Fibroblasts from human postmyocardial infarction scars acquire properties of cardiomyocytes after transduction with a recombinant myocardin gene

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ABSTRACT Myocardial scar formation impairs heart function by inducing cardiac remodeling, decreasing myocardial compliance, and compromising normal electrical conduction. Conversion of myocardial scar fibroblasts (MSFs) into (functional) cardiomyocytes may be an effective alternative treatment to limit loss of cardiac performance after myocardial injury. In this study, we investigated whether the phenotype of MSFs can be modified by gene transfer into cells with properties of cardiomyocytes. To this end, fibroblasts from postmyocardial infarction scars of human left ventricles were isolated and characterized by cell biological, immunological, and molecular biological assays. Cultured human MSFs express GATA4 and connexin 43 and display adipogenic differentiation potential. Infection of human MSFs with a lentivirus vector encoding the potent cardiogenic transcription factor myocardin renders them positive for a wide variety of cardiomyocyte-specific proteins, including sarcomeric components, transcription factors, and ion channels, and induces the expression of several smooth muscle marker genes. Forced myocardin expression also endowed human MSFs with the ability to transmit an action potential and to repair an artificially created conduction block in cardiomyocyte cultures. These findings indicate that in vivo myocardin gene transfer may potentially limit cardiomyocyte loss, myocardial fibrosis, and disturbances in electrical conduction caused by myocardial infarction.—van Tuyn, J., Pijnappels, D. A., de Vries, A. A. F., de Vries, I., van der Velde-van Dijke, I., Knaaën-Shanzer, S., van der Laarse, A., Schalij, M. J., Atsma, D. E. Fibroblasts from human postmyocardial infarction scars acquire properties of cardiomyocytes after transduction with a recombinant myocardin gene. FASEB J. 21, 000–000 (2007)

Key Words: conduction (block) · fibrosis · gene transfer · heart infarction · myocytes

After myocardial infarction (MI), the cardiomyocytes in the infarcted myocardium are replaced by fibrotic scar tissue composed of (myo)fibroblasts and a collagen-rich extracellular matrix (ECM; see ref. 1). These cells persist and remain metabolically active even in areas of long-standing fibrosis (2). Myocardial scars contribute to deleterious remodeling and increased mechanical stiffness of the heart resulting in impaired systolic and diastolic function, which may lead to symptoms of heart failure. In addition, excessive deposition of ECM components reduces electrical coupling between cardiomyocytes, thereby impairing impulse propagation (3). Stem cell transplantation is being explored as a new therapeutic modality to regenerate damaged myocardium (4). So far, most animal and clinical studies have demonstrated only a modest and sometimes even transient improvement of cardiac function after infusion of somatic stem cells in infarcted myocardium (5, 6). True myocardial regeneration via in vivo (trans)differentiation of somatic stem cells into cardiomyocytes seems at best to be a rare event (7). Therefore, alternative treatment strategies targeting cardiac fibroblasts have been proposed, including 1) inhibition of ECM synthesis by these cells, 2) their conversion into (cardio)myocytes, and 3) the use of these cells as a local source of cardioregenerative and/or angiogenic factors (8). Introduction of a gene encoding a developmental regulator of (cardio)myogenesis in myocardial scar fibroblasts (MSFs) may induce their transdifferentiation into (cardiac) muscle cells. Illustrating the feasibility of this concept, in vitro and in vivo experiments have shown that transfer of the gene coding for the skeletal muscle-specific transcription factor (TF) MYOD1 leads to skeletal muscle differentiation in a variety of nonmuscle cell types, including cardiac fibroblasts (9–11). Given the differences in ion channel composition between skeletal muscle cells and cardiomyocytes (12), however, it is unlikely that

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MYOD1-transduced fibroblasts can produce a cardiac action potential. It would therefore be preferable to use a gene encoding a TF involved in cardiomyogenesis to induce heart muscle differentiation in MSFs and to endow them with the desired electrical properties.

Several TFs, including Nkx2.5, GATA binding protein family members (e.g., GATA4), T box-containing protein family members (e.g., Tbx5), the heart- and neural crest derivative-expressed (Hand) 1 and 2 proteins, polypeptide C of the MADS box transcription enhancer factor 2 (Mef2) group of proteins (e.g., Mef2C), and serum response factor (SRF), have been found to be essential for proper heart development during embryogenesis (for reviews, see refs. 13, 14). Each of these cardiac TFs is directly involved in the transcriptional regulation of genes encoding the specialized structural components and channel proteins of cardiomyocytes by binding to well-established DNA sequences in the promoter/enhancer regions of these genes. Recently, muscle development in the roundworm Caenorhabditis elegans was found to be regulated by three functionally redundant genes: the MyoD family homologue hhl-1, the SRF/Mef2 homologue unc-120, and the HAN D homologue hnd-1 (15). In vertebrates, the corresponding genes have adopted more specific roles in muscle development, with the MyoD family members regulating skeletal muscle formation while the Hand genes are involved in smooth and cardiac muscle development; SRF and Mef2 family members play an important role in specifying all three muscle lineages (13–15). However, forced expression of the genes encoding the aforementioned TFs in somatic mammalian cells of nonmuscle origin has not yet been reported to result in the formation of cardiomyocytes. Recently, a new cardiac and smooth muscle-restricted TF, designated myocardin, was identified (16). This protein, which exerts most of its effects by forming a complex with SRF and Mef2c, appears to be a key regulator of cardiac and smooth muscle differentiation (16–18). Infection with a myocardin-encoding adenovirus vector caused ±10% of human dermal fibroblasts (hDFs) and human mesenchymal stem cells (hMSCs), as well as ±100% of human epicardial cells, to adopt a cardiomyocyte-like phenotype through activation of genes specifying a wide variety of cardiomyocyte-specific polypeptides, including sarcomeric components and heart muscle-specific TFs (19, 20).

