Capsid Engineering Overcomes Barriers Toward Adeno-Associated Viral Vector-Mediated Transduction of Endothelial Cells

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Endothelial cells (EC) are targets in gene therapy and regenerative medicine, but they are inefficiently transduced with adeno-associated viral (AAV) vectors of various serotypes. To identify barriers hampering efficient transduction and to develop an optimized AAV variant for EC transduction, we screened an AAV serotype 2-based peptide display library on primary human macrovascular EC. Using a new high-throughput selection and monitoring protocol, we identified a capsid variant, AAV-VEC, which outperformed the parental serotype as well as first-generation targeting vectors in EC transduction. AAV vector uptake was improved, resulting in significantly higher transgene expression levels from single-stranded vector genomes detectable already few hours post-transduction. Notably, AAV-VEC transduced not only proliferating EC but also quiescent EC, although higher particle-per-cell ratios had to be applied. Also, induced pluripotent stem cell-derived endothelial progenitor cells, a novel tool in regenerative medicine and gene therapy, were highly susceptible toward AAV-VEC transduction. Thus, overcoming barriers by capsid engineering significantly expands the AAV tool kit for a wide range of applications targeting EC.

Keywords: AAV vectors, endothelial cells, induced pluripotent stem cells, uncoating, uptake

INTRODUCTION

Adeno-associated viral (AAV) vectors are one of the most frequently applied gene delivery systems in preclinical research and in human clinical trials. Three AAV vector-based gene therapies, Glybera, Luxturna, and Zolgensma, have already received marketing authorization in the Western World. Further are expected to follow in the foreseeable future, because clinical benefit is reported for a wide range of monogenetic diseases. First-generation AAV vectors are based on naturally evolved serotypes or variants of adeno-associated viruses (AAV). AAV are replication-deficient and nonpathogenic members of the Parvoviridae, with AAV serotype 2 (AAV2) as the best characterized representative and vector prototype. AAV vectors deliver a single-stranded DNA genome into proliferating or post-mitotic cells. Cell transduction is mediated by the AAV capsid that is assembled from 60 monomers, which share the so-called common VP3 region. This region is identical in amino acid sequence to the major capsid protein, VP3 (60 kDa), and contains the receptor binding motifs. In contrast, the two minor capsid proteins, VP1 (90 kDa) and VP2 (72 kDa), contribute distinct domains such as those required for endosomal escape and
nuclear delivery located within their N-terminal extensions.\(^5\) Efficiency of in vivo gene transfer was greatly improved by using capsids of serotypes or variants others than AAV2 for packaging of recombinant AAV genomes.\(^4\) Nevertheless, still relatively high vector doses are required to obtain therapeutic effects,\(^6,7\) and distinct clinically relevant cell types such as keratinocytes or dendritic cells are refractory toward transduction.\(^3,9\)

Non-susceptibility due to lack of receptors is overcome by cell surface targeting.\(^5,10\) The same strategy is used to re-direct AAV's tropism toward a predefined cell type or a more precisely defined cell receptor.\(^5,10\) In particular, the variable loops at the peaks of the AAV capsid and the N-terminus of the VP2 proteins accept tropism-modifying alterations without impairing capsid assembly.\(^5,10\) Lack of knowledge on peptides directing the particle toward a receptor that initiates a productive infection without interfering with capsid assembly fostered the development of viral libraries composed of capsid-engineered AAV variants that are subjected to high-throughput selection screens. Although libraries were initially based on AAV2 and displayed 7mer peptides of random sequence at capsid position 587\(^1\) or 588,\(^12\) the portfolio has been expanded to other serotypes, to libraries displaying larger peptides or being composed of shuffled capsids.\(^13\) Through this powerful technology, a whole tool kit of AAV mutants with significantly improved gene delivery properties for in vitro and in vivo usage have become available. These efforts also increased our knowledge of AAV's infection biology and on cell surface molecules suited for cell entry targeting.\(^5,8,14\)

Here, we focused on endothelial cells (EC), a cell type highly relevant in translational and basic research, but poorly transduced by AAV vectors with natural-occurring serotype capsids. We performed an AAV2 peptide display library screening on primary human macrovascular EC in the absence of helper virus co-infection and in the presence of high selection pressure. In addition, production of sub-libraries was optimized through a novel next generation sequencing (NGS)-based amplification and cloning strategy. Thereby, AAV-V\(_{EC}\) was strongly selected. With \(>85\%\) of transduced cells, AAV-V\(_{EC}\) is far more efficient than the parental serotype or first-generation capsid-engineered vectors at transducing EC. Using AAV-V\(_{EC}\) and the second most enriched variant AAV-N\(_{EC}\) as tools, we identified distinct steps in AAV's infection path that limit transduction of EC. Finally, we demonstrated AAV-V\(_{EC}\)'s potency in modifying induced pluripotent stem cell (iPSC)-derived endothelial progenitor cells (EPC). As EPC home to damaged tissue or growing vessels,\(^15\) genetic modification of such cells might be envisioned as a valuable addition to current “Trojan horse” concepts in regenerative medicine and antitumor therapy.

**MATERIALS AND METHODS**

**Cell lines and primary cells**

The human embryonic kidney cell line HEK293 (ATCC number: CRL-1573) and human cervix carcinoma cell line HeLa (ATCC number: CCL-2) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with GlutaMAX-I (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 IU/mL of penicillin (Invitrogen), and 100 \(\mu\)g/mL of streptomycin (Invitrogen). AAV receptor knock-out (AAVR KO) cell lines based on HEK293 and HeLa, respectively, were generated before\(^16\) and cultivated as described earlier. Human umbilical vein EC (HUVEC) (PromoCell) was maintained in EC growth medium 2 (PromoCell) supplemented with 2% FBS, 5 ng/mL endothelial growth factor (EGF), 10 ng/mL basic fibroblast growth factor, 20 ng/mL insulin-like growth factor, 0.5 ng/mL vascular EGF (VEGF), 1 \(\mu\)g/mL ascorbic acid, 0.2 \(\mu\)g/mL hydrocortisone, 100 IU/mL of penicillin (Invitrogen), and 100 \(\mu\)g/mL of streptomycin (Invitrogen). Throughout the experiments, only low-passage HUVEC were used (passage 4–7). All cells were maintained in a humidified incubator with 5% \(CO_2\) at 37°C.

