Molecular Therapy
Brief report

Endothelial-to-mesenchymal transition enhances permissiveness to AAV vectors in cardiac endothelial cells

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A major obstacle in inducing therapeutic angiogenesis in the heart is inefficient gene transfer to endothelial cells (ECs). Here, we identify compounds able to enhance the permissiveness of cardiac ECs to adeno-associated virus (AAV) vectors, which stand as ideal tools for *in vivo* gene delivery. We screened a library of >1,500 US Food and Drug Administration (FDA)-approved drugs, in combination with AAV vectors, in cardiac ECs. Among the top drugs increasing AAV-mediated transduction, we found vatalanib, an inhibitor of multiple tyrosine kinase receptors. The increased AAV transduction efficiency by vatalanib was paralleled by induction of the endothelial-to-mesenchymal transition, as documented by decreased endothelial and increased mesenchymal marker expression. Induction of the endothelial-to-mesenchymal transition by other strategies similarly increased EC permissiveness to AAV vectors. *In vivo* injection of AAV vectors in the heart after myocardial infarction resulted in the selective transduction of cells undergoing the endothelial-to-mesenchymal transition, which is known to happen transiently after cardiac ischemia. Collectively, these results point to the endothelial-to-mesenchymal transition as a mechanism for improving AAV transduction in cardiac ECs, with implications for both basic research and the induction of therapeutic angiogenesis in the heart.

INTRODUCTION

Cardiac ischemia fails to elicit an efficient angiogenic response capable of restoring oxygen and nutrient supply to the hypoxic region, with progressive loss of cardiac function.[1] Therapeutic angiogenesis aims to promote the formation of new blood vessels, supporting cardiac function. The delivery of pro-angiogenic molecules in the form of either recombinant proteins or genes has been attempted by multiple pre-clinical and clinical studies, with minimal success,[2,3] consistent with recent evidence indicating that the adult mammalian heart has a low angiogenic potential.[4]

Genetic modification of cardiac endothelial cells (cECs) would allow for both the study of mechanisms responsible for their low angiogenic potential and the development of innovative strategies to induce EC proliferation and the formation of new blood vessels in the ischemic heart. Thus far, most transfection methods commonly used to deliver nucleic acids to primary cells in culture do not work in cECs. The same applies to viral vectors, such as adeno-associated viruses (AAVs), that are successfully used for *in vivo* gene transfer in other cell types.[3,5,6]

To overcome this limitation, we performed a high-throughput screening (HTS) using a library of US Food and Drug Administration (FDA)-approved drugs to identify compounds able to increase the efficiency of AAV vectors in transducing primary cECs, with the aim of both gaining insights into the mechanisms responsible for cEC resistance to gene transfer and exploring novel therapeutic solutions.

RESULTS

Vatalanib increases AAV transduction in primary cECs

We compared the efficiency of several viral vectors and commercially available transfection reagents expressing the green fluorescent protein (GFP) on primary cECs from adult mice. As shown in Figure 1A, only adenoviral vectors transduced cECs with > 50% efficiency. However, their use *in vivo* is fraught by the induction of a potent inflammatory response.[7] This result confirms the poor permissiveness of cECs to most gene delivery systems.

