Cellular and Molecular Mechanisms of Arrhythmias in Cardiac Fibrosis and Beyond:

From Symptoms to Substrates towards Solutions

Chapter VIII

Development of adeno-associated virus vectors for the transduction of myocardial fibroblasts

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In preparation

Abstract

Introduction: Scar formation after myocardial infarction is an important cause of cardiac dysfunction. This makes myocardial scar fibroblasts (MSFs) interesting targets for therapeutic intervention. One way of modulating the properties of MSFs is by genetic modification with viral vectors. Among the currently used viral gene delivery vehicles, adeno-associated virus (AAV) vectors stand out for their *in vivo* safety and ability to spread easily through tissues. The aim of this study was to compare the transduction of neonatal rat cardiomyocytes (nrCMCs) and of human, mouse and rat MSFs by AAV vectors with different capsids and with transgenes driven by different promoters.

Methods & Results: AAV vector shuttle plasmids containing a *LacZ* gene driven by 9 different promoters including the human, mouse and rat cytomegalovirus *immediate early* gene (CMV-IE) promoter were constructed. The AAV vector genomes specified by these plasmids were packaged in AAV serotype 2 (AAV2) capsids. β-galactosidase assays revealed that of all CMV-IE promoters, the human CMV-IE promoter outperformed the rodent CMV-IE promoters in human MSFs while the rodent CMV-IE promoters were more active in murine and rat MSFs. Furthermore, of all nine promoters tested the human *eukaryotic translation elongation factor 1 alpha 1* gene (hEF1α) promoter was most active in rodent MSFs while the Rous sarcoma virus (RSV) promoter gave the highest transgene expression in nrCMCs. A comparison of the transduction efficiency of rat MSFs and nrCMCs with AAV2 vectors carrying capsids of AAV serotypes 2, 5, 6, 8 and 9 showed that transduction of rat MSFs was most efficient with AAV2/2 vectors whereas AAV2/9 best transduced the nrCMCs. Substitution of the surface-exposed tyrosine residues at amino acid positions 444, 500 and 730 in the AAV2 VP1 protein resulted in a 6-fold increase in the transduction efficiency of rMSFs by AAV2/2 vectors but also quadrupled their ability to transduce nrCMCs.

Conclusion: The highest transgene expression in rat MSFs is obtained by transduction with an AAV2/2 vector containing an hEF1α promoter-driven transgene while transgene expression in nrCMC is highest after transduction with an AAV2/9 vector in which foreign gene expression is controlled by the RSV promoter.

Introduction

After myocardial infarction (MI), cardiomyocytes in the affected region die and are replaced by scar tissue consisting of fibroblasts and dense extracellular matrix (ECM). The scar tissue contributes to the stiffening of the myocardium, reducing the overall contractile performance of the heart.[1] Furthermore, the scar tissue prevents proper action potential propagation due to (i) poor electrical coupling of scar fibroblasts with each other and with adjacent cardiomyocytes and (ii) high electrical resistance of the ECM.[2]

To overcome the adverse mechanical and electrical effects of scar tissue on cardiac performance, several approaches have been tested. First of all, transplantation of unmodified autologous bone marrow-derived stem cells (BMSCs) has been attempted to regenerate the damaged myocardium.[3] Some minor improvement of cardiac function was observed in some studies,[4-6] with the therapeutic effect being predominantly attributable to paracrine factors released by the donor cells rather than to the differentiation of the injected BMSCs into cardiomyocytes.[7] To increase their therapeutic effect, it has been proposed to endow BMSCs with genes that can induce their cardiomyogenic differentiation before injecting them into the heart.[8] However, numerous issues remain with cardiac cell therapy, including the inefficient retention of the donor cells at the site of injection, the poor survival of the injected cells, and the low propensity of the surviving BMSCs to differentiate into cardiomyocytes.

A possible strategy to bypass at least some of the limitations associated with cell therapy is to directly target the resident myocardial scar fibroblasts (MSFs) using *in vivo* gene therapy with the aim to endow them with more favorable biosynthetic, contractile and/or electric properties. Via this approach, the number of cells available for therapeutic intervention would be much higher than by the injection of stem cells, provided that the MSFs can be efficiently transduced *in vivo* with the gene construct of choice. Of the various gene delivery vehicles available for cardiac gene therapy, adeno-associated virus (AAV) vectors constitute an attractive option due to their excellent *in vivo* safety record and small size (≈20 nm in diameter) allowing them to penetrate dense tissues better than other commonly used viral vectors. Other advantages of AAV vectors are their low immunogenicity and the long-term persistence of their genomes in target cells mainly as episomes leading to prolonged transgene expression with a low chance of insertional mutagenesis.[9] Vectors based on recombinant AAV serotype 2 (AAV2) genomes packaged in capsids of AAV serotypes 1 through 9 (i.e. AAV2/1 through AAV2/9 vectors) have been tested in healthy rodent hearts. [10-13] Unfortunately, in none of these *in vivo* experiments the identity of the transduced cardiac cells was investigated making it impossible to determine the contribution of the four major cell types in the non-diseased heart (i.e. cardiac fibroblasts, cardiomyocytes, vascular smooth muscle cells and endothelial cells) to the observed transgene expression. However, Qi et al. did test the ability of AAV2/2, AAV2/5, AAV2/7, AAV2/8 and AAV2/9 vectors to transduce cardiac fibroblasts and

cardiomyocytes of neonatal rats in culture[13] but thus far the transduction of MSFs by different AAV vector pseudotypes has not been investigated.

Depending on the therapeutic strategy that is being pursued to treat infarcted hearts by gene therapy, it may be important to develop AAV vectors that efficiently transduce MSFs and induce high-level therapeutic expression in these cells but do not cause appreciable transgene expression in cardiomyocytes. In an attempt to generate an AAV vector meeting these requirements, five different AAV vector pseudotypes were tested for the efficiency by which they transduce MSFs as compared to cardiomyocytes. These experiments revealed that AAV2/6 and AAV2/2 vectors best transduce neonatal rat cardiomyocytes (nrCMCs) and rat MSFs (rMSFs), respectively. In addition, MSFs of man, mouse and rat and nrCMCs were exposed to AAV vectors carrying nine different promoters, to determine which of these promoters is most specific for either of these cell types. Of all promoters tested, the human *eukaryotic translation elongation factor 1 alpha 1* gene (hEF1α) promoter was the most active in rodent MSFs while the human cytomegalovirus *immediate early* gene (hCMV-IE) promoter and Rous sarcoma virus (RSV) promoter performed best in human MSFs (hMSFs) and nrCMCs, respectively. We also generated two new helper plasmids based on the widely used AAV packaging construct pDG[14] for the production of AAV2/8 and AAV2/9 vectors in a two-plasmid system. Use of these new helper plasmids resulted in AAV vector yields similar to those obtained with commonly applied AAV2/8 and AAV2/9 vector production systems based on the transfection of host cells with three different plasmids.

Materials and Methods

Primary cell cultures

All animals were treated in accordance with national guidelines and with permission of the Animal Experiments Committee of the Leiden University Medical Center.

rMSFs were obtained from myocardial scar tissue of 16-week-old Wistar Kyoto rats 2 weeks after MI induction by ligation of the left anterior descending artery (LAD). The white scar tissue was dissected from the heart and cut into small pieces. Each of these pieces was then placed in a gelatin-coated 10-cm^2 cell culture dish (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen, Breda, the Netherlands) and covered with a glass coverslip. After 7 days, the scar tissue was removed and the adherent fibroblasts that had grown out from the tissue were passaged. rMSFs were further propagated in DMEM supplemented with 20% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

mMSFs were obtained from myocardial scar tissue of 10 week old NOD/Scid mice 6 days after MI induction by ligation of the LAD. The white scar tissue was dissected from the heart and used to obtain mMSFs following the same protocol as for the isolation of the rMSFs.

Cultures of nrCMCs and neonatal rat cardiac fibroblasts (nrCFBs) were prepared from 2-day-old rats as described before.[15] nrCMCs were cultured in a 1:1 mixture of DMEM and Ham's F10 (Invitrogen) supplemented with 5% HS, (Invitrogen) 100 U/mL penicillin and 100 µg/mL streptomycin. In order to determine the fraction of nrCFBs present in purified nrCMC cultures, these cultures were immunostained for the cardiomyocyte marker sarcomeric α-actinin and for the fibroblast marker collagen type I. To this end, the cells were incubated overnight at 4°C with mouse IgG1-anti-sarcomeric α-actinin antibody (clone EA-53; Sigma-Aldrich, St. Louis, MO) and with collagen type I-specific rabbit IgG (ab292; Abcam, Cambridge, MA) which were diluted 1:400 in phosphate-buffered saline (PBS) + 5% FBS. The next day, the cells were washed once with PBS and then incubated for 1 hour at 4°C with Alexa Fluor 568-conjugated goat-anti-mouse IgG and Alexa Fluor 488-conjugated goat-anti-rabbit IgG antibodies (both from Invitrogen) diluted 1:400 in PBS + 5% FBS. The percentage of nrCFBs in purified nrCMC cultures ranged from 5 to 10% of the total cell number. nrCFBs were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

hMSFs were obtained and cultured as described before.[16] All primary cells were cultured at 37°C in an atmosphere of 10% CO_2 in air saturated with water vapour.

