the transplant environment and acute versus chronic expression of exogenous genes may differentially affect engraftment. For example, overexpression of growth factors necessary to induce healing processes (vascular endothelial growth factor [VEGF], epidermal growth factor [EGF], transforming growth factor [TGF]- β , etc.) may be extremely

beneficial, but their dysregulated overexpression may incite abnormal tissue differentiation.³⁰ Thus, acute expression of a beneficial gene may confer beneficial effects while limiting potentially harmful ones.

The cell surface glycan profile varies widely between different cell types and animal species.^{31–34} Many viruses use a diversity of glycans as primary or coreceptors. Most AAV serotypes have distinct binding preferences and affinities for a variety of glycans (Table 1).^{18,21,35–38} For instance, AAV5 binds α -2,3-N-linked sialic acid. Because ASCs express high levels of sialic acid as indicated by MAL and wheat germ agglutinin (WGA) staining, it could have been predicted that rAAV5 would have a high level of transduction. Therefore, knowing the glycosylation profile of ASCs will aid future studies that will attempt to identify additional novel viral vectors able to transduce efficiently. Furthermore, it is expected that the glycosylation profile of stem cells will change as they differentiate toward distinct lineages such as fat, muscle, neural, or epidermal cell types.^{39,40} Thus, if *ex vivo* applications are to include a differentiation step, understanding the glycosylation profiles of ASC descendants will be invaluable in selecting the most efficacious AAV vector.

The therapeutic relevance of ASCs for the treatment of a myriad of diseases continues to grow.^{1–3} Other studies have investigated genetic modification of ASCs to create drug-eluting cells that enhance healing in diverse clinical settings such as in wound healing, diabetes, cancer, and cardiovascular disease.^{9–11,40–42} For example, transfection of mouse ASCs with a plasmid encoding VEGF followed by implantation into a murine excisional wound model demonstrated accelerated healing and improved collagen deposition and maturation in the wound bed.⁴¹ In an attempt to develop a novel cell therapy for type 1 diabetes, Bahrebar and colleagues used a lentiviral vector to stably deliver the pancreatic duodenal homeobox 1 (PDX-1) transcription factor gene to ASCs.¹⁰ Transduced ASCs then differentiated into insulin-producing cells that expressed several islet-related genes and secreted insulin in response to glucose. To our knowledge, only three other studies have investigated rAAV transduction in ASCs.^{26–28} Kang and colleagues²⁶ transduced ASCs with rAAV2 encoding the gene for bone morphogenic protein-7 (BMP-7) and investigated the effect on bone formation. In contrast to our study, they report high transduction efficiency ($\sim 99\%$) and long-term BMP-7 expression until day 57 that resulted in bone formation both *in vitro* and *in vivo*. Differences between our study and this

study include ASC passage number and vector preparation. Whereas our study investigated third-passage ASCs, this study employed fifth-passage cells. Moreover, rAAV was prepared in BHK-21 cells coinfecting with recombinant HSV-1 rather than the triple-plasmid transfection in HEK-293 cells used here. Finally, the prolonged BMP-7 expression may have been due to ASC differentiation into osteoblasts rather than prolonged gene expression. By contrast, and in agreement with our study, Locke and colleagues²⁷ compared alternative AAV serotypes and found that whereas rAAV2 decreased cell viability, rAAV5 was an excellent gene therapy vector for ASCs. Similar to our study, rAAV was produced by triple-plasmid transfection in HEK-293 cells. However, in contrast to our study, the study by Locke and colleagues only investigated transduction on day 3 and did not demonstrate the duration of gene expression or examine the correlation between ASC surface glycosylation and rAAV serotype transduction. Shevchenko and colleagues²⁸ used rAAV2 encoding human VEGF₁₆₅ to improve the angiogenic response in ischemic skeletal muscle. They achieved ~65% transduction and high GFP signal on days 2–4 and prolonged expression for up to 30 days. However, Shevchenko and colleagues used viral supernatant rather than purified virus. It has been found that AAV in supernatant is associated with microvesicles that significantly improve transduction over iodixanol-purified virus.^{43,44} Moreover, neither the MOI used in the experiments nor the ASC passage number was clear despite their critical importance in correlating the amount of transduction with the conditions of the ASCs. Future studies should directly compare ASC glycosylation and rAAV efficiency at various ASC passages, as well as the effect of different rAAV production methods in order to

optimize vector choice and timing of rAAV transduction.

In summary, despite the benefits of autologous ASC therapy, the complexity of the disease environment is likely to require multimodal treatments that will include ASCs modified to differentiate or produce therapeutically beneficial biologics. Given its safe track record and gene expression profile, rAAV5 is an excellent candidate and should be the vector of choice for creating novel ASC-derived treatments.

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