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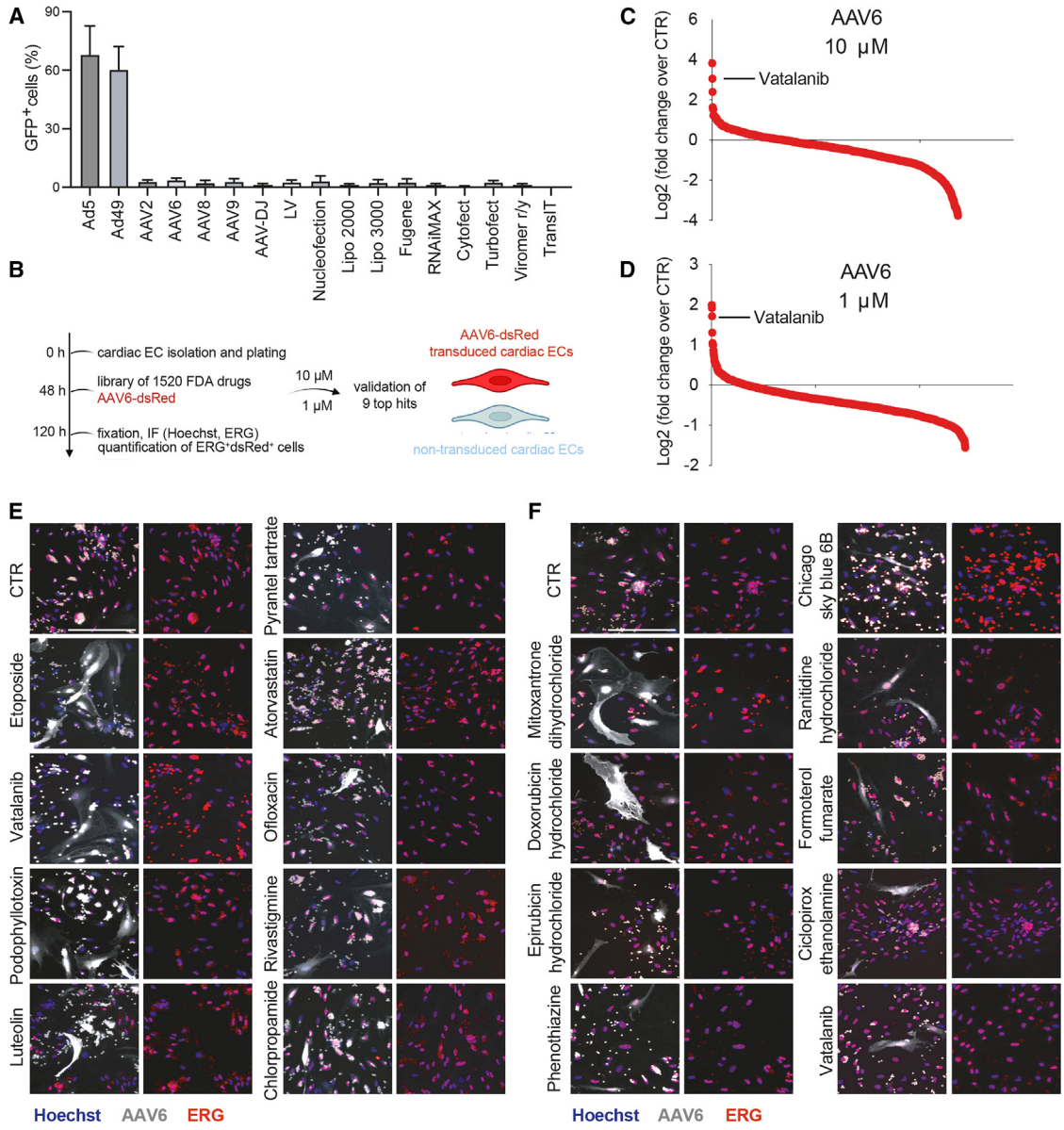


Figure 1. Vatalanib increases AAV transduction in primary cECs

(A) Quantification of cECs at 48 h after either transfection or transduction with the indicated vector encoding for GFP reporter gene. Data are shown as mean ± SEM.

(B) Schematic representation of HTS to identify FDA-approved drugs increasing AAV6-dsRed transduction.

(C) Results of the first HTS in which drugs were added at 10 µM, normalized to control (DMSO).