**Cell surface expression of AAV2 receptors**

Analyses of cell surface expression of AAV2 receptors on HUVEC (low passage) and HeLa cells were performed as described.\(^8,17\) Briefly, the following primary antibodies were used in this work: mouse anti-human \(\alpha\)v\(\beta\)5 antibody (MAB1961; Millipore), mouse anti-human \(\alpha\)5\(\beta\)1 antibody (MAB1999; Millipore), and mouse anti-human heparan sulfate delta antibody (USBiological). Goat anti-mouse IgG was used as secondary antibody (Southern Biotech). Cell surface expression was determined by flow cytometry (FACS Calibur; Becton Dickinson) after harvesting of cells by trypsin (HeLa; Invitrogen) or Accutase (HUVEC; Sigma) treatment. A minimum of 10,000 cells was counted for each sample.

**AAV peptide selection on HUVEC and AAV vector production**

AAV2 peptide display library\(^11\) was produced in HEK293 cells. Phenotype and genotype of variants was coupled. Low-passage HUVEC, 24 h post-seeding, were incubated with the AAV peptide library at a viral genome-per-cell ratio (GOD) of 1000
on ice for 1 h, followed by 3 h at 37°C. Particles that failed to enter the cells were removed by exchanging the medium. Cells were washed and then harvested by Accutase (Sigma) treatment. After a further washing step, total DNA was isolated (DNeasy Blood and Tissue kit; Qiagen). Amounts of particles that successfully entered cells were quantified by quantitative polymerase chain reaction (qPCR) using cap gene-specific primers. Cap sequences neighboring the insertion site were amplified by PCR and sub-cloned into pLG backbone with CLO primers (Supplementary Table S1). The sub-library was produced as described earlier. Meanwhile, one third of viral DNA was PCR amplified with NGS primers for sequencing. To raise the selection pressure, GOI was reduced in subsequent selection rounds (GOI 100 for 2nd and GOI 10 for 3rd round). The 3rd round of AAV peptide display selection was repeated. Cells were harvested, and DNA was isolated from cell nuclei after subcellular fractionation (Subcellular Protein Fractionation Kit for Tissues; Thermo Fischer Scientific).

NGS data obtained from that selection (Sub-Nuc) were compared with NGS data from round 1 to 3 of whole cell selection (Sub-Lib1–Sub-Lib3). Purity of fraction at the protein level was assayed by western blot analysis (Supplementary Fig. S2) using antibodies against AKT (cytosol; Cell Signaling Technologies, Inc.) and Lamin B (nucleus; Santa Cruz Biotechnology, Inc.).

Peptide sequences of the most prominent two variants were ordered as oligonucleotides and cloned into pRC99 by using MluI/AseI sites generating pRC99-V EC and pRC99-N EC, respectively. For production of AAV vectors, HEK293 cells were cotransfected with a total of 37.5 µg of pRC19, pRC99-N EC, pRC99-SIG18 or pRC99-NDVRA5, pscAAV/EGFP23 or pGFP, and pXX6.24 Of note, the AAV helper plasmid pRC99-NDVRA5 was cloned as described in Nicklin et al.16 by using the peptide sequence reported by Muller et al.12 Cells were harvested 48 h post-transfection, cell pellet was lysed, and vectors were purified by iodixanol step gradient centrifugation.20 Genomic particle titers were determined by qPCR (LightCycler System; Roche Diagnostics) using green fluorescent protein (GFP)-specific primers, whereas capsid titers were determined by A20-ELISA (Progen).

Quantification of cell entry and nuclear entry efficiency

For determining vector uptake, HUVEC were incubated with the indicated GOI for 30 min on ice, followed by a shift to 37°C and 5% CO2. Cells were harvested at the indicated time points post-transduction (p.t.) by Accutase or trypsin treatment and washing steps as described earlier. Total DNA was isolated (DNeasy Tissue Kit; Qiagen), and vector genomes were quantified by qPCR (Roche Diagnostics) using GFP-specific and plasminogen activator (PLAT)-specific primers.23 After confirmation of target specificity by melting curve analyses, GFP values were normalized to PLAT levels by using the LightCycler 480 software 1.5 (Roche Diagnostics). For determining nuclear entry efficiency, subcellular fractionation was performed as described earlier. The nuclear fractions were subjected to DNA isolation and analyzed as described earlier.

Cell binding assay

For determining the cell surface binding, HUVEC were seeded 24 h before transduction in 12-well plates with a density of 2.5 × 10³ cells per well. Cell number per well was determined before transduction and used to calculate the GOI. To synchronize cell transduction, cells were incubated on ice for 10 min, followed by a washing step with phosphate-buffered saline (PBS) (cold). Cold fresh media were added to cells, which were maintained on ice for a further 20 min. Then, indicated vectors (GOI 5 × 10³) diluted in cold media were added to the cells. After a further 1-h incubation on ice, one sample for each vector—corresponding to the time 0 h p.t.—was washed with PBS intensively (two times), harvested by trypsin treatment, and finally stored at −80°C. The remaining samples were incubated at 37°C until harvesting (4, 6, and 24 h p.t.). Again, supernatant was removed, and cells were washed with PBS. Trypsin treatment followed by two washing steps of the cell pellet was performed to remove membrane-bound particles. A part of the “24 h samples” was analyzed by FACS (CytoFLEX platform; Beckman Counter). All samples were then subjected to DNA isolation and qPCR analyses as described earlier.

Transduction of HUVEC, AAVR KO, and iPSC-derived EPC

For determining transduction efficiency, HUVEC, HEK293-AAVR-KO, HEK293, HeLa-AAVR-KO, and HeLa cells, respectively, were incubated with indicated vectors and GOI for 24 h followed by flow cytometric analyses (FACS Calibur; Becton Dickinson). Background fluorescence was set to 1% by using nontreated cells as reference. For transduction of quiescent EC, HUVEC were cultivated until they reached a confluency of ~90%, followed by incubation for another 24 h at 37°C and 5% CO2 with EC Basal Medium (PromoCell), which was...
devoid of all supplements and FBS. Finally, the status of quiescence was confirmed by staining with 5-ethynyl-2′-deoxyuridine (EdU) according to the manufacturer’s instruction (Invitrogen).

