"Triplet" polycistronic vectors encoding Gata4, Mef2c, and Tbx5 enhances postinfarct ventricular functional improvement compared with singlet vectors

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Objective: The in situ reprogramming of cardiac fibroblasts into induced cardiomyocytes by the administration of gene transfer vectors encoding Gata4 (G), Mef2c (M), and Tbx5 (T) has been shown to improve ventricular function in myocardial infarction models. The efficacy of this strategy could, however, be limited by the need for fibroblast targets to be infected 3 times—once by each of the 3 transgene vectors. We hypothesized that a polycistronic "triplet" vector encoding all 3 transgenes would enhance postinfarct ventricular function compared with use of "singlet" vectors.

Methods: After validation of the polycistronic vector expression in vitro, adult male Fischer 344 rats (n = 6) underwent coronary ligation with or without intramyocardial administration of an adenovirus encoding all 3 major vascular endothelial growth factor (VEGF) isoforms (AdVEGF-All6A positive), followed 3 weeks later by the administration to AdVEGF-All6A-positive treated rats of singlet lentivirus encoding G, M, or T (1×10^5 transducing units each) or the same total dose of a GMT "triplet" lentivirus vector.

Results: Western blots demonstrated that triplet and singlet vectors yielded equivalent GMT transgene expression, and fluorescence activated cell sorting demonstrated that triplet vectors were nearly twice as potent as singlet vectors in generating induced cardiomyocytes from cardiac fibroblasts. Echocardiography demonstrated that GMT triplet vectors were more effective than the 3 combined singlet vectors in enhancing ventricular function from postinfarct baselines (triplet, 37% ± 10%; singlet, 13% ± 7%; negative control, 9% ± 5%; $P < .05$).

Conclusions: These data have confirmed that the in situ administration of G, M, and T induces postinfarct ventricular functional improvement and that GMT polycistronic vectors enhance the efficacy of this strategy. (J Thorac Cardiovasc Surg 2014;148:1656-64)

Supplemental material is available online.

The studies by Takahashi and Yamanaka[1] in 2006 demonstrating that genetic cellular reprogramming could be used to generate induced pluripotent stem cells from adult somatic cells heralded a new era in tissue engineering. In 2010, Ieda and colleagues[2] advanced this work into the cardiac arena by demonstrating that a "cocktail" of transcription factors—Gata4 (G), Mef2c (M), and Tbx5 (T)—induced the conversion of cardiac fibroblasts directly into what they termed "induced cardiomyocyte" cells. This novel cell transdifferentiation strategy, subsequently used to genetically reprogram myocardial scar fibroblasts directly into cardiomyocyte-like cells in situ, offers the exciting new possibility of regenerating contractile tissue from otherwise unsalvageable infarcted myocardium.[3-16] Such direct cardiac cellular reprogramming would obviate entirely the challenges of exogenous stem cell delivery or induced pluripotent stem staging strategies.[17,18]

Given that current cellular reprogramming strategies require the coincident infection of target cells by multiple gene transfer vectors, each encoding a single transgene, we hypothesized that use of a polycistronic "triplet" vector encoding all 3 of the requisite (GMT) transdifferentiating factors would be more efficient than the use of single transgene vectors. We further postulated that this enhanced

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Abbreviations and Acronyms

Ad = adenovirus
AdNull = adenoviral vector that does not encode a transgene
cTnT = cardiac troponin T
CMV = cytomegalovirus
EF = ejection fraction
FACS = fluorescence activated cell sorting
FBS = fetal bovine serum
G = Gata4
GFP = green fluorescent protein
IMDM = Iscove's modified Dulbecco's medium
M = Mef2c
PBS = phosphate-buffered saline
T = Tbx5
TU = transducing units
VEGF = vascular endothelial growth factor

efficiency would result in improved postinfarct ventricular function compared with use of single transgene vectors. In the context of our previous demonstration that myocardial scar before vascularization enhances the efficacy of cardiac regeneration strategies,[11] we have demonstrated in a rat coronary ligation infarct model after scar prevascularization with vascular endothelial growth factor (VEGF) that triplet GMT vectors induce greater improvements in postinfarct ventricular function than do equivalent total doses of singlet vectors.

METHODS

Vectors and Cells

An adenovirus vector (AdVEGF-All6A$^+$) based on the human Ad5 serotype with E1a, partial E1b, E3 deletions that render the vector replication deficient was used to deliver all 3 major isoforms of VEGF (121, 165, and 189) to myocardial tissues.[19] This AdVEGF-All6A$^+$ vector expression cassette, under the control of the cytomegalovirus (CMV) promoter or enhancer and the SV40 polyadenylation signal, incorporates a cDNA/genomic hybrid of human VEGF with intron modifications to allow expression of the 121, 165, and 189 alternative splicing VEGF isoforms.[19] An adenoviral vector that does not encode a transgene (AdNull) was used as a control vector.