In this study we used reporter gene assays to compare the ability of the cardiogenic TFs (cTFs) GATA binding protein 4 (GATA4), heart- and neural crest derivative-expressed 1 (Hand1, also known as eHand), MADS box transcription enhancer factor 2C (Mef2c), myocardin (both cardiomyocyte-enriched isoforms), Nkx2.5, and Tbx5 to transactivate the promoters of the human natriuretic peptide precursor A (ANF), myosin heavy chain 6 (αMHC), and gap junction protein α1 (Cx43) genes. As myocardin outperformed all other cTFs as a transcriptional activator, we generated a self-inactivating (SIN) lentivirus vector (LV.CMV.myocL-HA) directing synthesis of the largest isoform of human myocardin (myocL). This lentivirus vector was subsequently used to impose a cardiomyocyte-like gene expression program on human MSFs (hMSFs), as evidenced by reverse transcription-polymerase chain reaction (RT-PCR) analyses and immunofluorescence microscopy (IFM). Furthermore, electrophysiological studies using multielectrode array (MEA) -containing culture dishes revealed that hMSFs transduced with LV.CMV.myocL-HA were highly effective at repairing an artificially created conduction block in monolayers of neonatal rat cardiomyocytes (rCMCs).

**MATERIALS AND METHODS**

**Isolation and culture of human myocardial scar fibroblasts**

hMSFs were isolated from multiple samples of human ventricular scar tissue obtained during surgical reconstruction of the left cardiac ventricle. Each sample was cut into small pieces, which were transferred to porcine gelatin (Sigma-Aldrich, St. Louis, MO, USA) -coated 10 cm² culture dishes and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS; all from Invitrogen). A glass coverslip was placed on top of the tissue pieces to prevent them from floating. The culture medium was refreshed every 3 days. Outgrowth of cells was visible 2 days after culture initiation. Three days later, the coverslip and tissue pieces were removed and the hMSFs were detached with trypsin-EDTA solution (Invitrogen), then reseeded in new culture dishes. When the cultures reached 3–6 × 10⁶ cells, aliquots of 2 × 10⁶ hMSFs were frozen to −80°C at −1°C/min in DMEM containing 40% FBS and 10% dimethyl sulfoxide (Sigma-Aldrich) and subsequently stored in nitrogen vapor.

**Cell culture and in vitro differentiation assays**

HeLa cells were propagated in DMEM supplemented with 10% FBS, hDFs were cultured as described before (19); the isolation and culture of rCMCs has been specified elsewhere (21). Induction and analysis of in vitro adipogenesis and osteogenesis have been carried out as described previously (19).

**Immunophenotyping**

The surface antigen expression profile of hMSFs was determined by flow cytometry as described for bone marrow-derived hMSCs (19).

**Promoter assays**

For comparing the transactivating activity of different cTFs, we generated the luciferase-encoding reporter constructs pANF.LUC, pMHC.LUC, and pCX43.LUC containing 2241, 5505, and 3009 bp fragments of the human ANF, αMHC, and Cx43 promoters, respectively. The nucleotide sequences of these pGL3-basic (Promega, Madison, WI, USA) derivatives have been deposited in the GenBank database under accession nos. EF186080, EF186081, and EF186082.

In the expression plasmids pU.CAG.hrGFP, pU.CAG.myocL, pU.CAG.myocS, pU.CAG.Hand1, pU.CAG.Nkx2.5, pU.CAG.GATA4, pU.CAG.Mef2C, and pU.CAG.Tbx5, coding...
sequences of the *Renilla reniformis* green fluorescent protein (Stratagene, San Diego, CA, USA), myocL, the small cardiomyocyte-enriched isoform of human myocardin (myocS), and the human versions of Hand1, Nkx2.5, GATA4, Mef2C, and Tbx5 are placed under the transcriptional control of the CAG promoter (22) and rabbit β-globin gene polyadenylation signal. For the nucleotide sequences of these constructs, see GenBank accession nos. EF186083, EF186084, EF208955, EF208956, EF186085, EF186086, and EF186087, respectively.