Cell lines

293T cells[17] were cultured in DMEM supplemented with 10% FBS at 37°C and 10% CO_2. 911E4 cells[18] were grown in DMEM supplemented with 10% FBS, 0.4 mg/mL geneticin (Invitrogen) and 0.1 mg/mL hygromycin B (Invitrogen) at 37°C and 10% CO_2. Upon seeding the 911E4 cells for end-point titration of AAV vector stocks, the cells were cultured in DMEM supplemented with 10% FBS, 0.4 mg/mL geneticin, 0.1 mg/mL hygromycin B and 100 ng/mL of doxycyclin (Sigma-Aldrich).

Plasmid constructs

The various AAV vector shuttle plasmids encoding *Escherichia coli* β-galactosidase were constructed as follows. The AAV vector shuttle constructs pDD1, pDD2 and pDD345 containing RSV, hCMV-IE and the hybrid, chicken *β-actin* gene-based CAG promoter, a multiple cloning site and the simian virus 40 (SV40) polyadenylation signal (pA) flanked by the AAV2 inverted terminal repeats (ITRs) were a gift of Dongsheng Duan.[19] First, the 3.1-kb XhoI×XbaI fragment from pCMVβ.LacZnls12co (Marker Gene Technologies, Eugene, OR) containing a *Lac*Z gene that is codon-optimised for expression in mammalian cells and encodes a nuclear-targeted version of β-galactosidase (LacZnls12co) was ligated to the vector backbone of XhoI- and XbaI-digested pDD345 using T4 DNA ligase to create pDD.CAG.nlsLacZco.SV40pA. Next, a 46-bp synthetic polyadenylation (synpA) signal[20] flanked by BamHI and NotI restriction enzyme recognition sequences was created by hybridizing two oligonucleotides (sense strand: 5'-GGCCGCAATAAAATATCTTTATTTTCATTACATCTGTGTGTTGGTTTTTTGTGTG-3'; antisense strand: 5'-GATCCACACAAAAAACCAACACACAGATGTAATGAAAATAAAGATATTTTATTGC-3'). The synpA site was subsequently cloned into BamHI- and NotI-digested pDD.CAG.nlsLacZco.SV40pA to make pDD.CAG.nlsLacZco.synpA. The latter construct was incubated with BamHI and HindIII and the 3.2-kb fragment containing the *Lac*Z gene and synpA was combined with the 4.7-kb fragment of BamHI- and HindIII-digested pDD2 to produce pDD.hCMV-IE.nlsLacZco.synpA.

The hybrid, SV40 *early* gene-based SRα promoter was excised from plasmid pMX1 (R&D Systems, Abingdon, UK) using XbaI and BglII and inserted in XbaI- and BamHI-digested pBluescriptSK(-) (Stratagene, Palo Alto, CA) to generate pBluescriptSK(-).SRα. The SRα promoter was subsequently excised from pBluescriptSK(-).SRα using EcoRV and inserted in reverse orientation in EcoRV-digested pBluescriptSK(-) to generate pBluescriptSK(-).SRα(-). The latter construct was treated with BcuI and HindIII and the resulting 0.7-kb fragment containing the SRα promoter was combined with the 7.2-kb BcuI×HindIII fragment of pDD.CAG.nlsLacZco.synpA using T4 DNA ligase. This led to the construction of AAV vector shuttle plasmid pDD.SRα.nlsLacZco.synpA.

The RSV promoter was obtained by digestion of pDD1 with XhoI and HincII and cloned into EcoRV- and SalI-digested pBluescriptSK(-) yielding pBluescriptSK(-).RSV. Next, the 0.7-kb BcuI×HindIII fragment from pBluescriptSK(-).RSV containing the RSV promoter was

combined with the 7.2-kb BcuI×HindIII fragment of pDD.CAG.nlsLacZco.synpA yielding pDD.RSV.nlsLacZco.synpA.

The mouse *S100 calcium binding protein A4* gene (mFSP1) promoter[21], rat *lysyl oxidase* (rLOX) promoter, hEF1α promoter, mouse *CMV-IE* gene (mCMV-IE) promoter and rat *CMV-IE* gene (rCMV-IE) promoter sequences were obtained by polymerase chain reaction (PCR). The mFSP1 and rLOX promoters were amplified from chromosomal DNA, whereas the hEF1α, rCMV-IE and mCMV-IE promoters were amplified from pLVET-KRAB[22], pSSHK3[23] and pMH5 (Microbix Biosystems, Mississauga, Ontario, Canada), respectively. The PCRs were performed in a total volume of 50 µL using 200 ng (for genomic DNA) or 10 ng (for plasmid DNA) template, 0.5 µmol/L of both forward and reverse primer (mFSP1: 5'-GCTGATATCGAAGCCTGGTTCCCAGAC-3' and 5'-CTGGATATCACCTTTCTGTGCCTCTCA-3'; rLOX: 5'-GCCGATATCCTGGGGTTTATTCGCTG-3' and 5'-GAAGATATCGATGATCTCCCGGCTCGTC-3'; hEF1α: 5'-GCTGATATCGGCTCCGGTGCCCGTCAG-3' and 5'-CTGGATATCTCACGACACCTGAAATGG-3'; mCMV-IE: 5'-CTAACTAGTGGGGTGGTGACTCAATGGCC-3' and 5'-GATAAGCTTAGTACCGACGCTGGTCGCGC-3'; rCMV-IE: 5'-GTACTAGTTCCCATATTAAGAATGAGTTATAG-3' and 5'-GTAAAGCTTCCGATCCGAAGACTGTGACTG-3'), 200 µmol/L of each dNTP, 1× Phusion HF buffer (Finnzymes, Espoo, Finland) and 1 U Phusion high-fidelity DNA polymerase (Finnzymes). The PCRs were performed according to the protocol provided with the DNA polymerase using an annealing temperature of 60°C and 35 amplification cycles. The 1.1-kb PCR fragment containing the mFSP1 promoter, the rLOX promoter PCR fragment of 0.8 kb and the 1.2-kb PCR fragment containing the hEF1α promoter were digested with EcoRV, purified from agarose gel using the JETSORB gel extraction kit (GENOMED, Löhne, Germany) and cloned into EcoRV-digested pBluescriptSK(-) to produce pBluescriptSK(-).mFSP1, pBluescriptSK(-).rLOX and pBluescriptSK(-).hEF1α, respectively. The mFSP1 promoter was released from pBluescriptSK(-).mFSP1 using EcoRV and combined with the 7.2-kb EcoRV fragment of pDD.SRα.nlsLacZco.synpA to generate pDD.mFSP1.nlsLacZco.synpA. The rLOX and hEF1α promoters were excised from pBluescriptSK(-).rLOX and pBluescriptSK(-).hEF1α, respectively, using BcuI and HindIII and combined with the 7.2-kb BcuI×HindIII fragment of pDD.CAG.nlsLacZco.synpA to produce pDD.rLOX.nlsLacZco.synpA and pDD.hEF1α.nlsLacZco.synpA. The 1.1-kb PCR fragments containing the mCMV-IE and rCMV-IE promoter were digested with BcuI and HindIII, purified from agarose gel and ligated to the 7.2-kb BcuI×HindIII fragment of pDD.CAG.nlsLacZco.synpA yielding pDD.mCMV-IE.nlsLacZco.synpA and pDD.rCMV-IE.nlsLacZco.synpA, respectively. The correctness of the cloned PCR fragments was verified by nucleotide sequence analysis using the BigDyeTerminator v3.1 cycle sequencing kit, the BigDye XTerminator purification kit and the 3730xl DNA analyzer (all from Applied Biosystems Life Technologies, Carlsbad, CA).

To create an AAV vector containing an hEF1α promoter-driven *Aequorea victoria enhanced green fluorescent protein* (eGFP) gene, the AAV vector shuttle plasmid

pTR.hCMV-IE.eGFP.WHVPRE.bGHpA, a derivative of pTR-CMVgfp containing the woodchuck hepatitis virus posttranscriptional regulatory element (WHVPRE)[24] was digested with BamHI and KpnI. Next, the 5'- and 3'-recessed ends were blunted using T4 DNA polymerase and the 1.6-kb fragment containing the *eGFP* gene and the WHVPRE was ligated to the 5.2-kb Klenow DNA polymerase-treated HincII×HindIII fragment of pDD.hEF1α.nlsLacZco.synpA to create pDD.hEF1α.eGFP.WHVPRE.synpA. The latter AAV shuttle plasmid was incubated with MunI and XhoI and the resulting 5.8-kb digestion product was combined with the 0.5-kb RSV promoter-containing MunI×XhoI fragment of pDD1 to produce pDD.RSV.eGFP.WHVPRE.synpA.