For transduction of iPSC-derived EPC (iEPC), iPSC clone H2E6C24 was differentiated into EC lineages by using a modified protocol based on a previously described procedure.25 Briefly, iPSC were cultured as monolayers in MEF-conditioned medium on Geltrix (Thermo Fischer Scientific) and mesodermal induction was initiated by the GSK3-inhibitor CHIR (Axon Medchem). Cells were further differentiated into EPC in VEGF (Peprotech) containing medium for 6 days. On day 7 of differentiation, EC were harvested by using StemPro Accutase (Thermo Fischer Scientific) and seeded on a gelatin (Sigma-Aldrich)-coated plate in EGM2 medium (Lonza). Transduction was performed at indicated days of differentiation with indicated vectors in EGM2 medium. GFP expression was analyzed 48 h p.t. by flow cytometry (FACS Calibur; Becton Dickinson).

**Heparin competition assay**

Overall, 2.5 × 10^4 HUVEC per well were seeded 24 h before transduction. This cell number was used for calculation of GOI. Cells were incubated for 10 min on ice; after a washing step (cold PBS), cold fresh media were added to the cells, which were maintained on ice for a minimum of 20 min. Viral vectors were diluted in heparin containing medium and added to the cells (final concentration indicated in figure). One hour post-vector application, samples were shifted to 37°C for 24 h. Percentage of transgene-expressing cells was determined by using flow cytometry (FACS Calibur; Becton Dickinson).

**Heparin affinity chromatography**

A HiTrap heparin affinity column (Amersham) was equilibrated with 1 × PBS/1 mM MgCl₂/2.5 mM KCl. Gradient-purified AAV vector preparation diluted in 1 × PBS/1 mM MgCl₂/2.5 mM KCl was collected. Flow-through and 4 × 5 mL wash fraction (1 × PBS/1 mM MgCl₂/2.5 mM KCl) were collected. Elution was performed with increasing ionic strength (1 × PBS/1 mM MgCl₂/2.5 mM KCl plus 100 mM NaCl up to 1 × PBS/1 mM MgCl₂/2.5 mM KCl plus 1 M NaCl). For each step, five column volumes were collected. Samples were analyzed by qPCR using transgene (GFP)-specific primers.

**Capsid thermal stability assay**

We performed the capsid thermal stability assay as described.26 Briefly, wells of qPCR plates were loaded with 5 × 10^8 vector genome containing particles of indicated AAV vector preparations diluted in PBS. The temperature gradient was generated by a LightCycler® 96 System (Roche Life Science). Then, PBS was used to dilute samples. Samples were transferred to a nitrocellulose membrane by using a vacuum blotter at native dot blot conditions. Membrane was blocked and then incubated with primary (A20, or B1; Progen) and then secondary (peroxidase-conjugated antibodies) antibodies. Finally, membranes were incubated with an enhanced chemiluminescence reagent (West Dura; Pierce) and analyzed by autoradiography film exposure or FusionFX device (Peqlab).

**Statistical analysis**

Statistical analyses and significance tests were performed by applying unpaired, two-tailed t-tests. p-values of <0.05 were considered significant.

**RESULTS**

**Insufficient levels of heparan sulfate proteoglycan may limit EC transduction**

At a vector GOI with which more than 80% of HeLa cells were transduced with a single-stranded AAV2 vector encoding for enhanced GFP, only 18.0 ± 4.95% of HUVEC expressed the reporter gene (Fig. 1A). This result is consistent with earlier

**Figure 1.** Vascular endothelial cells are poor targets for AAV2 transduction. (A) Transduction efficiency on low-passage HUVEC. HeLa cells or low-passage HUVEC were incubated with AAV2 vectors encoding for enhanced GFP controlled by the human CMV promoter in a single-stranded vector genome conformation at a GOI of 10,000. Percentage of GFP-expressing cells was determined by flow cytometry 24 h p.t. Shown are mean values of two (HeLa) or three (HUVEC) independent experiments. Error bars indicate SD. (B) Expression of AAV2 receptors. Low-passage HUVEC were stained with mouse anti-human heparan sulfate delta, α5β1 and αvβ5 integrin antibodies, respectively, followed by a phycoerythrin-labeled IgG secondary antibody. Percentage of cells positive for HSPG or indicated integrins was quantified by flow cytometry. Shown are mean values of three independent experiments. Error bars indicate SD. Insert: same stainings were performed on HeLa cells. Shown is the mean of two independent experiments. AAV2, adeno-associated viral serotype 2; CMV, cytomegalovirus; GFP, green fluorescent protein; GOI, genome-per-cell ratio; HSPG, heparan sulfate proteoglycan; HUVEC, human umbilical vein endothelial cells; p.t., post-transduction; SD, standard deviation.
reports showing that AAV2 and other AAV serotypes are inefficient in EC transduction.\textsuperscript{12,18,27,28}

Low permissiveness is frequently associated with inefficient provision of receptors; therefore, HUVEC were analyzed for the presence of AAV2’s primary receptor, heparan sulfate proteoglycan (HSPG),\textsuperscript{29} and the internalization receptors \(\alpha_{v\beta5}\) and \(\alpha_{5\beta1}\) integrin,\textsuperscript{30,31} respectively. As shown in Fig. 1B, HUVEC clearly stained positive for \(\alpha_{v\beta5}\) and \(\alpha_{5\beta1}\), whereas HSPG was barely detectable. In contrast, HeLa cells showed considerable expression of both, HSPG and the two integrins (Fig. 1B, insert).

Given the dependency of AAV2 on HSPG for subsequent steps in cell transduction, such as priming the capsid for co-receptor binding, endosomal escape, and uncoating,\textsuperscript{32} it is reasonable to postulate that the low HSPG expression level negatively impacts AAV2 transduction efficiency.

**AAV peptide selection of AAV capsid variants on HUVEC**

To develop an AAV variant that overcomes this pre- and maybe further post-entry barriers, an AAV peptide display screening was performed. The library was based on AAV2, displayed random 7mer peptides at position 587, and was screened on HUVEC by applying the following criteria: (1) Selections were performed in the absence of helper virus co-infection to avoid any assistance in cell infection caused either directly by helper virus proteins or indirectly by changing the intracellular milieu, (2) time of library exposure was limited to three hours, and (3) selection pressure was constantly increased by decreasing the GOI to select for highly infectious variants.

In total, three rounds of selection were performed on low-passage HUVEC. Each time, viral genomes were isolated from whole cell extract. Genomes served as the template for sub-library production by using a novel NGS-based protocol (Supplementary Fig. S1). In parallel, NGS was performed to monitor the selection procedure (Fig. 2). Cluster analysis revealed a strong selection of two viral variants, AAV-VSSSTPR and AAV-NNPLPQR (Fig. 2A). Both variants were recovered with a frequency of 0.03% from the 1st round of selection. Frequency of occurrence increased to 0.42% (VSSSTPR) and 0.88% (NNPLPQR) in the 2nd and finally to 2.93% (VSSSTPR) and 3.23% (NNPLPQR) in the 3rd round of selection.