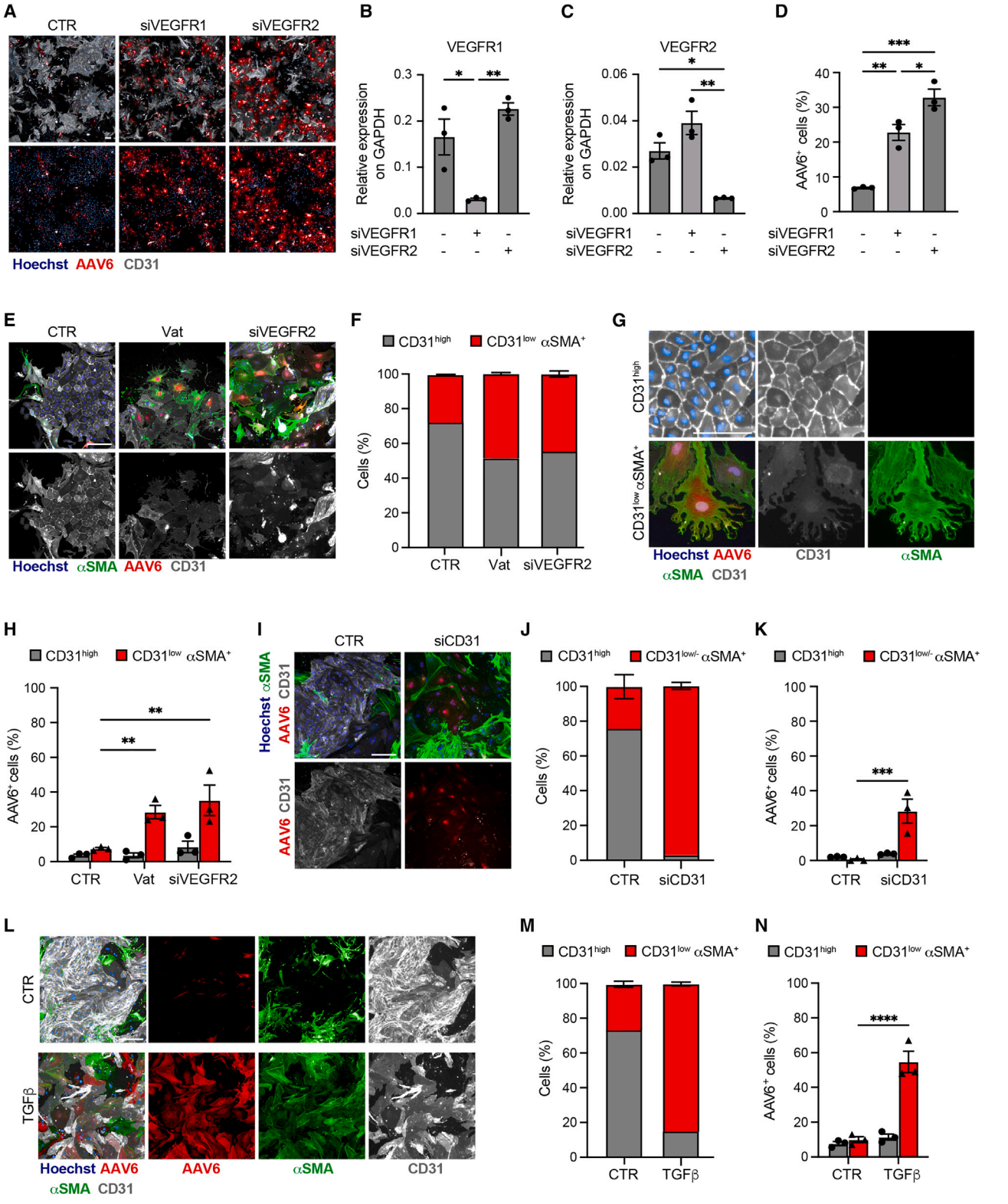
(D) Results of the second HTS in which drugs were added at 1 µM.

(E and F) Representative images of the nine top hits from the first (E) and second (F) rounds.

Scale bars: 100 µm.

Thus, we performed two independent screenings on >95% pure cECs using a library of 1,520 FDA-approved drugs at two different concentrations (10 and 1 µM), together with AAV6-dsRed, as this serotype performed best *in vitro* and shows high tropism for the heart[8,9] (Figure 1B).

The results of both screenings, showing the percentage of cells expressing dsRed, are available at https://doi.org/10.5281/zenodo.11191792. The first ten top hits increasing AAV6 transduction include several topoisomerase II inhibitors (etoposide, mitoxantrone dihydrochloride, doxorubicin hydrochloride, epirubicin hydrochloride), consistent with the known positive correlation between DNA damage and AAV transduction.[10] The only drug listed among the top hits in both screenings was vatalanib, a tyrosine kinase inhibitor targeting multiple vascular endothelial growth factor receptors



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(VEGFRs), platelet-derived growth factor receptors, and c-Kit, thus pointing toward a different mechanism, one not related to DNA damage (Figures 1C–1F).