One day before transfection, 3 × 10^4 HeLa cells were seeded per well of a 24-well plate (Greiner, Frickenhausen, Germany). The next day the cells were transfected using ExGen 500 (Fermentas, Hanover, MD, USA) with 0.2 μg pMHC.LUC DNA or the molar equivalent of pANF.LUC or pCX43.LUC supplemented with pUCBM21 (Boehringer Mannheim, Mannheim, Germany) to a total of 0.2 μg DNA and 0.2 μg pU.CAG.myocL DNA or the molar equivalent of pU.CAG.myocS, pU.CAG.Hand1, pU.CAG.Nkx2.5, pU.CAG.GATA4, pU.CAG.Mef2C, pU.CAG.Tbx5, or pU.CAG.hrGFP, again supplemented with pUCBM21 to a total of 0.2 μg DNA. Sixteen hours later, the transfection medium was replaced by fresh culture medium. At 48 h post-transfection, the luciferase activity in each cell sample was measured using Steady-Glo (Promega) and a Lumat LB9507 luminometer (EG & G, Berthold, Bad Wildbad, Germany). The induction factors of the various TFs were calculated for all three heart muscle-specific promoters by dividing the luciferase activity observed after cotransfection of one of the three reporter constructs and a TF-encoding plasmid by that found after cotransfection of pU.CAG.hrGFP and the same reporter construct. Each pU.CAG plasmid was tested in quadruplicate in three independent experiments to compensate for possible differences in transfection efficiency.

**Lentivirus vector production**

To generate SIN human immunodeficiency virus type I vectors encoding a recombinant myocL protein carrying an epitope of the human influenza virus A hemagglutinin (HA) at its C terminus (myocL-HA) or specifying a nuclear-targeted version of *Escherichia coli* β-galactosidase (nls-bGal), the shuttle plasmids pLV.CMV.myocL-HA and pLV.C-PGK.nls-bGal were made on the basis of construct pLV.CMV-IRESc-eGFP (23). Expression of myocL-HA from pLV.CMV.myocL-HA is controlled by the human cytomegalovirus immediate-early gene (CMV-IE) promoter and pLV.C-PGK.nls-bGal contains a hybrid promoter consisting of the enhancer of the CMV-IE fused to the human 3'-phosphoglycerate kinase gene promoter to drive transgene expression. Furthermore, to enhance transgene expression in both lentivirus vector shuttle plasmids, the 3' long terminal repeat is preceded by the woodchuck hepatitis virus post-transcriptional regulatory element (24). The nucleotide sequences of pLV.CMV.myocL-HA and pLV.C-PGK.nls-bGal have been deposited in the GenBank database under accession nos. EF186078 and EF186079, respectively. The lentivirus vector shuttle plasmid pLV.CMV.myocL.IRES.eGFP and pLV.CMV.bGal.IRES.eGFP were also derived from pLV.CMV-IRES.eGFP and specify bicistronic mRNAs encoding the enhanced green fluorescent protein and myocL or bGal, respectively. For the nucleotide sequences of these constructs, see GenBank accession nos. EF205035 and EF205034.

Vascular stromatosis virus G-protein-pseudotyped lentivirus vector stocks were produced by seeding six 175 cm^2 culture flasks with 6.6 × 10^4 293T per cm^2 and transfecting these producer cells the next day with 85 ng/cm^2 of either pLV.CMV.myocL-HA or pLV.C-PGK.nls-bGal together with 76 ng/cm^2 psPAX2 (Addgene, Cambridge, MA, USA) and 41 ng/cm^2 pLP/VSVG (Invitrogen) using the calcium phosphate-DNA coprecipitation method (25). Sixteen hours later, the transfection medium was replaced by new culture medium. At 48 h post-transfection, the culture fluid was collected and freed of cellular debris by centrifugation at room temperature for 10 min at 800 g and filtration through a 0.45 μm pore-sized cellulose acetate filter (Pall Corporation, East Hills, NY, USA). To concentrate the lentivirus vector particles, 51 ml of the cleared culture medium was loaded onto a 4 ml cushion of 20% sucrose in phosphate-buffered saline (PBS), then centrifuged for 90 min at 25,000 rpm and 10°C in an SW 28 rotor (Beckman Coulter, Fullerton, CA, USA). Next, the supernatant was discarded and the pellets with the vector particles were suspended in 200 μl PBS-10% FBS by gentle rocking overnight at 4°C.

An aliquot of both vector stocks was serially diluted in culture medium containing 8 μg/ml polybrene and used to infect HeLa cells. Four hours later, the inoculum was exchanged with fresh culture medium. At 2 days postinfection, the functional titer (in terms of HeLa cell-transducing units [HTUs]) of the LV.C-PGK.nls-bGal preparation was determined by 5-bromo-4-chloro-3-indolyl-beta-d-galactopyranoside staining of formaldehyde-fixed cells and that of the LV.CMV.myocL-HA stock was determined by immunostaining of ethanol-fixed cells with a 1:1000 dilution of monoclonal antibody (mAb) HA.11 (Covance, Princeton, NJ, USA) directed against the epitope tag.