![Figure 2. Heatmap of variants determined by NGS. (A) Sequences recovered from whole cell lysates. After each round of selection, viral genomes isolated from library-infected HUVEC were subjected to NGS. Between 18,000 (sub-library 2) and 26,000 (sub-library 1) reads were performed. Results of selection round 1 (GOI 1000), round 2 (GOI 100), and round 3 (GOI 10) are shown. Color code represents the frequency of occurrence for each clone according to the R-package heatmap (Pretty Heatmaps by Raivo Kolde). (B) Sequences recovered from nuclear fraction. Sub-library 2 was used to infect HUVEC (GOI 100), followed by isolation of the nuclear fraction (Supplementary Fig. S2) and NGS of isolated viral genomes. NGS, next generation sequencing.](https://example.com/figure2)
Isolation of viral variants from whole cell lysate indicates successful viral cell entry; however, it does not indicate the ability of the mutants to reach the nucleus. Consequently, the 3rd round of selection was repeated. When cells were harvested this time, nuclei were isolated (Supplementary Fig. S2) and then subjected to NGS sequencing to identify viral variants that accumulated in this cell compartment (Fig. 2B). Comparing the sequence pattern of this selection with those obtained for the whole cell lysate suggests that indeed not all variants reached the nuclear area with the same efficiency (Fig. 2A vs. B). VSSSTPR and NNPLPQR, however, remained the most frequently selected variants, although the ranking changed (3.27% for VSSSTPR vs. 2.62% for NNPLPQR). Analyzing genomes encoding for VSSSTPR and NNPLPQR, respectively, revealed at least three independent selection events, that is, selection of three independent clones for each of the two peptide motifs (Table 1).

AAV targeting vectors transduce HUVEC with higher efficiency

To characterize the tropism of AAV-VSSSTPR and AAV-NNPLPQR, variants were produced as vectors (AAV-VEC and AAV-NEC) displaying the respective peptide at position 587 in each of the 60 capsid subunits. Vectors again delivered GFP as reporter gene in a single-stranded vector genome conformation. AAV2 was prepared in parallel, to act as a control. The parental serotype and the newly developed variants showed a comparable packaging efficiency, revealing that ligand insertion did not interfere with vector production or with vector genome packaging (Supplementary Table S2).

Vectors were then tested on low-passage HUVEC (Fig. 3). Of the two mutants, AAV-VEC showed the highest transduction efficiency at all vector GOIs. At the lowest GOI (GOI 3600), AAV-VEC transduced 28.1 ± 6.8% of HUVEC cells. Transduction rate increased to up to 68.2 ± 6.9% at a GOI of 30,000, representing an up to 2.7-fold increase in transduction efficiency over AAV2. In contrast, although selected with a comparable efficiency as AAV-VEC, AAV-NEC was significantly less efficient in transducing HUVEC. However, AAV-NEC demonstrated a significantly improved EC transduction rate as compared with AAV2 at the highest vector dose tested.

Particularly in primary cells and in vivo, conversion of single-stranded AAV (ssAAV) vector genomes into a transcriptionally active double-stranded conformation has been identified as a post-entry barrier that can be overcome by usage of a self-complementary genome conformation.33 To evaluate whether EC transduction efficiency can be further improved by switching to a self-complementary genome design, we performed a side-by-side comparison of conventional single-stranded and self-complementary vector genomes delivered by AAV-VEC, our best performing variant (Fig. 4). Especially at lower GOI, self-complementary AAV (scAAV)-VEC showed a higher transduction efficiency compared with ssAAV-VEC. Of note, scAAV-VEC at a GOI of 2500 was already sufficient to achieve a transduction efficiency of 50%.

AAV-VEC is superior to previously developed capsid variants

AAV targeting vectors for EC transduction were previously developed.12,18,28,34–39 The first example—already described in 2001—is AAV-SIG,

<table>
<thead>
<tr>
<th>Motif</th>
<th>Sequence</th>
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<tr>
<td>VSSSTPR</td>
<td>GTG AGC AGC TCG ACC CCG AGG</td>
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<td>GTG AGC AGC TCA ACC CCG AGG</td>
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<tr>
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<td>AAC AAC CCG CTC CGG CAG CGG</td>
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<td></td>
<td>AAC AAC CCG CTC CCA CAG CGG</td>
<td>1.38</td>
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<tr>
<td></td>
<td>AAC AAC CCG CTC CGG CAA CCG</td>
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Table 1. Viral genomes encoding selected capsid-modified adeno-associated viral variants

Next generation sequencing revealed selection of the same peptide motif by different viral clones. The percentage represents the portion of the individual sequence coding for the corresponding motif.

![Figure 3](attachment:image.png)

**Figure 3.** Transduction efficiency of AAV variants compared with AAV2. Low-passage HUVEC were incubated with a serial dilution of AAV2 (black), AAV-NEC (light gray), or AAV-VEC (dark gray). Vectors encode for GFP controlled by CMV promoter in a single-stranded vector genome conformation. Numbers on X-axis indicate genome (vg)-per-cell ratio. The percentage of transgene (GFP)-expressing cells was determined by flow cytometry 24 h p.t. Experiments were performed three times independently. Error bars indicate SD. **p < 0.001; *p < 0.05. n.s., nonsignificant; vg, vector genome.
which displays the SIGYPLP peptide at position 587. Direct comparison of AAV-SIG with AAV-V_{EC} by incubating low-passage HUVEC at a GOI of 10,000, followed by quantification of GFP-expressing cells 24 h later, revealed AAV-V_{EC} to have a 13-fold greater transduction efficiency (Fig. 5A).

Although the SIGYPLP peptide was selected on HUVEC, the same cell type as AAV-V_{EC}, but by phage display and not by AAV peptide display, Muller et al. performed the first AAV2 peptide display on EC and identified AAV-NDVRAVS as an efficient variant for transducing human coronary artery EC. Comparison of AAV-NDVRAVS with AAV-V_{EC} again revealed the superiority of AAV-V_{EC} to have a 13-fold greater transduction efficiency (Fig. 5B).