Vatalanib increases AAV permissiveness by inducing EndMT

To assess whether the effect of vatalanib was due to its capacity to inhibit VEGFRs, we treated cECs with short interfering RNAs (siRNAs) for both VEGFR1 and VEGFR2, which are highly expressed by cECs. As shown in Figures 2A–2D, silencing of either VEGFR, but mostly VEGFR2, significantly increased AAV transduction, consistent with the effect of vatalanib.

By staining cells for the additional EC marker CD31, we observed that both vatalanib and siVEGFR2 induced the appearance of two subpopulations expressing CD31 at different intensities (herein referred as $CD31^{low}$ and $CD31^{high}$, respectively; Figures 2E and 2F). $CD31^{low}$ cECs display features of endothelial-to-mesenchymal transition (EndMT), such as loss of their typical cobblestone morphology, acquisition of an elongated shape, and expression of the mesenchymal marker α-smooth muscle actin (αSMA),[11] (shown in Figure 2G for siVEGFR2). Notably, AAV6-dsRed mostly transduced $CD31^{low}$ αSMA^{+} cells upon either vatalanib treatment or VEGFR2 silencing (Figure 2H).

Next, we assessed whether the induction of EndMT by alternative methods similarly increases cEC permissiveness to AAV. First, we silenced CD31 using a specific siRNA (siCD31), which resulted in increased αSMA expression, confirming the acquisition of an EndMT phenotype (Figures 2I and 2J). Loss of CD31 significantly increased AAV6-dsRed transduction in $CD31^{low/-}$ αSMA^{+} cells undergoing EndMT (Figure 2K).

Finally, we treated cECs with transforming growth factor β2 (TGF-β2), the main inducer of EndMT,[11] prior to AAV6 transduction. A significant increase in AAV6 efficiency was selectively observed in $CD31^{low}$ αSMA^{+} cells undergoing EndMT (Figures 2L–2N).

Collectively, these data indicate that the EndMT favors permissiveness of cECs to AAV6 transduction.

Transient EndMT after MI allows EC transduction by AAV vectors *in vivo*

Next, we compared additional AAV serotypes, namely AAV2 and AAV9, known to be more effective *in vivo*. Exposure of cECs to TGF-β increased the efficiency of all serotypes but mostly AAV9, which was chosen for *in vivo* validation, due to its tropism for the heart (Figures 3A and 3B).

It was recently demonstrated that transient EndMT occurs in the heart 3 days after myocardial infarction (MI).[12] Thus, we expected that AAV vectors transduce cECs during this time window. To experimentally test this hypothesis, we induced MI in membrane Tomato/membrane GFP (mT/mG) reporter mice, in which Tomato fluorescence is switched to GFP upon Cre-mediated recombination. At day 3 after MI, we administered AAV9-Cre by intracardiac injection (Figures 3C and 3D). After 7 additional days, we collected the hearts, in which AAV9-transduced and non-transduced cells appeared green (GFP^{+}) and red ($mTomato^{+}$), respectively (Figures 3C and 3D). By focusing on the border between healthy myocardium and the scar, we observed several GFP^{+} cells within circular structures with a typical vessel-like morphology that scored positive for CD31 (Figures 3E–3H). Some of these transduced cells were still positive for the EndMT marker αSMA, consistent with partial reacquisition of an endothelial phenotype at day 10 after MI (Figure 3I). Thus, transient EndMT after MI allows cEC transduction by AAV vectors *in vivo*.