**Lentivirus vector transduction**

hMSFs were seeded at a density of 2 × 10^4 cells/cm^2 in 10 cm² culture dishes with (for IFM) or without (for RT-PCR analysis) glass coverslips. The next day, the culture fluid was replaced by 1 ml fresh growth medium containing 20 μg/ml diethylaminoethyl-dextran sulfate (GE Healthcare) and 1 HTU of LV.CMV.myocL-HA or LV.C-PGK.nls-bGal per cell. Four hours later, the cells were washed three times with PBS and the inoculum was replaced by fresh culture medium. One week after transduction, cells were analyzed by IFM or RT-PCR analysis.

**Reverse transcription-polymerase chain reaction analysis**

Semiquantitative RT-PCR analyses were performed as previously reported (20) with the addition of primer pairs targeting transcripts encoding the α1C and α1D subunits of the voltage-dependent L-type calcium channels (CACNA1C or Cav1.2; 5'-AGGAGGAGTTTGGTTGGT3'-3' and 5'-TG-GAGCTGACTTGAGATG3'-3' and CACNA1D or Cav1.3; 5'-GCAAGATGACAGCCTAGAAG3'-3' and 5'-ATGGTGTATG-GTTAGAC3'-3'), respectively, the cardiac pacemaker channel (hyperpolarization-activated cyclic nucleotide-gated potassium channel 4; HCN4; 5'-CGCCTATTGATTATATCG3'-3' and 5'-GCCGAGGATGACTGCTCTC3'), and the inwardly rectifying potassium channel J3 (KCNJ5 also known as Kir3.1; 5'-TCCCCCTTGACCAACTTGAAC3'-3' and 5'-AG-CACATGAGAAGCTCTCC3'). For these new primer pairs, 35 PCR cycles and a annealing temperature of 60°C were applied. As internal controls for the quantity and quality of the RNA specimens, RT-PCR amplifications targeting transcripts of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) were performed in parallel. PCR reactions carried out on cDNA derived from total RNA samples of human atrium, ventricle, vascular smooth muscle, and skeletal muscle served as positive controls, and PCRs in which the cDNA was replaced by water were included as negative controls. For comparing relative mRNA levels, only PCR samples from within the linear range of amplification were used.
used. Quantitative RT-PCR (qPCR) was performed in triplicate using the qPCR&GO kit (Qbiogene, Irvine, CA, USA) and the LightCycler 480 real-time PCR system (Roche, Nutley, NJ, USA), according to the manufacturers’ specifications. To determine PCR efficiencies, standard curves were generated by 2-fold serial dilution of the cDNA template derived from MSFs treated with myocardin (series of 8). Both GAPDH and the β-actin (ACTB) gene were used to normalize transcription levels. Melting curve analysis was carried out to verify that only a single product was formed during the qPCR reaction. Normalization and statistical analysis were performed using the Relative Expression Software Tool (REST) (26). Additional primers used in the qPCR reactions were 5'–AGAAGGATTCCTATGTGGGCG-3' and 5'–AACCTGAGGGAGCGGTACTTC-3' for ACTB, 5'–TGTCACAATGCGGTGTCCAC-3' and 5'–TTTCTCTGCGGCTACTGAG-3' for ANF, 5'–TCTAGTATGCCGACTTGC-3' and 5'–ACTGACAGCCACCTTTGC-3' for Cx43, 5'–CCGTTTCCAGCTGACCGG-3' and 5'–CGAGAAAATCCGTTGACTCC-3' for GAPDH, and 5'–AACCTGAGGGAGCGGTACTTC-3' for smMHC.

**Immunofluorescence microscopy**

Immunofluorescent labeling of cells was performed as described previously (20). Nuclei were stained with Hoechst 33342 (Molecular Probes, Carlsbad, CA, USA). HA-tagged myocardin was stained with a 1:10,000 dilution of mAb HA.11 in PBS-5% FBS. The mAb directed against sarcromeric tropomyosin (clone CH1; IgG1) and the rabbit polyclonal Ab recognizing Cx43 were obtained from Sigma-Aldrich. Goat polyclonal Ab’s specific for gap junction proteins α5 (Cx40) and α7 (Cx45), CACNA1C and the α subunit of the voltage-gated sodium channel type V (SCN5a, also known as Nav1.5) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used at a 100-fold dilution (for properties of the other cardiac and smooth muscle protein-specific Ab’s, see refs. 19, 20). Ten randomly distributed recordings were made of each channel protein staining at 200-fold magnification and signal intensities were quantified using Image-Pro Plus (Version 4.1.0.0, Media Cybernetics, Silver Spring, MD, USA). All samples were stained at the same time and processed equally. The mean intensities of samples were considered equally. The mean intensities of samples were considered significantly different when P < 0.05 using a Student's t test.