**Barriers toward AAV2-mediated transduction of vascular EC**

AAV peptide display selects from a pool of capsid variants those that comply best with the selection criteria. Our results argue that, in particular, not only AAV-V_{EC} but also AAV-N_{EC} possess features that are beneficial for EC transduction that are not only superior to other variants in our library but also superior to AAV2 (Fig. 3). Based on this, we characterized the vector-EC interaction in more detail.

The kinetics and efficiency of cell entry as an indicator of successful uptake were determined by quantifying intracellular vector genomes at indicated time points by qPCR (Fig. 6). Highest vector

**Figure 5.** Improved transduction efficiency compared with previously developed capsid-engineered AAV vectors. Low-passage HUVEC were incubated with indicated capsid-modified vectors encoding for GFP in a single-stranded vector genome conformation at GOI of 10,000. The percentage of GFP-expressing cells was determined by flow cytometry 24 h p.t. (A) Mean value of two independent experiments; (B) mean of three independent experiments ± SD. *p < 0.05.

**Figure 6.** AAV targeting vectors differ in pre-entry and intracellular steps. Low-passage HUVEC were incubated with AAV2 (black), AAV-N_{EC} (light gray), or AAV-V_{EC} (dark gray), at a GOI of 10,000. Cells were harvested at the indicated time points (p.t.), and unbound and membrane-bound particles were removed. (A) Intracellular particles in whole cell lysate: total DNA was isolated, and number of intracellular vector genomes was quantified by qPCR using GFP- and PLAT-specific primers. Values obtained for vector genomes were normalized to values for PLAT. Shown are results of three independent experiments ± SD. *p < 0.05. (B) Particles in nuclear fraction: Vector-treated cells were subjected to cell compartmentalization 8 h p.t. Total DNA was isolated from the nuclear fraction. Vector genomes were quantified by qPCR using GFP-specific primers and normalized to PLAT. Shown are results of three independent experiments ± SD. **p < 0.001; *p < 0.05. PLAT, plasminogen activator; qPCR, quantitative polymerase chain reaction.
copy numbers (VCN) were detected for AAV-VEC at all time points. AAV-VEC and the parental serotype AAV2 reached a plateau at 4 h p.t., whereas VCN continuously increased with time in the case of AAV-NEC, reaching a VCN comparable to AAV-VEC at 8 h p.t. To discriminate whether improved binding or internalization is responsible for the improved entry efficiency, we measured cell binding in the cold and again intracellular vector particles on shifting samples to 37°C (Supplementary Fig. S3). Although AAV-VEC and AAV2 did not differ in cell binding, we again observed significantly higher intracellular VCN for AAV-VEC compared with AAV2. Overall, the results indicate that AAV-VEC is internalized into HUVEC with a significantly higher efficiency than AAV2.

Determination of intranuclear vector genomes at 8 h p.t. by isolating cell nuclei of vector-treated cells followed by qPCR measurements revealed that AAV-VEC and AAV-NEC showed a comparable VCN, which again was significantly higher than the VCN of AAV2 (Fig. 6). Measuring VCN does not allow to discriminate between vector genomes that have been released from the capsid (uncoating) and those that are still contained in the capsid and are thus not available for transcription. As an indirect measure for uncoating efficiency, we therefore determined the onset and level of transgene expression. Interestingly, our two targeting vectors that reached the nuclear area with comparable efficiency differed with regard to the onset of transcriptional activity and transgene expression level, arguing for an improved uncoating efficiency of AAV-VEC compared with AAV-NEC (Fig. 7). Not only AAV-NEC but also AAV2 demonstrated a significantly later onset of transcriptional activity (Fig. 7A), a difference confirmed by flow cytometry (Fig. 7B). However, in case of AAV2, it remains to be shown whether the lower transcriptional activity is, indeed, due to a lower uncoating efficiency or caused by the lower of number of particles since significantly fewer AAV2 particles were internalized and accumulated in the nuclear area. An argument for the former, however, is the lower (thermal) stability of AAV-VEC compared with AAV2 (Supplementary Fig. S4).

AAV-VEC binds heparin, a soluble analogue of HSPG

Insertion of peptides in position 587 of the AAV capsid proteins destroys the HSPG-binding motif of wild-type AAV2,40 which hampers the natural infection process. This is an advantage for cell surface targeting approaches aiming at re-directing AAV’s tropism.40 However, on insertion of overall positively charged peptides or of peptides that form an HSPG-binding motif, targeting vectors regain the ability to bind to HSPG although affinity might differ.41,42

Interestingly, our selection on HUVEC, which were performed without any selection pressure regarding specificity of cell transduction, yielded exclusive variants that present an arginine (R) residue at position 7 of the peptide insertion (Fig. 2). This arginine might form together with the two linker amino acids (AA in our case) and R588 of the wild-type AAV2 sequence a heparin binding motif (RXXR)41,42 that contributes to cell transduction. To test whether AAV-VEC is able to bind to HSPG, we performed a heparin affinity chromatography and eluted viral particles with increasing salt concentration (Fig. 8A). For comparison, AAV2 was subjected to the same test. AAV2 was eluted as a single peak when adding 300 mM NaCl. In contrast, lower salt concentrations were sufficient to compete with the binding of AAV-VEC. In line, the addition of increasing concentrations of heparin to AAV2 and AAV-VEC before cell transduction revealed that a lower concentration of heparin is Figure 7. AAV-VEC does show a fast onset of transgene expression. HUVEC were incubated with AAV2 (black), AAV-NEC (light gray), or AAV-VEC (dark gray) encoding for GFP in a single-stranded vector genome conformation at a GOI of 10,000. (A) Quantification of transgene-specific mRNA. Total RNA was isolated at the indicated time points p.t., and GFP expression levels were determined by qPCR using GFP-specific primers. Values were normalized to GAPDH. (B) Quantification of transgene-expressing cells. Cells were harvested at the indicated time points p.t., and percentage of transgene expressing cells was determined by flow cytometry. Shown are results of three independent experiments ± SD. *p < 0.05.
sufficient to interfere with AAV-V EC-mediated transduction of HUVEC (Fig. 8B, C).