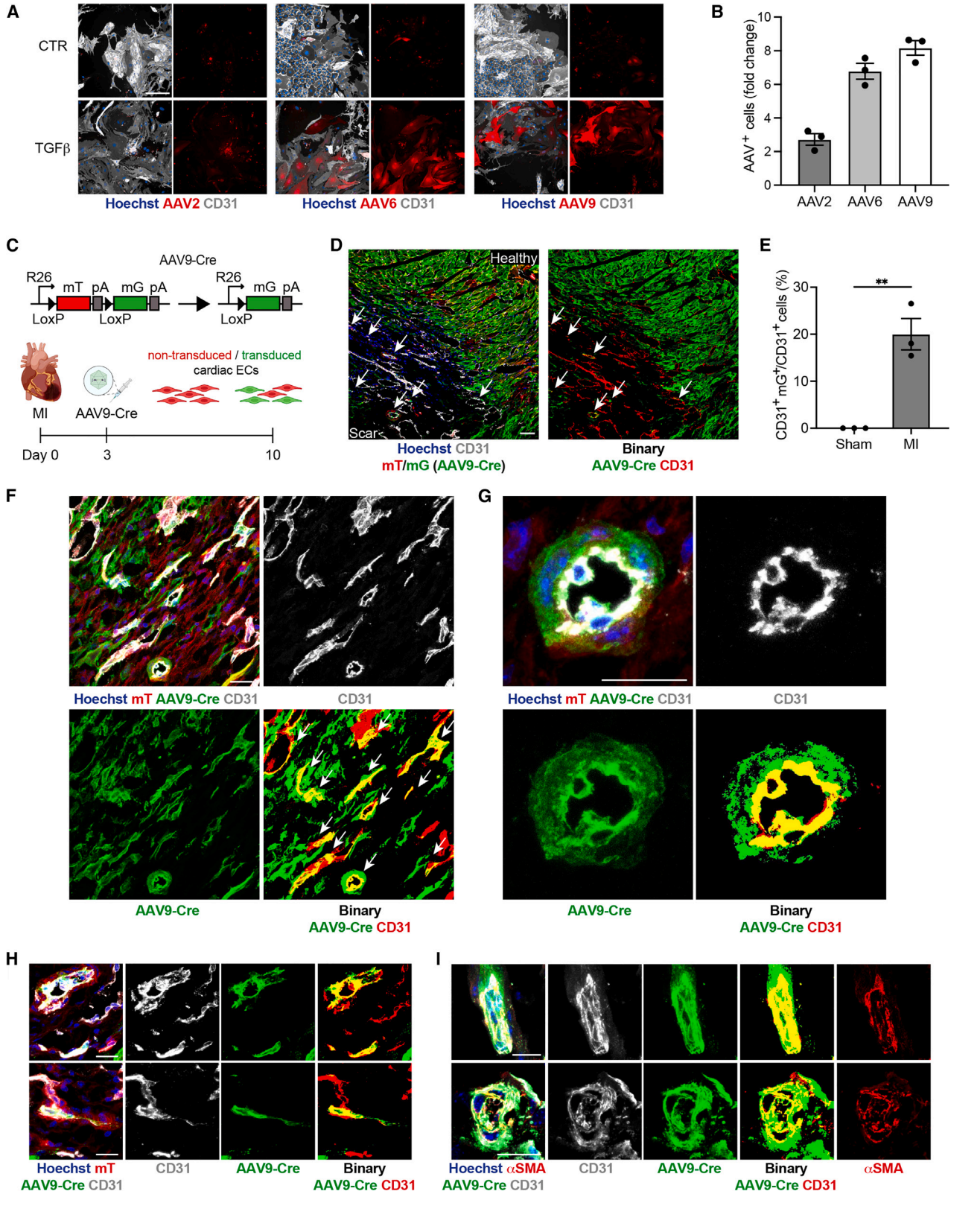
DISCUSSION

HTS is usually employed to either discover new therapeutic molecules or decipher specific biological pathways.[13,14] In the context of AAV-based gene therapy, HTS has been used to trace the path of AAV vectors inside mammalian cells and discover major determinants of AAV transduction[15,16]

This is the first HTS aiming at identifying FDA-approved drugs able to increase the efficiency of transduction using AAV vectors, specifically in cECs. This has dual value: on the one hand, it sheds light on the mechanisms that mediate AAV entry and processing in cECs, while on the other, it identifies new tools to improve the efficacy of cardiac gene therapy.