**Induction and restoration of artificial conduction block**

Experimental conduction blocks were created in monolayers of rCMCs as described before (21). In brief, 2 × 10⁶ freshly isolated rCMCs were seeded on pretreated MEA culture dishes (MultiChannel Systems, Reutlingen, Germany) with 60 titanium nitride electrodes (electrode diameter: 30 μm, interelectrode distance: 200 μm) to generate confluent monolayers of synchronously contracting cells. After 2 days of culture, a 330 μm gap spanning the entire diameter of the culture dish was created by a laser dissection microscope (P.A.L.M. Microlaser Technologies GmbH, Bernried, Germany) that was either left free of cells (n = 10) or filled with 5 × 10⁴ rCMCs (positive control; n = 9), LV.CMV.bGal.IRES.eGFP-transduced hMSFs (negative control; n = 10), or LV.CMV.myocL.IRES.eGFP-transduced hMSFs (n = 9). The infection of hMSFs with LV.CMV.bGal.IRES.eGFP or LV.CMV.myocL.IRES.eGFP took place 2 days before application to the channel. After 7 days of culture, electrogams were recorded and color-coded activation maps were generated.

**RESULTS**

Transactivation of heart muscle-specific promoters by cardiogenic transcription factors

To identify the most suitable cTF for endowing hMSFs with properties of cardiomyocytes, expression plasmids were generated encoding human GATA4, Hand1, Mef2C, the two myocardin splice variants myocL and myoC, Nkx2.5, and Tbx5, then transient transfection experiments in HeLa cells were performed to test their ability to transactivate the promoters of the human genes specifying 1) the atrial peptide hormone ANF, 2) the cardiac sarcomeric protein αMHC, and 3) the gap junction protein Cx43, which is abundantly expressed in cardiomyocytes. Although many promoter activation studies focus on proximal promoter elements, we also included distal promoter elements in our reporter gene constructs to more closely mimic endogenous gene regulation. Functional GATA4, Hand1-E12 complex, Mef2, and Tbx5 binding sites are present in all three promoters, whereas only the ANF and Cx43 promoter regions contain known Nkx2.5 recognition sequences. CARG boxes, which are required for DNA binding of the myocardin/SMRT complex, are present only in the ANF and αMHC promoters.

As shown in Fig. 1, both myocardin isoforms strongly stimulated the activity of all three heart muscle-specific

**Figure 1.** ANF, αMHC, and Cx43 promoter activation by the cTFs GATA4, Hand1, Mef2C, myoC, Nkx2.5, and Tbx5 in HeLa cells. The average induction factors and ses are shown (n = 12).

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promoters. GATA4 failed to induce ANF promoter activity but did activate the αMHC and Cx43 promoters, albeit to a lesser extent than myocardin. All other cTFs significantly activated only the Cx43 promoter, with Hand1 and Tbx5 showing the lowest transactivating activity toward this promoter. Based on these screenings, myocardin was used in the rest of the study.

Isolation and characterization of human ventricular scar fibroblasts

In vitro culture of human myocardial scar tissue resulted in the outgrowth of cells with a spindle-shaped morphology (Fig. 2). After one round of subculturing, the cells stained negative for sarcomeric α-actinin, sarcomeric MHC, and smooth muscle MHC (smMHC), indicating the cultures were free from contaminating cardiac and smooth muscle cells. The surface antigen profile of the hMSFs as determined by fluorescence-activated flow cytometry was similar to that of hDFs (Table 1). Both cell types expressed at their surface large amounts of the hyaluronate receptor (CD44), the major T cell antigen (Thy-1; CD90), and human leukocyte class I antigens (HLA-ABC), as well as low numbers of membrane cofactor protein (MCP also known as CD46), a component of the complement system. In contrast, typical MSC markers such as neural cell adhesion molecule (NCAM;CD56), β3 integrin (CD61), P-selectin (CD62P), transferrin receptor (CD71), and vascular cell adhesion molecule 1 (VCAM-1; CD106) were not present at their plasma membranes. The surface of hMSFs and hDFs also stained negative for the hematopoietic markers CD11A, CD14, CD15, CD19, CD34, and CD45 as well as for the endothelial markers platelet-endothelial cell adhesion molecule 1 (PECAM-1; CD31), vascular endothelial growth factor receptor 2 (VEGFR-2; Flk-1), and VE-cadherin. Neither cell type expressed the CXC motif chemokine receptor 4 (CXCR4; fusin), human leukocyte class II subtype DR antigens (HLA-DR), the Cox-sackie and adenovirus receptor (CAR), or the human and murine stem cell markers CD133 and Sca-1 at their surface or contained the cardiac progenitor cell marker Isl-1. Finally, in contrast to hMSFs, hDFs displayed neutral endopeptidase (NEP; MME; CD10) at their plasma membrane.