AAVR is an essential receptor for the large majority of AAV serotypes to transduce human cells and mouse tissues.16,43 Recently, two groups mapped for AAV2 the AAVR binding site to the spike region adjacent to the threefold axis of symmetry.44,45 Since our capsid modification concerns the tip of the second highest spike, we were interested in determining whether AAV-V EC differs from AAV2 regarding the dependency on AAVR for cell transduction. In the absence of an EC-specific AAVR knockout cell line, we used HEK293-AAVR-KO and HeLa-AAVR-KO as model systems. These cells, and as control parental AAVR wild-type cells, were transduced with increasing doses of AAV-V EC and AAV2, respectively (Supplementary Fig. S5). As expected, in the absence of AAVR, the transduction efficiency of AAV2 was reduced to background. AAV-V EC also transduced both parental cell types, but neither in HEK293-AAVR-KO nor in HeLa-AAVR-KO transgene expression above background was observed, revealing that at least for these otherwise highly permissive cell lines AAVR is an essential factor for AAV2 as well as for AAV-V EC.

Overall, these results indicate that AAV-V EC binds heparin/HSPG, although with a lower affinity compared with AAV2. Based on the strong selection for R in position 7 of inserted peptides, we might argue that binding to HSPG or other negatively charged cell surface molecules is an advantage for transducing EC. However, binding to HSPG is also correlated with a broad tropism (Uhrig et al.42 and Supplementary Fig. S5) and accumulation in liver tissue when vectors are applied intravenously.41 Thus, AAV-V EC might be best suited for local in vivo application or ex vivo transduction of EPC that are subsequently infused in case an EC-selective transduction is desired.

AAV-V EC transduces quiescent EC with higher efficiency than AAV2 or AAV-N EC

Proliferating EC as used in this study are located at the tip of sprouting vessels under physiological

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Figure 8. AAV-V EC does bind heparin and thus HSPG. (A) AAV-V EC shows lower affinity to heparin compared with AAV2. Heparin affinity chromatography was performed for AAV2 (black) and AAV-V EC (dark gray) vector stocks. Ten fractions were collected by using elution buffers with increasing ionic strength. For each fraction, five column volumes were collected. VCN were measured by qPCR using GFP-specific primers. Percentage of VCN was calculated as relative vector copies by setting total VCN to 100%. Shown are results of a single experiment. (B, C) Cell transduction by AAV-V EC compared with AAV2 is competed by lower concentration of heparin. HUVEC were incubated with AAV2 (black) or AAV-V EC (dark gray) delivering an sc vector genome encoding for GFP controlled by a CMV promoter with a GOI of 10,000, in the presence of different concentrations of heparin. Percentage of GFP-expressing cells was determined 24 h p.t. by flow cytometry. The statistical analysis comparing the transduction efficiencies between AAV2 and AAV-V EC is shown in (A). The statistical analysis comparing the different concentrations of heparin to control, for AAV2 (upper panel) and AAV-V EC (lower panel) is shown in (B). Shown are results of four independent experiments using two different batches for each vector – SD. *p < 0.05; **p < 0.01; ***p < 0.001. VCN, vector copy numbers.
conditions (i.e., wound healing) and during tumor-induced angiogenesis, whereas the majority of EC lining the inner part of vessels are nonproliferating. To mimic these conditions, quiescence was induced by depleting supplements and FBS from culture medium as indicated by reduced incorporation of EdU (Fig. 9A vs. B). Side-by-side comparison of AAV2, AAV-V_EC, and AAV-N_EC demonstrated that AAV-V_EC was the most efficient vector with exception of transduction at GOI 2000 (Fig. 9C). For AAV-N_EC, an advantage compared with AAV2 was again only observed at the highest vector dose. However, transduction efficiency for AAV2, AAV-VEC, and AAV-N_EC on quiescent cells was lower compared with proliferating cells.

**iPSC-derived EPC are highly susceptible toward AAV-VEC transduction**

*Ex vivo* genetic modification of EPC followed by re-infusion might represent a promising alternative to direct *in vivo* modification, because EPC home to sites of tissue damage or tumor growth. Consequentially, we tested iPSC-derived EPC (iEPC), which are generated in a patient-specific manner, but they also hold promise as a future source for off-the-shelf EPC, regarding their transducibility with AAV vectors. Specifically, a human fibroblast-derived iPSC clone was differentiated into iEC by using a forward reprogramming protocol. At days 8 and 14 of differentiation, iEC were incubated with AAV-V_EC and AAV2 vectors at a GOI of 200, followed by transduction efficiency analyses 48 h later (Fig. 10).

At both stages of differentiation, iEC were susceptible toward AAV2 and AAV-V_EC. AAV-V_EC, however, transducing iEC with, for example, 76.1 ± 1.9% of transduced iEC (d14) was clearly superior versus 20.8 ± 1.3% for AAV2. This high efficiency of transduction prompted us to assay even lower GOI (Supplementary Fig. S6). Interestingly, with a GOI as low as 50, >40% of AAV-V_EC treated cells showed reporter gene expression clearly above background. Transduction efficiency of AAV-V_EC could be increased to nearly 100% with a GOI of only 1000 (Supplementary Fig. S6).

**DISCUSSION**

EC form the inner layer of vessels controlling vasomotor tone, transport, and permeability. In addition, EC contribute to the innate immune response, possess key functions in vascular homeostasis, and secrete proteinous factors into the blood stream. Consequently, disruption of the EC layer or of vascular homeostasis triggers disease processes, making EC attractive targets for therapeutic interventions. The latter include genetic modification in gene therapy or regenerative medicine settings employing the homing capability of EC to repair damaged tissue or to target growing (tumor) vessels. EC are, however, only weakly susceptible to the most common viral vectors. Here, we focused on the AAV vector system and developed an AAV variant, AAV-V_EC, for improving transduction of proliferating, quiescent, 

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**Figure 9.** Quiescent HUVEC are permissive, in particular, for AAV-V_EC (A, B) EdU staining of HUVEC. (A) Proliferating HUVEC and (B) HUVEC after induction of quiescence through depletion of supplements and fetal bovine serum were fixed. Proliferation is marked by EdU incorporation (red stained cells). Cell nuclei were stained by DAPI (blue stained cells). (C) Transduction of quiescent cells. Low-passage HUVEC were incubated with a serial dilution of AAV2 (black), AAV-N_EC (light gray), or AAV-V_EC (dark gray). Vectors encode for GFP controlled by CMV promoter in a single-stranded vector genome configuration. GOI is indicated. Percentage of transgene (GFP)-expressing cells was determined by flow cytometry 24 h.p.t. Experiments were performed three times independently. Error bars indicate SD. **p < 0.001; *p < 0.05. EdU, 5-ethynyl-2’-deoxyuridine.