Figure 2. EndMT increases permissiveness to AAV vectors in cECs

(A) Representative images of cECs transduced with AAV6-dsRed upon silencing of VEGFR1 and VEGFR2.
(B and C) Quantification of VEGFR1 and VEGFR2 mRNA upon transfection with the indicated siRNAs.
(D) Quantification of $AAV6\text{-}dsRed^{+}$ cECs upon silencing of VEGFR1 and VEGFR2.
(E) Representative images of cECs transduced with AAV6-dsRed upon the indicated treatments and stained for both CD31 and αSMA.
(F) Quantification of $CD31^{high}$ and $CD31^{low}$ αSMA^{+} cells in response to the indicated treatments.
(G) Representative images of the morphology of two cEC sub-populations ($CD31^{high}$ images from CTR cells, $CD31^{low/-}$ αSMA^{+} images from siVEGFR2 cells).
(H) Quantification of AAV6-transduced cells within each population shown in (F).
(I) Representative images of cECs transduced with AAV6-dsRed upon silencing of CD31.
(J) Quantification of $CD31^{high}$ and $CD31^{low/-}$ αSMA^{+} cells in response to CD31 silencing.
(K) Quantification of AAV6-transduced cells within each population shown in (J).
(L) Representative images of cECs transduced with AAV6-dsRed upon treatment with TGF-β.
(M) Quantification of $CD31^{high}$ and $CD31^{low}$ αSMA^{+} cells in response to TGF-β.
(N) Quantification of AAV6-dsRed efficiency in the two populations shown in (M).
Scale bars: 100 μm. Data in (B), (C), (D), (F), (H), (J), (K), (M), and (N) are shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.



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Our work identified vatalanib as the most efficient drug enhancing AAV transduction in cECs. Among the top hits, most were DNA-damaging compounds, previously described as able to recruit the DNA damage response machinery at the site of DNA damage, thereby relieving its inhibition on AAV long terminal repeats.[8,10] While very effective *in vitro*, their clinical use is limited by obvious side effects.

Vatalanib stood out, as it is not genotoxic and was the only tyrosine kinase inhibitor selected in both screening rounds. Not only did vatalanib increase AAV transduction, but it also changed the cEC phenotype, resulting in the loss of endothelial markers and the acquisition of mesenchymal features, consistent with the role of VEGFR2 in maintaining EC identity.[17] This prompted us to hypothesize that the mechanism that makes cECs more permissive to AAVs was the EndMT, which was confirmed by our data.

Would this mean that cECs have to lose their endothelial identity to be transduced by AAV vectors? Recently, transient EndMT has been described to occur early after MI. Our data confirmed that cECs in the fibrotic region exhibited both endothelial and mesenchymal characteristics and were successfully transduced by AAV vectors. Interestingly, the acquisition of a mesenchymal identity is reversible, and cECs reacquire their endothelial phenotype at day 14,[12] indicating that our findings could be valuable in a translational setting to therapeutically transduce cECs impacted by MI. While the mechanism by which the EndMT favors AAV transduction remains elusive, multiple lines of evidence suggest that ECs undergoing the EndMT deregulate the expression of DNA repair genes,[18–21] which are known to control AAV transduction efficiency.[10,16]

Collectively, our results have unveiled a new mechanism that favors AAV transduction in ECs, with important implications for cardiovascular gene therapy.

MATERIALS AND METHODS

AAV preparation

AAV vectors were produced by the AAV Vector unit at ICGEB Trieste (http://www.icgeb.org/RESEARCH/TS/COREFACILITIES/AVU.htm)[22] and used at concentrations of 1×10^6 vg/cell and 2.6×10^{10} vg/injection for *ex vivo* and *in vivo* applications, respectively.

Isolation of cECs and HTS

ECs were isolated from the hearts of C57BL/6 mice using anti-CD31 magnetic beads[23] and cultured in EC growth medium-2 (EGM-2; Lonza, CC-3162) on fibronectin/gelatin-coated plates.