Helper plasmids for making AAV2/8 and AAV2/9 vectors in a two-plasmid production system were generated by replacing the AAV2 cap gene in pDG by the cap gene of AAV serotype 8 (AAV8) and AAV serotype 9 (AAV9), respectively. To this end, pDG (obtained from Jürgen Kleinschmidt; [14]) was digested with Bsu15I, treated with the Klenow fragment of DNA polymerase I and subsequently digested with SalI. The AAV8 and AAV9 cap genes were released from pAAV2/8 or pAAV2/9 (both obtained from James Wilson; Penn Vector Core, Philadelphia, PA), respectively, by digesting these plasmids with MssI and SalI. The resulting 3.1-kb fragments containing the cap gene of either AAV8 or AAV9 were combined with the 18.7-kb Bsu15I×SalI fragment of pDG to create pDP8 and pDP9, respectively.

To make, in a three-plasmid production system, wild-type AAV2 vectors and AAV2 vectors in which the surface-exposed tyrosine residues at positions 444, 500 and 730 of the VP1 protein are replaced by phenylalanines, we first generated plasmid pUCBM21.SmaI.Cap2 by combining the 2.6-kb HindIII×Klenow polymerase-blunted Bsu15I fragment from pDG (Grimm et al., 2002) with the 2.7-kb HindIII×SmaI fragment of pUCBM21PSXSP. pUCBM21PSXSP is a derivative of pUCBM21 (Boehringer Mannheim) containing extra non-spaced MssI, SmiI and XhoI restriction enzyme recognition sites (adaptor molecule contained HindIII overhang, MssI, SmiI and XhoI site, complete NcoI site and Eco32I half-end) in between the HindIII and NcoI target sequences and extra non-spaced SmiI and MssI recognition sites (adaptor molecule contained BamHI overhang, complete SmaI site, SmiI and MssI sites and EcoRI overhang) in between the SmaI and EcoRI target sequences. Plasmid pUCBM21.SmaI.Cap2 was used as template for site-directed mutagenesis of the codons corresponding AAV2 VP1 amino acid residues 444, 500 and 730 essentially as described by Liu and Naismith (2008) (reference: Liu H, Naismith JH. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. BMC Biotechnol 2008;8:91) except for the use of VELOCITY DNA polymerase (Bioline) instead of Pfu DNA polymerase. The oligodeoxyribonucleotide pairs for introducing the tyrosine-to-phenylalanine substitutions were 5' CTGTATTTCTTGAGCAGAACAAACACTCCAAGTGGAAC 3' and 5' CTGCTCAAGAAATACAGGTACTGGTCGATGAGAGGATTC 3' for Tyr^{444} 5' GTGAATTCTCGTGGACTGGAGCTACCAAGTACCACC 3' and 5' GTCCACGAGAATTCACTGTTGTTGTTATCCGCAGATG

3' for Tyr^{500} and 5' ACCAGATTCCTGACTCGTAATCTGTAATTGCTTGTTAATCAA 3' and 5' of AGTCAGGAATCTGGTGCCAATGGGGCGAG 3' for Tyr^{730}. The plasmid encoding a version of AAV2 VP1 carrying all three tyrosine-to-phenylalanine mutations was designated pUCBM21.SmaI.Cap2Y444500730F. After having confirmed the correctness of the AAV2 sequences in pUCBM21.SmaI.Cap2 and in pUCBM21.SmaI.Cap2Y444500730F by dideoxy nucleotide sequence analysis, both plasmids were incubated with SmiI and the resulting 2.3-kb digestion products were combined with the 4.9-kb SmiI×Eco32I fragment of pAAV2/8 to generate pAAV2/2 and pAAV2/2Y444500730F, respectively.
All restriction and DNA modifying enzymes as well as the dNTPs were purchased from Fermentas (St. Leon-Rot, Germany) and the oligodeoxyribonucleotides were provided by Eurofins MWG Operon (Ebersberg, Germany).

AAV vector production, purification and quantification
AAV vector particles were produced in 293T cells. Per AAV vector production, 10^6 (small scale) or 7×10^7 (intermediate scale) cells were seeded at a density of 10^5 cells/cm^2 and cultured in DMEM supplemented with 10% FBS 1 day before transfection. Two hours prior to transfection, the cell culture medium was refreshed with DMEM + 10% FBS. The cells were transfected with 71 ng AAV vector shuttle plasmid and 214 pDG[14], pDF5, pDG6[25], pDP8 or pDP9 per cm^2 of cells (two-plasmids production system) or 71 ng AAV vector shuttle plasmid, 71 ng pAAV2/2, pAAV2/2Y444500730Y, pAAV2/8 or AAV2/9 and 143 ng pAd.ΔF6 (provided by James Wilson; Penn Vector Core)[26] per cm^2 of cells (three-plasmids production system) using 3 ng linear 25-kDa polyethylenimine (Polysciences Europe, Eppelheim, Germany) per ng DNA as transfection agent. After 16 hours, the medium was replaced by DMEM + 5% FBS. Sixty-four hours after transfection, the 293T cells from four 175 cm^2 flasks were collected in 10 mL PBS + 1 mM $CaCl_2$ + 1 mM $MgCl_2$. The cells were then subjected to 3 cycles of snap freezing in liquid nitrogen and thawing in a water bath of 37°C to release the AAV vector particles from the cells. The AAV vector preparation was then treated for 30 min with 100 U of OmniCleave Endonuclease (EPICENTRE Biotechnologies, Madison, WI) per mL sample at 37°C. Next, the cell debris was pelleted at 250 × *g* and the supernatant was collected.
The AAV vector particles were purified using an iodixanol block gradient. To this end, 17.5 mL of the cleared vector suspension was loaded into a 39-mL polyallomer tube (Quick-Seal, 25 × 89 mm; Beckman Coulter, Fullerton, CA, USA). Next, 9 mL of 15%, 5 mL of 25%, 5 mL of 40% and 3 mL of 60% iodixanol solution (OptiPrep, Sigma-Aldrich) in 1 mM $MgCl_2$, 2.5 mM KCl was sequentially layered under the AAV vector preparation, which was then centrifuged for 70 min at 69,000 revolutions per min in a 70 Ti rotor (Beckman Coulter). Thereafter, the bottom of the centrifuge tube was punctured and the AAV vector particles at the 40-60% iodixanol interface were collected.

Finally, the AAV vector particles were washed with PBS + 5% sucrose and concentrated in three steps to approximately 200 µL per AAV vector in an Amicon Ultra-15 filter unit with a nominal molecular weight limit of 100 kDa (Millipore, Amsterdam, the Netherlands) following the instructions of the filter supplier. AAV vector stocks were stored in 50 µL aliquots at -80°C.

Titration of AAV vector stocks

The titers of AAV vector stocks were determined by dot-blot assay and are based on DNase-resistant AAV genome copies (GC). Two µL of each AAV vector stock was treated for 30 min at 37°C with 10 U OmniCleave in 30 µL 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$ to digest any unpackaged DNA. Then, 10 µL proteinase K solution (50 mM Tris-HCl [pH 8.0], 8 M urea, 4 mM $CaCl_2$ and 1 µg/µl proteinase K [Fermentas]) was added and the sample was incubated at 37°C for 1 hour to release the AAV vector genomes. Next, the samples was boiled for 5 min in a water bath and immediately chilled on ice to denature the vector DNA. The sample was subsequently diluted 1:1 with 20× SSC buffer (3 M NaCl, 300 mM trisodium citrate [pH 7-8]) and blotted onto a Hybond-N+ nylon membrane (GE Healthcare, Diegem, Belgium) using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Veenendaal, the Netherlands). The vector DNA was then fixed to the membrane by incubation for 2 hours at 80°C. The AlkPhos direct labelling and detection system with CDP-*Star* (GE Healthcare) was used to quantify the DNA. As a probe either a 501-bp fragment specific for *nlsLacZco* (primers: 5'-CGTGACCGTGTCCCTGTGGC-3' and 5-'GGCACCATGCCGTGGGTCTC-3') or a 438-bp fragment binding to the WHVPRE (primers: 5'-TCAGGCAACGTGGCGTGGTGTG-3' and 5'-AAGACCAAGCAACACGGACCGG-3' was obtained by PCR and isolated from agarose gel. The membrane was prehybridized for 30 min at 55°C after which 150 ng of the thermostable alkaline phosphatase-labelled probe was added to hybridize overnight at 55°C. Stringency washes and signal detection were performed following the recommendations of GE Healthcare. The ChemiDoc XRS+ system and Quantity One software (both from Bio-Rad Laboratories) were used to quantify the DNA. The titers typically ranged from 10^{11} to 10^{12} GC/mL.

To compare the amount of pTR.hCMV-IE.eGFP.WHVPRE.bGHpA-based AAV vectors produced with the two- and three-plasmid AAV2/8 and AAV 2/9 vector production systems, unconcentrated vector preparations were subjected to end-point titration in 911E4 cells using flow cytometric analysis of *eGFP* expression at 6 days post transduction as readout. Vector yields were expressed in transducing units (TU)/producer cell.