Lentivirus vectors were constructed to provide expression of GMT, with either "singlet" or "triplet" expression cassettes coupled to a green fluorescent protein (GFP) reporter transgene. Singlet GMT vectors were constructed, as previously described.[11] In brief, the GMT transcription factor cDNAs (Table E1) were amplified from either commercially available sequences (Addgene) in the case of rat Gata4 (Genbank NM_144730) and rat Tbx5 (Genbank NM_001009964) or from rat heart tissue cDNA in the case of Mef2c (Genbank NM_001009964). Amplified sequences were cloned independently into the pENTR3C plasmid (Invitrogen, Life Technologies, Carlsbad, Calif). These plasmids were used to generate the singlet lentivirus vectors and as a template for assembling the triplet construct.

For the triplet construct, G, T, and M were amplified from the pENTR-Gata4, pENTR-Tbx5, and pENTR-Mef2c plasmids and assembled by overlapping polymerase chain reactions with 2A self-processing viral peptide bridges downstream of a furin cleavage site (furin 2A) between each transcription factor gene. The *Thosea asigna* virus 2A sequence was used as a bridge between Gata4 and Tbx5 proteins and the equine rhinitis A virus 2A between the Tbx5 and Mef2c sequences (Figure E1). The assembled GMT construct was cloned into the pENTR3C dual selection vector (Invitrogen) and incorporated into the FG12-CMV vector (Addgene), which includes the eGFP transgene under the control of the ubiquitin-C promoter for lineage efficiency analysis.

The F12-CMV-GMT plasmid containing the "triplet" expression cassette was co-transfected into the 293T human embryonic kidney cell line (ATCC, Manassas, Va), along with plasmids pMD2G and psPAX from the gateway system (Invitrogen) using lipofectamine 2000 reagent (Invitrogen). After 72 hours, supernatants containing virus were collected, clarified by centrifugation, and syringe filtered (0.45-μm pore size; Sarstedt, Nümbrecht, Germany). The virus was further pelleted from clarified supernatants by centrifugation for 2 hours at 10,000g and resuspended in viral diluent (3% sucrose, 10 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl).

To generate a control lentivirus vector, a homologous lentivirus vector with an expression cassette without a transgene but with an eGFP cassette under the control of the ubiquitin promoter was generated by co-transfecting the F12-CMV plasmid with the lentivirus packaging plasmids into 293T cells, after which, the virus was purified as described in the preceding paragraphs.

Transduction units were established by quantitative polymerase chain reaction assay, as previously described.[20] In brief, 1×10^5 293 T cells were plated in a 24-well plate with 0.5 mL of Dulbecco's modified Eagle medium, 10% fetal bovine serum (FBS), polybrene (8 μg/mL), and 20 μL of serially diluted lenti vector. After 48 hours in a carbon dioxide incubator, the cells were harvested, and the cellular DNA was purified using a DNA extraction kit (Qiagen, Venlo, Limburg, The Netherlands). Lenti RNA genome reverse transcribed into DNA was quantified using real time polymerase chain reaction and Stratagene Mx3005P (Life Technologies). SYBR Green SuperMix (Quanta Biosciences, Gaithersburg, Md) was used. The following primers located in the group-specific antigen gene were used: 5′- AGCGTCAGTATTAAGCGGGG-3′ and 5′-AGGCCAGGATTAACTGCGA-3′. The transducing unit was calculated as RNA genome/diploid cell/μL.

Adult cardiac fibroblasts were harvested from Fisher 344 adult male rats (weight 250-275 g; Harlan, Indianapolis, Ind) using protocols approved by the Baylor College of Medicine institutional animal care and use committee. These were prepared as described by Crabos and colleagues.[20] In brief, the hearts excised from 2 to 3 rats were minced, washed in Hank's Balanced Salt Solution (Gibco, Life Technologies) supplemented with antibiotics (penicillin and streptomycin, 100 U/mL and 100 mg/mL, respectively), and incubated at 37°C in the presence of 0.1% trypsin and 110 U/mL collagenase for 15 minutes. Disaggregated cells were serially pelleted, resuspended in Iscove's modified Dulbecco's medium (IMDM) with GLUT Max (Life Technologies) supplemented with 10% FBS and 1% penicillin and streptomycin antibiotics, and then seeded onto a 75-cm^2 flask and incubated in humidified 5% carbon dioxide/95% air at 37°C. After 120 minutes, the unattached cells were discarded, and the attached cells were cultured further in IMDM containing 10% FBS for 4 days. The cells were then rinsed in phosphate-buffered saline (PBS) and harvested by enzymatic disaggregation (0.05% trypsin), centrifuged, and resuspended in IMDM containing 10% FBS at a concentration of 100 cells/mL. The cells were then seeded onto 24- or 6-well culture dishes and infected with the appropriate vectors after 48 hours.

In Vitro Immunofluorescence Studies

Triplet GMT lentivirus vectors (9×10^5 transducing units [TU]), singlet G, M, and T vectors (9×10^5 TU each) or GFP control lentivirus vectors (9×10^5 TU) were added to rat cardiac fibroblast culture media (IMDM plus 10% FBS) for 48 hours in the presence of 1 μg/mL polybrene

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