**Figure 10.** Transduction of iEC at days 8 and 14 of differentiation. The human fibroblast-derived iPSC clone H2E6C24 was differentiated into iEC. At days 8 and 14 of differentiation, iEC were incubated with AAV2 (black) or AAV-V_EC (dark gray). Vectors delivered a self-complementary vector genome encoding for GFP controlled by a CMV promoter. Transduction efficiency and transgene expression level was determined by flow cytometry 48 h.p.t. (A) Percentage of GFP-expressing cells and (B) MFI. In B, values obtained for AAV2 are set to one. Shown are results of three independent experiments ± SD. **p < 0.001; ***p < 0.001. iEC, iPSC-derived endothelial progenitor cells; iPSC, induced pluripotent stem cell; MFI, mean fluorescence intensity.
and iPSC-derived EC. As this variant clearly outperformed the parental serotype, we used this variant (and AAV-NEC) to identify barriers that limit transduction of EC.

AAV vector transduction is a multistep process that is initiated by binding to cell surface glycans such as sialic acids in case of AAV1, -4, -5, and -6, or HSPG in case of AAV2, the serotype we used as the basis for our study. Antibody staining confirmed the presence of both integrins, whereas HSPG was barely detectable (Fig. 1B). This condition did not impair vector uptake since AAV2 vector particles were detected intracellularly but seemed to limit efficacy of AAV2 internalization (Fig. 6, Supplementary Fig. S3), because with AAV-VEC, a vector that uses its inserted peptide for cell entry, significantly higher VCN were detected (Fig. 6, Supplementary Fig. S3). AAV2, AAV-VEC, and AAV-NEC were trafficked to the nucleus, although significantly more capsid-engineered AAV than AAV2 particles reached the nucleus (Fig. 6). Interestingly, nuclear accumulation did not correlate with transduction efficiency. Specifically, AAV-VEC and AAV-NEC differed remarkably regarding the onset of transgene expression as well as the level of transgene expression, although both vectors exhibited a comparable extent of nuclear trafficking (Figs. 3, 6, and 7). Transgene expression requires availability of vector genomes for transcription. As vectors delivered identical vector genomes, AAV-V EC seems to be more efficient in releasing vector genomes. This feature is may be due to the specific peptide insert, VSSSTPR, or the receptor and respective downstream signaling pathways activated on binding of this engineered capsid to the EC surface or a combination of both mechanisms. Once released from the capsid, single-stranded AAV vector genomes need to be converted into a double-stranded DNA for initiation of transcription. Since AAV-V EC applied in the expression kinetics experiment (Fig. 7) delivered a single-stranded AAV vector genome from which transcribed mRNA is detectable by qPCR already at early time points p.t., EC—at least HUVEC—possess all necessary components to assist AAV in second-stranded synthesis. Nevertheless, using a self-complementary instead of the natural single-stranded vector genome conformation is advantageous, particularly when only a few vector genomes are present, that is, in conditions of low GOI (Fig. 4).

Thus, regarding the main steps in AAV infection—cell surface attachment, internalization, trafficking toward the nuclear area, release from the endosomal compartment, nuclear delivery, uncoating, and second-strand synthesis—vector uptake and uncoating appear as the most critical steps that impact EC transduction, whereas use of a self-complementary vector genome conformation can further improve transduction efficiency.

AAV-V EC, selected by an AAV peptide display screen on HUVEC, differs from AAV2 in the 7mer peptide VSSSTPR inserted at position 587 of each of the 60 subunits of the AAV capsid. Peptides inserted at that position were demonstrated to mediate receptor binding and determine vector tropism. The receptor for AAV-V EC is currently unknown, a general caveat of directed evolution approaches in which variants are selected for tropism for a predefined target cell population without the option to redefine the target receptor. The latter—if required—needs to be determined afterward, for example, by comparative gene analyses. Despite this drawback, high-throughput selection screens of AAV capsid libraries have—since their first application more than 15 years ago—demonstrated their potency to tailor the AAV-target cell interaction without the need to know barriers that limit respective target cell transduction. As mentioned earlier, the adaptation in AAV-EC interaction regarding AAV-V EC concerned vector uptake (compared with AAV2) and uncoating (compared with AAV-N EC). Comparison of AAV-V EC with AAV-SIG, a first-generation capsid-engineered vector that displayed a peptide identified by phage display selection on HUVEC, revealed a 13-fold higher transduction efficiency for AAV-V EC (Fig. 5A). This is likely due to the fact that selection of the SIGYLP peptide was based on overcoming only one barrier, that is, the uptake process, since the SIGYLP peptide was selected for improved binding to EC without tackling further downstream barriers. Our capsid variant selected here also demonstrated superior transduction efficiency compared with AAV-NADARVS (Fig. 5B). Since we did not perform a side-by-side comparison of AAV-V EC and AAV-NADARVS for the various steps of EC transduction, we can only speculate on the reason(s) for this observation. It is possible that the NADARVS peptide is underestimated in our case, as we cloned this peptide into position 587, whereas Muller et al. selected the peptide from an AAV peptide display library displaying peptides at position 588, the neighboring position. This peptide, however, mediated EC targeting with a comparable efficiency when displayed at an AAV9 capsid, which argues against a positional effect. Alternatively, improved efficiency by AAV-NADARVS may be restricted to human coronary EC, the EC subtype this
variant has been selected on, and/or requires cofactors since selection of AAV-NADARVS was performed in the presence of adenovirus, whereas we decided against adenovirus superinfection.

We did not perform extensive screenings for EC subtypes that were susceptible to AAV-V_EC, but we demonstrated AAV-V_EC’s ability to transduce HUVEC independent of their proliferation state as well as iPSC-derived EPC (Figs. 3, 9, and 10). The latter are currently entering the arena of drug screening as they allow patient-specific disease modeling. In addition, EPC home to damaged vasculature, making them promising targets for genetic modification to develop novel therapeutic strategies in the context of regenerative medicine, gene therapy, organ transplantation, and cardiovascular disease as well as antitumor therapy. Given the recent advancement in iPSC technology, in particular, with regard to “off the shelf cell products,” iPSC-derived EPC will likely replace conventional EPC in the clinical setting. With its impressive efficacy in transducing iPSC-derived EPC, AAV-V_EC may represent a highly potent delivery system also in this setting.