Semiquantitative RT-PCR analyses and IFM were used to investigate the expression of cardiac, smooth, and skeletal muscle genes in hMSFs. These experiments revealed that hMSFs naturally express the GATA4 and Mef2C genes at low levels (Fig. 3 and Fig. 5A, lane U). In the majority of hMSFs the GATA4 protein is localized in the cytoplasm, whereas in rCMCs this cTF is found exclusively in the nucleus (Fig. 3). In addition, hMSFs abundantly express Cx43 (Fig. 3 and Fig. 5A, lane U). In these cells, the Cx43 protein is mainly localized around the nucleus, with small amounts being present at intercellular borders; in rCMCs, Cx43 is largely confined to areas of cell-cell contacts (Fig. 3). hMSFs also contain low amounts of CACNA1C- and SCN5a-specific mRNAs (Fig. 5A, lane U).

Human ventricular scar fibroblasts can undergo adipogenesis but not osteogenesis

To investigate whether hMSFs have characteristics of hMSCs, their capacity to differentiate into osteoblasts and adipocytes was tested. Under conditions that induced osteogenesis in hMSCs, hMSFs as well as hDFs failed to differentiate into osteoblasts (Fig. 4). When exposed to adipogenic differentiation medium, hMSCs and hMSFs underwent adipogenesis, in contrast to hDFs (Fig. 4).
Forced myocardin expression results in the activation of cardiomycocyte-specific genes in human ventricular scar fibroblasts

Using a combination of RT-PCR analyses and IFM, we investigated the presence of cardiac, smooth, and skeletal muscle-specific transcripts in hMSFs infected with the SIN lentivirus vectors LV.C-PGK.nls-bGal or LV.CMV.myocL-HA. After transduction of the cells with the control vector LV.C-PGK.nls-bGal, no changes in the expression of the analyzed genes were observed as compared with uninfected hMSFs (compare columns U and L in Fig. 5A). In contrast, infection of hMSFs with the myocardin-encoding SIN lentivirus vector LV.CMV.myocL-HA led to activation of the genes encoding the cTFs Hand1 and Hand2, and caused an increase in GATA4 (Fig. 5A and Fig. 6C) and Mef2C (Fig. 5A) expression. Nkx2.5-specific transcripts were not detected in LV.CMV.myocL-HA-transduced hMSFs (Fig. 5A, lane M). Furthermore, myocardin induced the expression in hMSFs of the genes encoding the cardiac sarcomeric components, cardiac troponin T (cTnT), atrial and ventricular myosin light chain 2 (Mlc2a and Mlc2v), the α and β isoforms of cardiac myosin heavy chain (αMHC and βMHC; Fig. 5), α-actinin, and tropomyosin (Fig. 6C) as well as of the ANF gene (Fig. 5 and Fig. 6C). LV.CMV.myocL-HA infection of hMSFs did not result in an accumulation of detectable amounts of cardiac troponin I (cTnI) -specific mRNA, normally present only in mature cardiomyocytes, but did induce expression of slow-twitch skeletal troponin I (ssTnI), which encodes the major isoform of troponin I in immature heart muscle cells (27) (Fig. 5A and Fig. 6A). Forced myocardin expression also led to the expression in hMSFs of genes specifying the sarcoplasmic reticulum Ca2+-ATPase 2a (SERCA2a) and the cardiac ion channel proteins CACNA1D, HCN4, and KCNJ3 and caused a large increase in the intracellular levels of the gap junction proteins Cx40 and Cx45 (Fig. 6A, B). In addition, myocardin up-regulated the expression of CACNA1C and of the gene encoding SCN5a at both the mRNA and protein level (Fig. 5 and Fig. 6A, B). These effects were seen in essentially all LV.CMV.myocL-HA-transduced hMSFs as judged by IFM (Fig. 6). Besides activating cardiac genes, myocardin also induced the expression of the smooth muscle genes encoding aortic smooth muscle actin (ASMA), calponin h1 (CNN1), transgelin (SM22), and smMHC (Fig. 5 and Fig. 6A). However, no synthesis of the skeletal muscle-specific transcripts for fast-twitch skeletal troponin I (fTnI)
and skeletal myosin heavy chain (skMHC) was observed after infection of hMSFs with LV.CMV.myocl-HA. The presence of cardiac and smooth muscle markers in hMSFs was always associated with myocardin gene expression, as detected by immunostaining for the HA tag attached to myocardin (Fig. 6A). Double labeling for smMHC and either ANF or sarcomeric α-actinin confirmed that cardiac and smooth muscle marker genes were expressed in the same cells (Fig. 6D). Spontaneous beating of LV.CMV.myocl-HA-transduced MSFs was never observed, consistent with the absence of properly organized sarcomeres as evidenced by the lack of striation after immunostaining with Ab’s directed against sarcomeric components (Fig. 6A, C, D).