AAV transductions

To test the ability of different AAV vector pseudotypes to transduce MSFs and nrCMCs and to compare the activity of the different promoters in MSFs and nrCMCs, cells were seeded in fibronectin (Sigma-Aldrich)-coated cell culture dishes at a density of 6.25×10^4 cells/cm^2.

For measuring β-galactosidase activity by chemiluminescence assay, cells were cultured in 96-well plates (Corning Life Sciences, Amsterdam, the Netherlands). Chromogenic detection of β-galactosidase activity was done with cells maintained in 24-well plates (Greiner Bio-One). To test the ability of the AAV vectors AAV2/2.hEF1α.eGFP.WHVPRE.synpA, AAV2/2Y444500730Y.hEF1α.eGFP.WHVPRE.synpA (hereinafter referred to as AAV2/2mt.hEF1α.eGFP.WHVPRE.synpA) and AAV2/9.RSV.eGFP.WHVPRE.synpA to achieve cell type-specific transgene expression in rMSFs and nrCMCs, cells were seeded on fibronectin-coated round glass coverslips (14 mm Ø) in 24-well cell culture plates.

All nrCMC cultures were treated with the anti-proliferative agent mitomycin C (10 µg/mL; Sigma-Aldrich) at day 1 of culture for 2 hours to inhibit the proliferation of the remaining nrCFBs in these cultures. The next day, the cells were transduced with 100,000 GC of AAV vector per cell in 500 µL (2-cm^2 well) or 100 µL (0.32-cm^2 well) of a 1:1 mixture of DMEM and Ham's F10 without FBS. After 2 hours, an equal volume of 1:1 mixture of DMEM and Ham's F10 supplemented with 10% HS, 200 U/mL penicillin and 200 µg/mL streptomycin was added to the cells. The MSFs were transduced at day 1 of culture with 100,000 GC of AAV vector per cell in 500 µL (2-cm^2 well) or 100 µL (0.32-cm^2 well) DMEM without FBS. After 2 hours, an equal volume of DMEM + 20% FBS, 200 U/mL penicillin and 200 µg/mL streptomycin was added to the cells.

At 7 days post transduction, the cultures were either lysed and the lysates stored at -80°C for future β-galactosidase activity measurements using a chemiluminescence assay or the cells were fixed for subsequent cytochemical visualisation of β-galactosidase activity.

β-galactosidase activity assays

To quantify β-galactosidase levels in whole cell cultures, cells in a single well of a 96-well plate were lysed in 50 µL lysis buffer (25 mM Tris-HCl [pH 7.8], 2 mM 1,2-diaminocyclohexanetetraacetic acid, 2 mM dithiothreitol, 10% glycerol and 1% Triton X-100). The Galacto-Light β-galactosidase reporter gene assay system (Applied Biosystems Life Technologies) was used to quantify β-galactosidase activity using a Wallac 1420 VICTOR 3 multilabel plate reader (Perkin Elmer Nederland, Groningen, the Netherlands). These experiments were performed in triplicate to allow for statistical analysis.

To visualize β-galactosidase in individual cells, cell cultures were incubated for 10 min with 0.25% (v/v) glutaraldehyde (grade II; Sigma-Aldrich) in PBS. The cells were then washed twice with PBS to remove the fixative and incubated overnight with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution (1 mg/mL X-gal [Sigma-Aldrich], 100 mM Na_2HPO_4/NaH_2PO_4 [pH 7.0], 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$) at 37°C. After two 5-min washes with PBS to remove the X-gal solution, the cells were viewed using an inverse microscope (IX51; Olympus, Zoeterwoude, the Netherlands) equipped with a

Peltier-cooled digital camera (XC30) and CellF software (both from Olympus) for image capture and processing/analysis, respectively.

Immunofluorescent staining

Cell cultures exposed to 10^5 GCs of AAV2/2.hEF1α.eGFP.WHVPRE.synpA, AAV2/2mt.hEF1α.eGFP.WHVPRE.synpA or AAV2/2.RSV.eGFP.WHVPRE.synpA per cell were fixed 7 days after transduction by a 20-min incubation on ice with phosphate-buffered 4% formaldehyde (Mallinckrodt Baker, Phillipsburg, NJ). After fixation, the cells were washed 3 times with PBS containing 10 mM glycine (PBSG) and permeabilized by incubation for 5 min on ice with PBS containing 0.1% Triton X-100. After 3 more washes with PBSG, the cells were incubated for 1 hour with 5% 0.22-µm filter-sterilized FBS in PBS to block potential aspecific antibody binding sites. To distinguish nrCMCs from nrCFBs, the nrCMC cultures were stained for the cardiomyocyte marker sarcomeric α-actinin or for the fibroblast marker collagen type I as described above except for the use of 400-fold diluted Alexa Fluor 568-conjugated donkey-anti-mouse IgG or Alexa Fluor 568-conjugated donkey-anti-rabbit IgG antibodies (both from Invitrogen) as secondary antibodies. Next, the cells were incubated for 10 min at room temperature with 10 µg/ml Hoechst 33342 in PBS. After washing the cells 4 times for 5 min with PBS, staining results were analyzed with the aid of the aforementioned Olympus IX51 inverse fluorescence microscope. The intensity of the fluorescent signals was determined using ImageJ software (http://rsb.info.nih.gov/ij/index.html). Image analysis was performed by a blinded investigator using pictures with a resolution of 1280x1024 pixels and of equal exposure time. Quantification of green fluorescent signal was performed in a fixed area or interest (AOI) of 52 by 47 pixels once per cell and expressed in arbitrary units of the mean grey value. Placement of the AOI in the cytoplasm was at a distance between 0 and 2 AOIs from the nucleus. Picture background was determined at sites without apparent fluorescence, averaged and subtracted from the fluorescent signal of cells in that picture. For quantifications performed on nrCMC cultures that contain endogenous nrCFBs, mean fluorescence of all cells was divided by the average fluorescent signal of nrCFBs to derive the fold difference in transgene expression between cell types per viral vector. Quantification was performed in ≥5 pictures per group and an average of 177 cells.

Western blot analyis

Western blot analysis of eGFP levels was performed on whole-cell protein extracts of purified nrCMC, nrCFB or rMSF cultures exposed to 10^5 GCs of AAV2/2.hEF1α.eGFP.WHVPRE.synpA, AAV2/2mt.hEF1α.eGFP.WHVPRE.synpA or AAV2/9.RSV.eGFP.WHVPRE.synpA per cell. At 7 days post transduction, cells were lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50

mM Tris-HCl [pH 8.0] supplemented with protease inhibitors [cOmplete, Mini Protease Inhibitor Cocktail Tablets from Roche Diagnostics Nederland, Almere, the Netherlands]). After clearance of the cell lysates by centrifugation for 20 min at 4°C and 21.130 × *g*, the protein concentration in each sample was determined using the Pierce BCA Protein Assay Kit Total (Thermo Scientific, Rockford, IL). Next, per sample, 4 µg total protein was applied to individual lanes of NuPage 12% Bis-Tris gels (Invitrogen) and electrophoresis was performed for 2 h at 150 V, after which proteins were transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare) overnight using a wet blotting system. After blocking for 1 h at room temperature with 10 mmol/L Tris-HCl (pH7.6), 0.05% Tween-20 and 150 mmol/L NaCl (TBST) containing 2% ECL Advance blocking agent (BA; GE Healthcare), the membranes were incubated for 1 h at room temperature with primary antibodies diluted in TBST-2% BA. Primary antibodies were rabbit anti-eGFP (ab290; Abcam; 1:1,000), mouse anti-cardiac troponin I (clone 19C7; 4T21; Hytest, Turku, Finland; 1:1,000), mouse anti-vimentin (clone V9; V6630; Sigma-Aldrich; 1:200). After washing the blots for 4 times 5 min with TBST, membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated rabbit or mouse IgG-specific goat secondary antibodies (sc-3837 and sc-2005 from Santa Cruz Biotechnology, Santa Cruz, CA) diluted 15,000-fold in TBST-2% BA. Following 4 additional washing steps, blots were developed using ECL Prime Western Detection Reagent (GE Heathcare). The chemiluminescence signals were captured using a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Veenendaal, the Netherlands). Chemiluminescence signals were quantified using ImageJ.

Statistics

The significance of differences between means was calculated by one-way analysis of variance followed by the Bonferroni's *post hoc* test whenever appropriate using Graph Pad Prism 4 (Graph Pad Software, La Jolla, CA). Comparison between two groups was performed using Student's t-test. Differences were considered significant if $p<0.05$. Results are expressed as mean ± standard deviation.