In summary, we here report on the development of AAV-V_EC, a capsid-engineered AAV vector, that is internalized and consequently transported to the nucleus with significantly higher efficacy than the parental serotype. AAV-V_EC showed efficient transduction of conventional EC at different states of differentiation and also demonstrated an impressive efficacy in iPSC-derived EPC. Of note, efficient and fast onset in transgene expression was observed even when delivering the vector genome in its natural single-stranded DNA conformation. This option is particularly promising for clinical application, as the full coding capacity of the AAV vector system is thereby available for delivery of transgene expression cassettes of ~5 instead of 2.5 kb, the upper limit of AAV vectors with a self-complementary vector genome design.

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AUTHOR DISCLOSURE
No competing financial interests exist.

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SUPPLEMENTARY MATERIAL
Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Figure S6
Supplementary Table S1
Supplementary Table S2

REFERENCES


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Supplementary Figure S1. NGS-based monitoring and cloning procedure. Total DNA isolated from library-treated cells. NGS primers are designed to recognize unique cap sequence flanking the random sequence coding for the 7mer peptide and restriction enzyme recognition sites. (left panel) Molecular cloning of sub-library. The forward NGS primer has been modified with biotin, whereas the backward NGS primer remained unmodified. After the PCR amplification step, DNA amplicons are carrying a biotin residue at the 5'-end and contain the random peptide encoding sequences flanked by AscI and NotI restriction enzyme recognition sites. These two restriction sites are the same that were used for insertion of the random oligonucleotides for the initial library cloning. DNA amplicons are treated with NotI and are purified by streptavidin microbead-mediated pull-down followed by AscI-mediated release of the amplicons from the beads. The latter were then used for ligation and generation of the sub-library. (right panel) Monitoring of AAV library selection process. The same NGS primers (left panel, but now both unmodified) were used to monitor the selection process as such and the enrichment of specific clones to identify candidates for further characterization. AAV, adeno-associated viral; NGS, next generation sequencing; PCR, polymerase chain reaction.
Supplementary Figure S2. Subcellular compartmentalization. HUVEC were subjected to cell compartmentalization according to the manufacturer’s protocol. Proteins extracted from the cytosolic membrane and nuclear fractions were analyzed by western blotting using antibodies against AKT and Lamin B. HUVEC, human umbilical vein endothelial cells.

Supplementary Figure S3. AAV-VEC is internalized with higher efficiency, despite a similar binding ability compared with AAV2. HUVEC were incubated with AAV2 (black) or AAV-VEC (dark gray) delivering an sc vector genome encoding for GFP controlled by CMV promoter at a GOI of 5,000. Transduction was synchronized on ice for 1 h. (A) Transduction efficiencies. Percentages of GFP-expressing cells were determined 24 h.p.t. by flow cytometry. (B) Binding efficiencies. Before shifting to 37°C, unbound particles were removed and cells were harvested. Total DNA was isolated, and VCN was quantified by qPCR using GFP primers and normalized against PLAT, which served as housekeeping gene. (C, D) Entry efficiencies. At the indicated time points, membrane-bound and unbound particles were removed, total DNA was isolated, and VCN was quantified by qPCR using GFP primers and normalized against PLAT. Statistical analysis comparing vectors is shown in (C), the one comparing the different time points for AAV2 (upper panel) and AAV-VEC (lower panel), is shown in (D). Shown are results of four independent experiments using two different batches for each vector ± standard deviation. *p < 0.05; **p < 0.01; ***p < 0.001. AAV2, AAV serotype 2; CMV, cytomegalovirus; GFP, green fluorescent protein; n.s., non-significant; PLAT, plasminogen activator; p.t., post transduction; qPCR, quantitative PCR; VCN, vector copy numbers.
**Supplementary Figure S4.** AAV-VEC capsids show lower thermal stability compared with AAV2. AAV2 and AAV-VEC were submitted to a temperature gradient followed by a native dot blot analysis using A20 and B1 antibodies, allowing to discriminate intact and disassembled AAV capsids, respectively. Shown are results of a single experiment.

**Supplementary Figure S5.** AAV-VEC requires AAVR for efficient transduction of HeLa and HEK-293 cells. AAV2 (upper panels) or AAV-VEC (lower panels) delivering an sc vector genome encoding for GFP controlled by CMV promoter were applied to HeLa (left panels) or HEK-293 (right panels) cell lines that express (wt) or not AAVR (AAVR-KO). Percentage of GFP-expressing cells was determined 24 h p.t. by flow cytometry. Shown are results of two independent experiments (using two batches of both vectors; mean value). AAVR-KO, AAV receptor knock-out.
Supplementary Figure S6. Transduction of iEC with increasing vector GOI. iEC at day 8 of differentiation were incubated with indicated GOI of AAV-V ec and AAV2, respectively. AAV vector encoded for GFP controlled by CMV promoter in a self-complementary vector genome conformation. GOI, genome-per-cell ratio; iEC, induced pluripotent stem cell-derived endothelial progenitor cells.

Supplementary Table S1. NGS primer sequences

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<th>Sequences</th>
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MID, NGS identifier sequence; NGS, next generation sequencing.

Supplementary Table S2. Characterization of AAV vector preparations

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<th>Capsid titer ($\times 10^9$/μL)</th>
<th>Capsid-to-genomic ratio</th>
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<td>16.61 ± 8.88</td>
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Indicated viral vectors were produced at least three times independently. Genomic titer (vector genome containing particles per microliter), capsid titer (intact capsids per microliter), and packaging efficiency were determined. Packaging efficiency is defined as the ratio of capsid titer-to-genomic titer. Wild-type phenotype: below 50. Values are mean of three independent experiments ± standard deviation.

AAV2, adeno-associated viral serotype 2; EC, endothelial cells.

SUPPLEMENTARY REFERENCE

AUTHOR QUERY FOR HUM-2019-027-VER9-ZHANG_1P

AU1: Please note that gene symbols in any article should be formatted as per the gene nomenclature. Thus, please make sure that gene symbols, if any in this article, are italicized.

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AU5: The Publisher requests for readability that no paragraph exceeds 15 typeset lines. Please check for long paragraphs and divide where needed.

AU6: Please fix the expansion of the acronym “AAV”: “adeno-associated viruses” or “adeno-associated viral”?

AU7: Please expand “pLG.”

AU8: Please expand “CLO.”

AU9: Supplementary Figure S2 citation is not in sequential order in the text. Please check.

AU10: Please expand “MEP.”

AU11: Please mention the appropriate figure number in “figure.”

AU12: Please define “SIGYPLP.”

AU13: Please expand “RXXR.”

AU14: Please provide the grant numbers for funding agency(ies)/institution(s).

AU15: Please expand “GAPDH.”

AU16: Please define “DAPI.”