**DISCUSSION**

Key findings of this study are 1) myocl gene transfer leads to the induction of a (partial) cardiac phenotype in hMSFs from adult patients by activating genes encoding, among others, cardiomyocyte-specific TFs, sarcomeric components, and channel proteins involved in cell-to-cell communication and action potential propagation between heart muscle cells; and 2) myocl-transduced hMSFs are able to conduct an electrical impulse over considerable distances at relatively high velocities, thereby restoring an artificially created conduction block in cardiomyocyte cultures.

**Forced myocardin expression endows human ventricular scar fibroblasts with the capacity to repair an artificially created conduction block in vitro**

Using MEA culture dishes (Fig. 7A), we recorded extracellular electrogamograms throughout a spontaneously and synchronously beating monolayer of rCMCs (21). Electrical coupling between the lower and upper part of the culture dishes was lost after establishment of a 330 ± 20 μm-wide cell-free gap, resulting in two fields of asynchronously beating rCMCs (n=10; Fig. 7B, C). Filling the channel with LV.CMV.bGal.IRES.eGFP-transduced hMSFs (n=10) did not restore electrical conduction between both rCMC fields. In contrast, seeding of rCMCs in the gap (n=9) restored action potential transmission across the channel to normal levels (conduction velocity 19±0.8 cm/s), thereby resynchronizing the 2 rCMC fields (Fig. 7B, C). Application of LV.CMV.myocl.IRES.eGFP-transduced hMSFs into the gap (n=9) also resulted in restoration of conduction across the channel and consequently in resynchronization of both rCMC fields (Fig. 7B, C). The conduction velocity across the hMSFs was only 30% lower than that across the rCMCs (13±1.1 cm/s and 19±0.8 cm/s, respectively; P<0.01).
In vitro characterization of hMSFs

The in vitro characterization of hMSFs performed in this study revealed that these cells can transdifferentiate into adipocytes in vitro. This finding may have clinical significance since substitution of compact myocardial scar tissue by fat tissue is regularly observed in human patients at late stages of heart failure (28, 29). The ability of hMSFs to undergo adipogenesis in culture could be used to develop an in vitro model system to investigate the molecular mechanisms underlying the lipomatous changes occurring in diseased hearts. We also found that hMSFs are positive for GATA4, but that in most cells this cTF is found outside of the nucleus, precluding a direct role in transcription activation.

Transactivating capacity of cardiogenic transcription factors

A comparative analysis of the ability of several human cTFs to transactivate three different human heart muscle-specific promoters corroborated previous experiments demonstrating that myocardin is an exceptionally strong transcription activator acting directly or indirectly on a wide variety of different cardiac genes (16). In fact, none of the other cTFs were able to induce the human ANF, αMHC, or Cx43 promoters to a similar degree as either myocS or myocL (Fig. 1). Furthermore, in this first-time comparison of the two main cardiomyocyte-enriched isoforms of myocardin, we failed to detect significant differences in their transactivating capacity, leaving the biological role of the amino acid sequence contributed by myocardin exon 11 undefined. The activation of pANF.LUC and pMH-C.LUC by myocardin is compatible with the presence in the ANF and αMHC promoters of SRF binding sites. Although the Cx43 promoter lacks SRF binding sites, it does contain recognition sequences for activating protein-1 (AP-1) family members such as c-Fos and c-Jun. Transcription of the c-Fos and c-Jun genes is activated by SRF, thereby indirectly regulating Cx43 gene expression (31). It is likely that regulation of the Cx43 gene by SRF-myocardin complexes is indirect as well.
with these observations, Wu et al. recently identified a
common precursor for cardiac and smooth muscle cells
in the mammalian heart, which initially possesses both
cardiac and smooth muscle markers (40). However,
expression of genes encoding definitive cardiac and
smooth muscle marker genes like cTnT and smMHC
was not observed until the bipotential precursor cells
started to differentiate into heart or smooth muscle
cells.

Electrical properties of (myocardin-transduced)
human ventricular scar fibroblasts

In our in vitro electrophysiological studies, hMSFs
transduced with a control lentivirus vector were unable to
conduct an electrical impulse over a distance of 330 ±
20 μm (Fig. 7) even though they contained high levels
of Cx43 (Figs. 3, 5, 6A, B). The rapid decline of the
electrical signal (Fig. 7B) is consistent with previous in vitro
experiments showing that the maximum distance
over which cardiac fibroblasts can conduct an electrical
impulse from neighboring cardiomyocytes is ~300 μm
(41). Conduction under these circumstances is charac-
terized by its low speed (4.6±1.8 mm/s) and electro-
tonic nature. Because fibroblasts do not generate an
action potential, they function as an electrical load that
reduces the speed of action potential propagation in
bordering cardiomyocytes (42, 43).