Results

Comparison of two- and three-plasmid AAV vector production systems

AAV vector production protocols that do not require infection of the producer cells with a helper virus have improved the safety of AAV vectors for *in vivo* use. In addition, the development of the AAV helper plasmids pDG, pSH3 and pSH5 encoding not only the AAV Rep and Cap proteins but also adenoviral helper functions has reduced efforts and costs to produce AAV2 vector particles compared to three- or four-plasmid based production systems.[14,27] Two-plasmid-based systems for the production of AAV2 vectors pseudotyped with capsids from AAV serotypes 1, 3, 4, 5 and 6 have also been generated[25] but are not yet available for generating other AAV vector pseudotypes. Since AAV vectors carrying capsids of AAV8 or AAV9 have been shown to efficiently transduce the hearts of rodents [10,11,13,28], we set out to generate pDG-based helper plasmids for the production of AAV2/8 and AAV2/9 vectors. To this end, the *AAV*2 *cap* gene in pDG was replaced by the *cap* gene of AAV8 or of AAV9, resulting in construction of pDP8 and pDP9, respectively.

To compare the AAV vector yields obtained with these new helper plasmids to those achieved with conventional three-plasmid-based AAV2/8 and AAV2/9 vector production methods, crude AAV2/8.hCMV-IE.eGFP.WHVPRE.bGHpA and AAV2/9.hCMV-IE.eGFP.WHVPRE.bGHpA vector preparations were made using both production systems. Limiting dilution assays in 911E4 cells revealed that the amounts of functional AAV vector particles generated with pDP8 and pDP9 did not differ significantly from those obtained with a combination of pAd.ΔF6 and either pAAV2/8 or pAAV2/9 (8.60±3.28 TU/cell vs. 11.75±3.81 TU/cell, $p>0.05$, and 20.70±3.72 TU/cell vs. 24.18±2.68 TU/cell, $p>0.05$, respectively; n=6; Figure 1). These results show that the new helper plasmids pDP8 and pDP9 can be used to produce high-titered stocks of functional AAV2/8 and AAV2/9 vector particles.

Comparison of promoter activity in MSFs and nrCMCs

To compare the efficiency by which MSFs of man, mouse and rat can be transduced by AAV2/2 vectors, cultures of these cells were exposed to equal doses of AAV2/2.hCMV-IE.nlsLacZco.synpA, AAV2/2.mCMV-IE.nlsLacZco.synpA or AAV2/2.rCMV-IE.nlsLacZco.synpA. X-gal staining at 7 days post infection revealed that hMSFs were transduced with a higher efficiency than mMSFs and rMSFs (Figure 2). Furthermore, in hMSFs *LacZ* expression was highest when driven by the hCMV-IE promoter, while in mMSFs and rMSFs the *immediate early* gene promoters of the rodent cytomegaloviruses were more active. These results show that there is some level of species specificity in the activity of CMV-IE promoters.

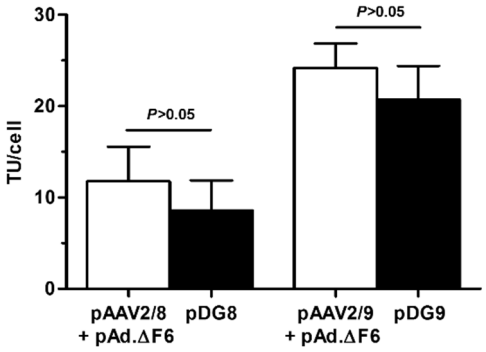

Figure 1. *Titers of small-scale AAV2/8 and AAV2/9 vector stocks generated with the aid of two- or three-plasmid AAV vector production systems. AAV vectors were produced using the eGFP-encoding AAV vector shuttle plasmid pTR.hCMV-IE.eGFP.WHVPRE.bGHpA and titered by serial dilution in 911E4 cells. Titers are based on the number of eGFP-positive cells as assessed by flow cytometry at 6 days post infection and given as the number of TU obtained per producer cell. The titers of AAV vector stocks made with the two-plasmid method (pDG8 or pDG9) did not differ significantly from those produced with the corresponding three-plasmid approach (pAd.ΔF6 and pAAV2/8 or pAAV2/9). Pseudotransduction did not contribute to the observed gene transfer activities as eGFP-positive 911E4 cells were undetectable when the AAV vector productions were carried out with helper plasmids lacking the AAV cap gene (data not shown).*

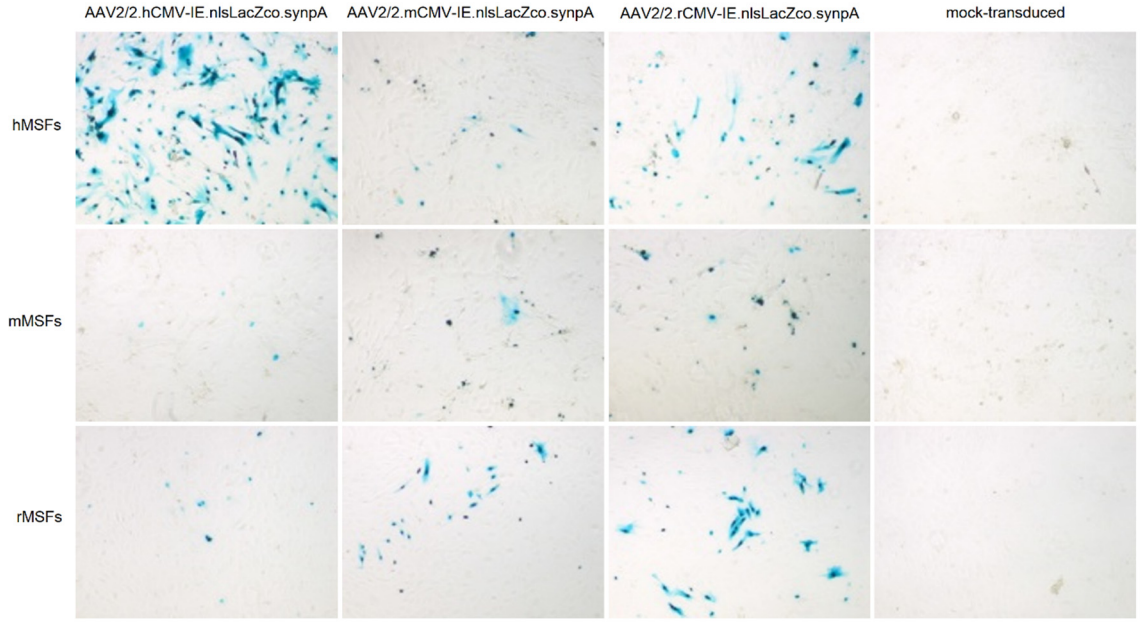

Figure 2. *β-galactosidase staining of AAV2/2 vector- or mock-transduced MSFs. The hCMV-IE promoter induces high-level transgene expression in hMSFs but is hardly active in mMSFs or rMSFs. The mCMV-IE and rCMV-IE promoters are more active in mMSFs and rMSFs than the hCMV-IE promoter and give similar low levels of LacZ expression in hMSFs.*

Next, the activity of the three CMV-IE promoters in MSFs was compared with that of other ubiquitous promoters and of two fibroblast-specific promoters (Table 1). For this purpose, MSF cultures were transduced in quadruplicate by AAV2/2 vectors carrying the *nlsLacZco* gene driven by one of nine different promoters. After 7 days, the cells were lysed and the β-galactosidase activity in the cell lysates was measured to assess the transcription rate of each promoter. The results showed that in hMSFs the hCMV-IE promoter displays the highest transcriptional activity, resulting in significantly higher β-galactosidase levels compared to those found with the other promoters ($p<0.001$; Figure 3A). Furthermore, in mMSFs, rMSFs and nrCFBs the hEF1α promoter showed the highest transcription rate, which resulted in significantly higher β-galactosidase levels than were obtained with the other promoters ($p<0.001$; Figure 3B, C and D).