For HTS, cECs were plated in 384-well plates (3.5×10^3/well). After 48 h, a library of 1,520 drugs (Prestwick Chemical) in DMSO was added at either a 10 (1st round) or 1 μM (2nd round) concentration. After an additional 72 h, cECs were fixed and stained with anti-ERG antibodies and Hoechst 33342. Images were acquired using an Operetta epifluorescence microscope (PerkinElmer) and analyzed by Harmony software (PerkinElmer).

EndMT induction

CD31, VEGFR1, and VEGFR2 were silenced using mouse-specific si-GENOME SMARTpools (Dharmacon M-048240-01-0005, M-040636-01-0005, and M-040634-00-0005) and RNAiMAX (Invitrogen, 13778-150). Silencing efficiency was >80%. Alternatively, cECs were stimulated with TGF-β2 (10 ng/mL; PeproTech; 100-35B-10UG), added twice every 48 h.

Immunofluorescence

Cells and tissues were fixed in 4% paraformaldehyde and blocked in 5% bovine serum albumin (Roche) supplemented with 0.1% Triton X-100 in PBS. Primary antibody was diluted 1:100, while secondary antibody (Invitrogen) was diluted 1:500. Nuclei were counterstained with Hoechst 33342 (Invitrogen, H3570). Primary antibodies were anti-CD31 (R&D/Bio-Techne, AF3628), anti-αSMA (Sigma-Aldrich, #C6198; Dako, M0823), anti-ERG (Abcam, ab92513), and anti-GFP (Abcam, ab5450; Abcam, ab290). Images were acquired using a Nikon Eclipse Ti-E epifluorescence microscope and a Zeiss LSM 880 confocal microscope.

Animal studies

Animals were housed in compliance with institutional national and international policies. Experimental procedures were approved by the ICGEB Animal Welfare Board, as required by the EU Directive 2010/63/EU, and by the Italian Ministry of Health (authorization no. 621/2019). MI was induced by coronary artery ligation in Rosa26-mT/mG.[4,24] After 3 days, AAV9-Cre was injected under echocardiographic guidance in infarcted and sham hearts.

Figure 3. Transient EndMT after MI increases permissiveness to AAV vectors *in vivo*

(A and B) Representative images (A) and quantification (B) of the percentage of cECs transduced with AAV2, AAV6, and AAV9 in response to TGF-β.

(C) Schematic representation of *in vivo* EndMT induction model. AAV9-Cre was administered to mT/mG reporter mice 3 days after MI, resulting in the switch from mTomato to mGFP in Cre^+ cells.

(D) Low-magnification image showing scar and healthy myocardium; mG(AAV9-Cre)$^+$CD31$^+$ transduced vessels are indicated by white arrows.

(E and F) Quantification (E) and representative images (F) of cECs transduced by AAV9-Cre in the MI border region.

(G and H) High-magnification images showing AAV9-transduced cECs (G) in the region shown in (F) and (H) in different animals.

(I) Representative images of cECs transduced by AAV9 and expressing the EndMT marker αSMA.

Scale bars: 100 μm in (A) and (D) and 20 μm in (F)–(I). Data in (B) and (E) are shown as mean ± SEM. $^{**}p < 0.01$.

Statistical analysis

Data are shown as mean ± standard error of the mean (SEM). Statistical analysis was performed by unpaired t test or one- or two-way ANOVA comparison test, followed by Welch and Bonferroni correction, using GraphPad Prism 9. A p value < 0.05 was considered statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). $n \geq 3$.

DATA AND CODE AVAILABILITY

The results of the screenings are available at https://doi.org/10.5281/zenodo.11191792.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.V., R.V., and S.Z.; investigation, N.V. and R.V.; methodology, N.V., R.V., and S.Z.; project administration, N.V., R.V., and S.Z.; visualization, N.V., R.V., and S.Z.; writing – original draft, N.V., R.V., and S.Z.; writing – review & editing, N.V., R.V., A.C., and S.Z.; supervision, A.C. and S.Z.; data curation for HTS, M.C.V., M.M., N.A.R.R., and L.B.; methodology for *in vivo* experiments, S.V.; resources, L.Z., M.G., and S.Z.; funding acquisition, S.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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