After transduction with a lentivirus vector encoding
myocardin, hMSFs abundantly expressed the genes for
the gap junction components Cx40, Cx43, and Cx45
and the ion channel proteins CACNA1C, CACNA1D,
HCN4, KCNJ3, and SCN5a (Fig. 5, Fig. 6A, B). These
data suggest that myocardin-transduced hMSFs obtained
components essential for impulse propagation (12).
Although the functionality of the aforementioned
channel proteins has not been tested, myocardin-trans-
duced hMSFs were able to resynchronize in vitro
two fields of rCMGs > 300 μm apart (Fig. 7). The conduc-
tion velocity across the cell-filled gap (13±1.1 cm/s)
approached that of rCMCs (19±0.8 cm/s), which
makes purely electrotonic conduction unlikely. How-
ever, it cannot be ruled out completely that increased
Cx40 and Cx45 levels are important contributors to the
improved conduction velocity of these cells.

Study limitations

Neonatal rat cardiomyocytes were used in our in vitro
electrophysiological studies. Although the use of hu-
man heart muscle cells is preferable, the limited avail-
ability of this cell type and their tendency to dediffer-
entiate during culture prevented the use of human
cardiomyocytes. Furthermore, although forced myocardin
expression endows hMSFs with 1) the three major
connexins characteristic for rCMCs (Fig. 6A), 2) cardiac
ion channel proteins necessary for the propagation of
Na+, K+, and Ca2+ currents (Fig. 5 and Fig. 6A), and 3)
the ability to conduct electricity at high velocities and to
repair an artificially created conduction block in vitro

Similarities between myocardin-transduced human
ventricular scar fibroblasts and embryonic
cardiomyocytes

Despite the induction of numerous cardiac genes on
transduction with the myocardin-encoding lentivirus
vector, hMSFs did not acquire the phenotype of mature
cardiomyocytes, as evidenced by the absence of Nkx2.5
and cTnI, coexpression of smooth muscle marker
genes, and the absence of properly structured sarco-
meres. However, the observed phenotype does resemble
that of early cardiomyocytes. Like neonatal cardiomyo-
cytes, myocardin-transduced hMSFs display sTnI (Fig.
5A and Fig. 6A) instead of cTnI expression (Fig. 5A)
(32, 33). Furthermore, the expression in reprogrammed
hMSFs of HCN4 and CACNA1D (Fig. 5A),
both required for cardiac pacemaker activity, is consist-
tent with the phenotype of immature cardiomyocytes
(34, 35), which are spontaneously active. It should be
noted, however, that automaticity in myocardin-trans-
duced hMSFs was never observed. Other features shared by
myocardin-transduced hMSFs and cardiomyocyte precursor
cells are the presence of heart muscle-specific sarco-
meric components, which are not organized into sar-
comeres (36), and the coexpression of cardiac and
smooth muscle genes (37–39) (Fig. 6D). In accordance

Figure 7. Electrical conduction across monolayers of rCMCs
after establishment of a cell-free gap and its filling with
different cell populations. A) Arrangement of the MEA in cell
culture dishes. The electrodes in rows 2 and 3 cover the area
in which a cell-free gap is created; those in rows 1 and 4 are
positioned underneath bordering cardiomyocytes. B) Typical
electrograms from electrodes bordering and spanning the
gap region in rCMCs cultures in which the channel was left
open or filled with either LV.CMV.bGal.IRES.eGFP-trans-
duced hMSFs (hMSFs + control), LV.CMV.myocL.IRES.eGFP-
transduced hMSFs (hMSFs + myocardin), or rCMCs. Electro-
grams were recorded 7 days after the cell-free gap was made
and filled with cells. C) Isochronous color-coded activation
maps showing two electrically separated rCMC fields after
generation of the cell-free gap or after the establishment of a
cellular conduction block using LV.CMV.bGal.IRES.eGFP-
transduced hMSFs. Restoration of electrical conduction
across the gap and consequently electrical synchronization of
the culture occurred after filling the channel with LV.CMV.
myocL.IRES.eGFP-transduced hMSFs or control rCMCs.

Components essential for impulse propagation (12).
Although the functionality of the aforementioned
channel proteins has not been tested, myocardin-trans-
duced hMSFs were able to resynchronize in vitro
two fields of rCMGs > 300 μm apart (Fig. 7). The conduc-
tion velocity across the cell-filled gap (13±1.1 cm/s)
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makes purely electrotonic conduction unlikely. How-
ever, it cannot be ruled out completely that increased
Cx40 and Cx45 levels are important contributors to the
improved conduction velocity of these cells.
(Fig. 7), an in-depth electrophysiological characterization of these cells is required to prove that they truly become excitable.

**Perspectives**

Genetic modification of MSFs may provide a means of repairing conduction defects in damaged hearts. However, the transduction of MSFs with proper doses of all genes needed to produce a cardiac action potential represents a formidable technical challenge. Overexpression of myocardin activates an extensive cardiac gene expression program in hMSFs, leading to the production of key sarcomeric and electrical components of cardiomyocytes. This coincides with the appearance of electrical conduction in these cells and the restoration of an artificially created cellular conduction block in vitro. With further refinements, this technology holds the promise to transdifferentiate MSFs into cardiomyocyte-like cells in situ, thereby potentially improving myocardial mechanical performance and repairing electrical conduction defects.

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