To achieve the selective transduction of myocardial scar tissue, a promoter should ideally not only direct high levels of transgene expression in MSFs, but also be show low or no activity in cardiomyocytes. Thus, the activity of the nine promoters was also determined in purified nrCMC cultures. Quantification of β-galactosidase levels in each cell culture showed that the RSV promoter had a significantly higher transcriptional activity in nrCMCs than the other promoters ($p<0.001$), whereas the hEF1α promoter was only weakly active in these cells (3E). Together, these results indicate that of the promoters tested, the hEF1α promoter is preferred when targeting myocardial scar tissue in mouse or rat models, since it causes high-level transgene expression in MSFs but is relatively weakly active in nrCMCs.

promoter	promoter length (in nts)	AAV genome length (in nts)
Rous sarcoma virus long terminal repeat (RSV)	715	4503
Simian virus 40 early + R-U5 from human T cell leukemia virus I long terminal repeat (SRα)	741	4368
Human cytomegalovirus immediate-early (hCMV-IE)	1099	4711
Mouse cytomegalovirus immediate-early (mCMV-IE)	1126	4733
Rat cytomegalovirus immediate-early (rCMV-IE)	1177	4824
hCMV-IE enhancer + chicken β-actin core promoter + rabbit β-globin intron (CAG)	1110	4774
Human elongation factor 1α (hEF1α)	803	4484
Mouse fibroblast-specific protein 1 (mFSP1)	522	4284
Rat lysyl oxidase (rLO)	616	4354

Table 1.*Overview of the promoters used to drive LacZ expression in the AAV vectors.*

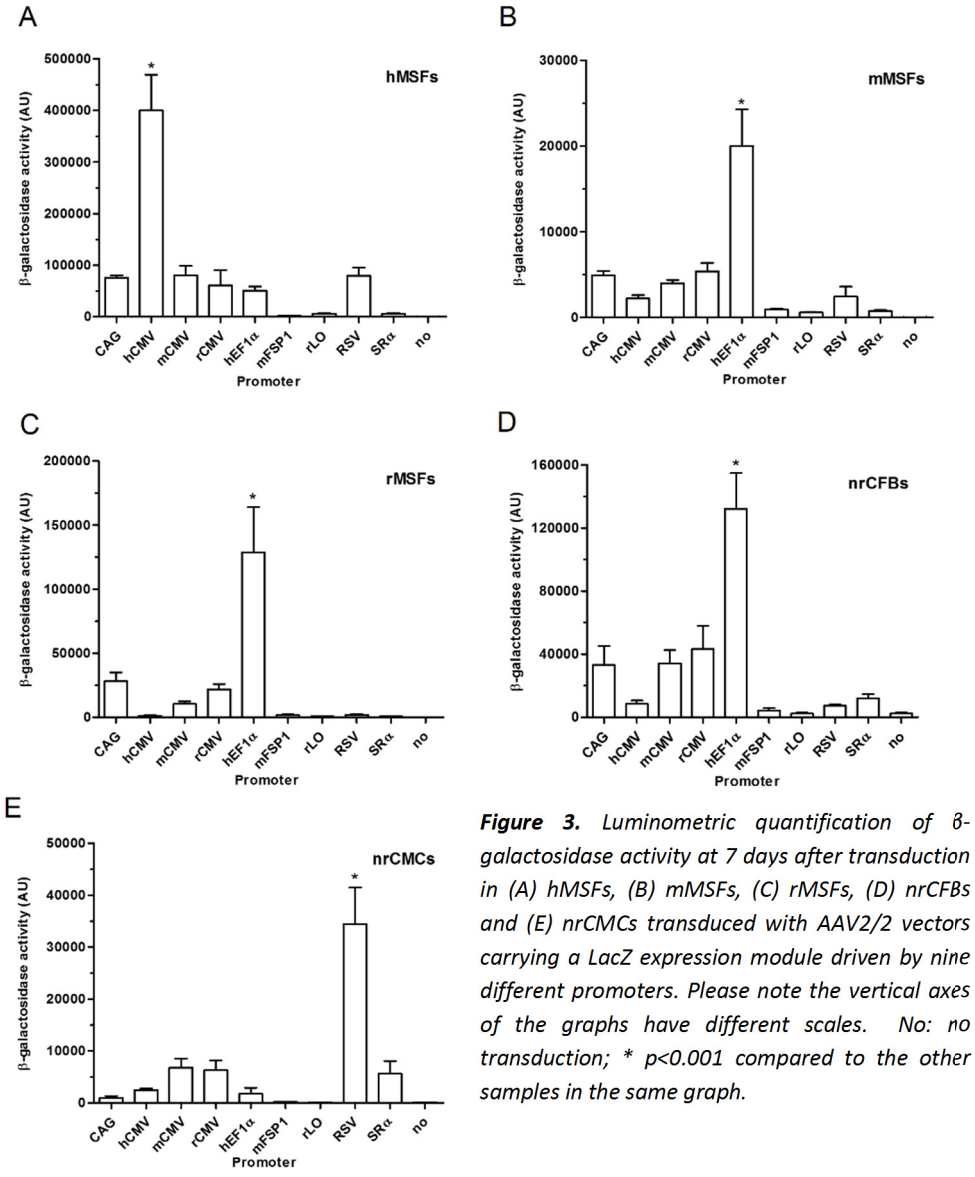

Figure 3. *Luminometric quantification of β-galactosidase activity at 7 days after transduction in (A) hMSFs, (B) mMSFs, (C) rMSFs, (D) nrCFBs and (E) nrCMCs transduced with AAV2/2 vectors carrying a LacZ expression module driven by nine different promoters. Please note the vertical axes of the graphs have different scales. No: no transduction; * p<0.001 compared to the other samples in the same graph.*

Comparison of the gene transfer activity of five different AAV vector pseudotypes in rMSFs and nrCMCs

Beside through transcriptional targeting, cell type-specific transgene expression in MSFs may also be accomplished by transductional targeting. To determine which AAV vector pseudotype transduces MSFs most specifically, rMSF and purified nrCMC cultures were exposed to AAV2 vectors with capsids of AAV serotypes 2, 5, 6, 8 or 9. To achieve the highest possible transgene expression levels, MSFs were transduced with vectors carrying an hEF1α promoter-driven *nlsLacZco* expression unit, whereas the cardiomyocytes received vectors in which the β-galactosidase-coding sequence was preceded by the RSV promoter.

In rMSFs, transduction with AAV2/2 vectors resulted in higher β-galactosidase levels than those obtained with the other AAV vector pseudotypes ($p<0.001$; n=6; Figure 4A). In addition, AAV2/6 vectors showed superior transduction of rMSFs compared to AAV2 vectors with capsids of AAV serotypes 5, 8 and 9 ($p<0.001$ vs. AAV2/8 and AAV2/9 vectors, $p<0.01$ vs. AAV2/5 vectors). In contrast, nrCMCs were most efficiently transduced by AAV2/6 vectors ($p<0.001$ vs. the other AAV vector pseudotypes), followed by AAV2/2 and AAV2/9 vectors ($p<0.001$ vs. AAV2/5 and AAV2/8; Figure 4A).

Based on these results, rMSFs are best transduced with AAV2/2 vectors while AAV2/9 vectors are a good candidate for the selective transduction of nrCMCs since they induce high-level transgene expression in these cells and poorly transduce MSFs, in contrast to AAV2/2 and AAV2/6 vectors (Figure 4B).

Although AAV2/2 vectors better transduced rMSFs than the other AAV vector pseudotypes tested, AAV2/2-mediated gene transfer to these cells was still rather inefficient. Recently, Li et al. (2010) reported that transduction of murine embryonic fibroblasts by AAV2/2 could be greatly improved by substituting the surface-exposed tyrosine residues at positions 444, 500 and 730 of the AAV2 VP1 protein for phenylalanines. To investigate whether the same would hold true for rat cardiac (myo)fibroblasts, AAV2 genomes carrying a recombinant *eGFP* gene under control of the hEF1α promoter were packaged in wild-type AAV2 capsids or AAV2 capsids carrying each of the three aforementioned amino acid substitutions. Next, nrCFBs were exposed to 10^5 GCs/cell of both vectors. As is evident from Figure 5A, the nrCFBs were more efficiently transduced by the vectors with the tyrosine-mutated AAV2 capsids.

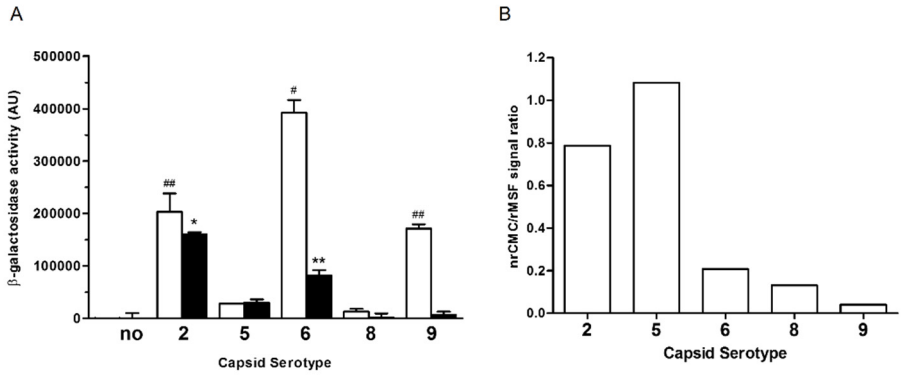

Figure 4. *(A) Comparison of the ability of five different AAV vector pseudotypes to transduce nrCMCs (white bars) or rMSFs (black bars). Transduction efficiencies were determined by luminometric quantification of β-galactosidase levels in lysates of each sample prepared at 7 days after infection (n=6 samples per cell type and per AAV vector pseudotype). The nrCMCs were transduced with AAV vectors carrying LacZ driven by the RSV promoter while the rMSFs were transduced by AAV vectors in which the β-galactosidase-coding sequence was preceded by the hEF1α promoter. No: no transduction; # p<0.001 vs. all other AAV vector pseudotypes in nrCMCs; ## p<0.001 vs. AAV2/5 and AAV2/8 vectors in nrCMCs; * p<0.001 vs. all other AAV vector pseudotypes in rMSFs; ** p<0.001 vs. AAV2/8 and AAV2/9 vector pseudotypes, p<0.01 vs. AAV2/5 vector pseudotypes in rMSFs. (B) Ratio between the β-galactosidase activity in rMSFs and in nrCMCs for AAV2.hCMV-IE.nlsLacZco.synpA vectors packaged in AAV2, AAV5, AAV6, AAV8 or AAV9 capsids.*

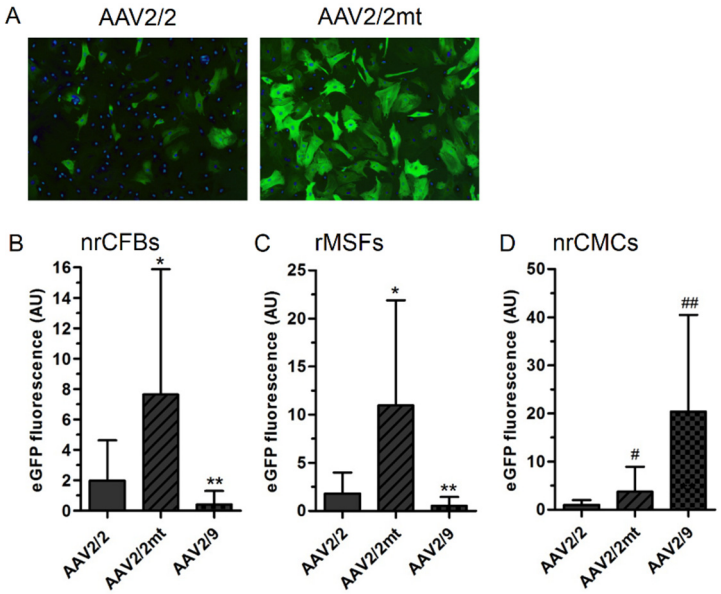

Figure 5. *Representative fluoromicrographs of nrCFB cultures taken at 7 days after transduction with 10^5 GCs/cell of AAV2/2.hEF1α.eGFP.WHVPRE.synpA (AAV2/2) or AAV2/2mt.hEF1α.eGFP.WHVPRE.synpA (AAV2/2mt). (B-D) Quantification of eGFP signals from fluoromicrographs of (B) nrCFB, (C) rMSF, and (D) nrCMC cultures transduced with 10^5 GCs/cell of AAV2/2.hEF1α.eGFP.WHVPRE.synpA (AAV2/2), AAV2/2mt.hEF1α.eGFP.WHVPRE.synpA (AAV2/2mt) or AAV2/9.RSV.eGFP.WHVPRE.synpA (AAV2/9). *:P<0.05 vs. AAV2/2 and AAV2/9. **:P<0.05 vs. AAV2/2 and AAV2/2mt.*

Transduction of nrCMCs and rat cardiac (myo)fibroblasts with optimized AAV vectors

Based on the previous results, we generated an AAV2/9 vector in which *eGFP* expression is driven by the RSV promoter and compared its ability to transduce nrCMCs, nrCFBs and rMSFs with that of AAV2/2.hEF1α.eGFP.WHVPRE.synpA and of AAV2/2mt.hEF1α.eGFP.WHVPRE.synpA. Both nrCFBs and rMSFs were poorly transduced by AAV2/9.RSV.eGFP.WHVPRE.synpA (Figure 5B, C) while this vector greatly outperformed the wild-type and tyrosine-mutant AAV2/2 vector in purified nrCMC cultures (Figure 5D). Substitution of the surface-exposed tyrosine residues at amino acid positions 444, 500 and 730 in the VP1 protein of AAV2/2.hEF1α.eGFP.WHVPRE.synpA yielded 3.9-, 6.1- and 3.6-fold higher transduction levels (in terms of mean eGFP signals) in nrCFBs, rMSFs and nrCMCs (Figure 5B, C and D). The presence in purified nrCMC cultures of 5-10% nrCFBs allowed us to compare in single cultures the efficiency with which both cell types could be transduced by AAV2/2.hEF1α.eGFP.WHVPRE.synpA, AAV2/2mt.hEF1α.eGFP.WHVPRE.synpA or AAV2/9.RSV.eGFP.WHVPRE.synpA through immunostaining for the cardiomyocyte marker α-actinin together with direct eGFP

fluorescence recordings. This experiment confirmed the preferential transduction of cardiac fibroblasts by the AAV2/2 vectors (Figure 6A, B, D and E) and of cardiomyocytes by AAV2/9.RSV.eGFP.WHVPRE.synpA (Figure 6C, F). It also illustrates the increased gene transfer activity of the AAV vectors with the tyrosine mutant AAV2 capsids in comparison to those possessing the wild-type AAV2 capsids (compare the green fluorescence intensity of the α-actinin-negative cell in Figure 6A with that of the α-actinin-negative cell in Figure 6B). Moreover, selectivity for nrCFBs was higher for the AAV2/2 vector with the mutant capsid (Figure 6D, E). These data were supported by western blot analyses (data not shown). Collectively, our results demonstrate that through rational design it is possible to make AAV vectors for the preferential transduction of cardiac (myo)fibroblasts or cardiomyocytes and provide a basis for determining the transduction profiles of AAV2/2(mt).hEF1α.eGFP.WHVPRE.synpA and AAV2/9.RSV.FP650.WHVPRE.synpA in infarcted rat hearts.

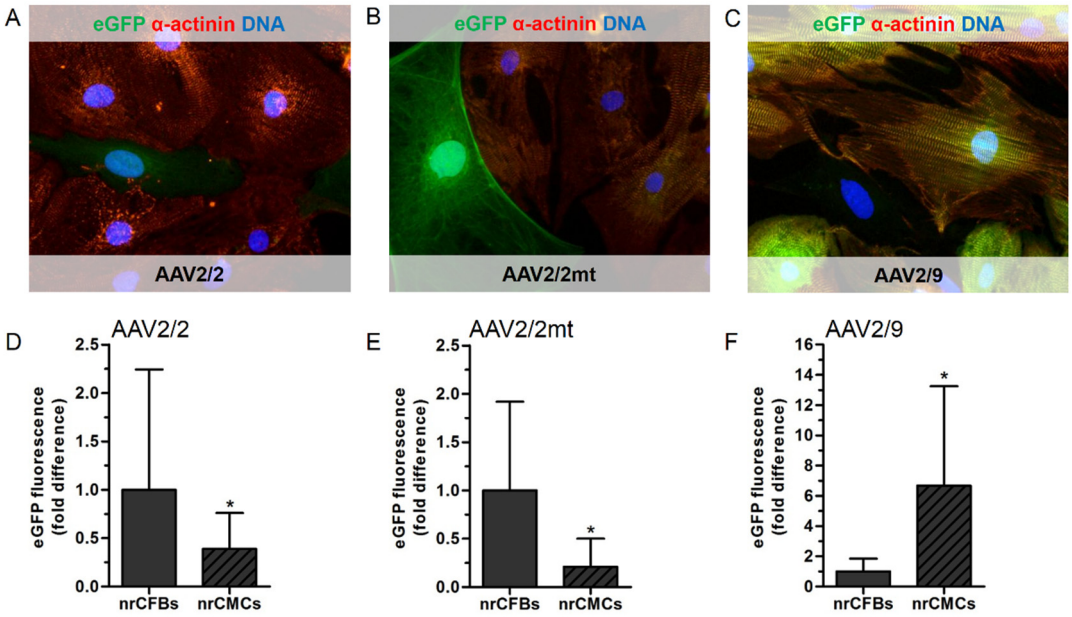

Figure 6. *(A-C) Representative fluoromicrographs of (A) AAV2/2.hEF1α.eGFP.WHVPRE.synpA (AAV2/2)-, (B) AAV2/2mt.hEF1α.eGFP.WHVPRE.synpA (AAV2/2mt)- and (C) AAV2/9.RSV.eGFP.WHVPRE.synpA (AAV2/9)-transduced cultures of nrCMCs containing endogenous nrCFBs. The cells were fixed at 7 days post transduction and stained for α-actinin to discriminate nrCMCs from nrCFBs and with Hoechst 33342 to visualize cell nuclei. (D-F) Quantification of the eGFP signal per cell type in nrCMCs containing endogenous nrCFBs following their transduction with 10^5 GCs/cell of (D) AAV2/2.hEF1α.eGFP.WHVPRE.synpA (AAV2/2), (E) AAV2/2mt.hEF1α.eGFP.WHVPRE.synpA (AAV2/2mt) or (F) AAV2/9.RSV.eGFP.WHVPRE.synpA (AAV2/9). The mean eGFP signal in nrCFBs was set to 1. *:P<0.05 vs. nrCFB.*

Discussion

The aim of this study was to develop AAV vectors conferring MSF-specific transgene expression. To this purpose, nine different promoters were tested for their activity in MSFs and cardiomyocytes. Furthermore, five distinct AAV vector pseudotypes were assessed for the efficiency with which they transduce MSFs and cardiomyocytes. Of the AAV vectors showing sufficiently high absolute transduction levels the ones with capsids of AAV2 and a transgene driven by the hEF1α promoter resulted in most specific transgene expression in rMSFs compared to nrCMCs. In addition, we found that among the effective AAV vectors the one with AAV9 capsids and the RSV promoter was least specific for rMSFs and would therefore be ideal to use when nrCMCs but not rat cardiac (myo)fibroblasts are the preferential target.

Although recombinant baculovirus-based AAV vector production systems are rapidly gaining popularity[29], co-transfection of 293 cells or derivatives of these cells with an AAV vector shuttle plasmid containing the AAV ITRs, a construct encoding AAV Rep and Cap proteins and a plasmid providing adenovirus helper functions (e.g. pAd.ΔF6 or pAd Helper 4.1)[30,31] still represents the most common means of producing AAV vectors. The production of AAV vectors by plasmid transfections has been simplified by the development of packaging constructs such as pDG and later pDP1 and pDP3 to pDP6, in which the AAV *rep* and *cap* genes are combined with the *E2A*, *E4orf6* and *VAI* and *VAII* genes of adenovirus to provide in one plasmid all *trans*-acting functions that are required for efficient AAV vector production and that are not encoded by the producer cells. Similar packaging plasmids have thus far not been reported for the production of AAV vectors with capsids of more recently discovered serotypes. Since several studies comparing different AAV vector pseudotypes for their capability to transduce rodent hearts have shown that AAV2/8 and AAV2/9 vectors are superior compared to the other AAV vector pseudotypes that were tested[10,11,13,28], we constructed two pDG-based helper plasmids for the production of AAV2 vectors with capsids of AAV8 or AAV9. The yields of AAV vectors produced with these new helper plasmids are similar to those of AAV vectors produced using pDG or one of its previously constructed derivatives.[25] pDG8 and pDG9 may thus be useful to facilitate the production of AAV2/8 and AAV2/9 vectors in quantities sufficient for animal experiments.

Cardiac fibrosis or scar formation after MI negatively affect cardiac function and predispose the heart to the development of arrhythmias.[32] To treat the effects of cardiac fibrosis, the electrical properties of the MSFs may be genetically altered to either improve the conduction through these cells or to functionally uncouple them from the surrounding cardiomyocytes thus reducing the risk of early afterdepolarizations. Since coupling between cardiac (myo)fibroblasts and cardiomyocytes results in the partial depolarization

of the latter cells, it may also be beneficial to increase the resting membrane potential of the former cells. Alternatively, it may be possible to endow the MSFs with properties of cardiomyocytes by their genetic reprogramming with cardiac transcription factor genes. However, cardiac (myo)fibroblast-directed gene therapy may have a detrimental effect on the function of neighboring healthy cardiomyocytes when these cells become transduced in parallel. The gene delivery vectors to be used for this purpose should therefore ideally transduce cardiac (myo)fibroblasts with high specificity. Thus far, in papers about the transduction of the heart by AAV vectors, no distinction was made between cardiomyocytes and cardiac (myo)fibroblasts. In this study, we aimed to generate an AAV vector for the preferential transduction of MSFs in comparison to cardiomyocytes. Five distinct AAV vector pseudotypes were compared to determine which of them is most suited for the selective transduction of MSFs. Also, nine different promoters were tested for their transcriptional activity in MSFs versus nrCMCs. In rMSFs and mMSFs, the hEF1α promoter was superior to the other promoters tested, whereas in hMSFs the hCMV-IE promoter was most active. The high transcriptional activity of the hEF1α promoter in the rodent cells is in concurrence with a study by Smolenski et al., who showed by serial analysis of gene expression in mouse cardiac fibroblasts that the *hEF1α* transcript was the most abundant of all RNA polymerase II transcripts. It is conceivable that the rat or mouse EF1α promoter may even be more active in the rodent MSFs than the hEF1α promoter. This will, however, only be beneficial if these rodent promoters not also display an undesirable increase in transgene expression in the nrCMCs. The high and low activity of the RSV promoter in nrCMCs and rat cardiac (myo)fibroblasts, respectively, is consistent with previous findings[33] and suggests that this promoter is very useful to induce high-level transgene expression in cardiomyocytes but not in cardiac (myo)fibroblasts. The fibroblast-specific promoters that were tested displayed only very low activity in the rodent MSFs. Possibly, crucial cis-acting signals and enhancer sequences are missing from these promoters, which had to be kept short due to the limiting packaging capacity of the AAV capsid. A challenge for future research would therefore be to construct a small but strong fibroblast-specific promoter.

Comparison of five different AAV vector pseudotypes showed that only AAV2/2 vectors transduce rMSFs efficiently, whereas cardiomyocytes are best transduced by AAV2/2, AAV2/6 and AAV2/9 vectors. The only other study that investigated the transduction by AAV vectors of cardiac fibroblasts and cardiomyocytes separately yielded similar results, although AAV2/6 vectors were not tested in that study[13]. Since the goal of the aforementioned study was to identify an AAV vector pseudotype that displayed a preferential tropism for cardiomyocytes, they did not investigate other ways to achieve cardiomyocyte-specific transgene expression. However, our findings and those of Qi et al. show that AAV2/2 vectors, whilst being the only option to efficiently transduce MSFs, also targets nrCMCs. Thus, in order to obtain specific transgene expression in MSFs multiple

targeting strategies need to be combined. The combination of transductional and transcriptional targeting to preferentially transduce either rMSFs or nrCMCs was tested with AAV2/2 vectors expressing *eGFP* under control of the hEF1α promoter and with AAV2/9 vectors expressing *eGFP* under control of the RSV promoter. The results indeed show some degree of specificity for rMSF of the AAV2/2.hEF1α.eGFP.WHVPRE.synpA vector that can be attributed to the differential promoter activity. The vector AAV2/9.RSV.eGFP.WHVPRE.synpA, which was designed for the selective transduction of cardiomyocytes, showed a more pronounced difference in transgene expression between rMSFs and nrCMCs, which is due to the combination of both transductional and transcriptional targeting. Ideally, we would like to target the rMSFs with the same level of specificity as the nrCMCs. This may be achieved by incorporating cardiomyocyte-specific microRNA target sites into the untranslated region(s) of the transcripts encoded by the transgene whose expression should occur in cardiac (myo)fibroblasts but not in cardiomyocytes.[34] This method exploits the naturally occurring microRNA-based gene regulatory mechanisms of cells to posttranscriptionally silence expression of genes that contain the target sequence of such a microRNA in their mRNAs. Selective suppression of transgene expression in cardiac muscle cells depends on the identification of cardiomyocyte-specific microRNAs. MicroRNA-1, -133, -206 and -208 may all qualify for this purpose as they have been shown to be (cardiac) muscle cell-specific.[35,36]

Another interesting finding of this study is the difference in the capability of AAV vectors to transduce MSFs from different species. This may be caused by many factors including differences in receptor availability or amino acid sequence or differences in the fate of the vector particles after cellular uptake. In a recent study, Bhrigu and Trempe showed that nuclear entry of AAV2 particles is impaired in murine fibroblasts as compared to that in human cervical carcinoma cells.[37] Furthermore, it was shown that tyrosine-mediated ubiquitination and subsequent proteasomal degradation of internalized AAV particles limits the transduction of cells by AAV2 vectors.[38] Mutation of three tyrosines in the AAV2 capsid into phenylalanines greatly reduced the susceptibility of AAV2/2 vectors to proteasomal degradation and caused a 130-fold increase in transgene expression in mouse fibroblasts transduced with these vectors.[39] These results indicate that it might be beneficial to equip the MSF-specific AAV2/2 vectors with AAV2 capsids containing the three tyrosine-to-phenylalanine substitutions. In addition to increasing gene transfer to both nrCMCs and nrCFBs, our study shows that these capsid mutations also improved the specificity of transduction of cardiac fibroblasts, as the difference in transduction level between nrCMCs and nrCFBs was bigger for the tyrosine mutant AAV2/2 vector than for the wild type AAV2/2 vector. Such selectivity may be beneficial for situations where genetic modification of just one cardiac cell type is desirable.

In conclusion, this study showed that in order to efficiently transduce cardiac (myo)fibroblasts with AAV vectors, an AAV2/2 vector containing a transgene driven by the hEF1α promoter should be used. In addition, in nrCMCs the highest transgene levels are obtained using an AAV2/9 vector that has the RSV promoter to drive the transgene. In the absence of a strong, small-sized fibroblast-specific promoter, some level of specificity for MSFs can be obtained by a combination of transductional and transcriptional targeting, but other (e.g. microRNA-based) targeting principles should be added to achieve truly MSF-specific transgene expression in rMSFs. Our optimized AAV vectors should next be tested *in vivo* to confirm our *in vitro* findings.

Acknowledgments
We thank Joost Verhaagen and Erich Ehlert for sharing their knowledge on the production of AAV vectors.

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