Regenerative medicine in cardiovascular disease: from tissue engineering to tissue regeneration
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FORCED MYOCARDIN EXPRESSION ENHANCES THE THERAPEUTIC EFFECT OF HUMAN MESENCHYMAL STEM CELLS AFTER TRANSPLANTATION IN ISCHEMIC MOUSE HEARTS

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ABSTRACT

Background  Human mesenchymal stem cells (hMSCs) only have a limited differentiation potential towards cardiomyocytes. Forced expression of the cardiomyogenic transcription factor Myocardin may stimulate hMSCs to acquire a cardiomyogenic phenotype thereby improving their possible therapeutic potential.

Methods  hMSCs were transfected with green fluorescent protein (GFP) and Myocardin (hMSCmyoc) or GFP and empty vector (hMSC). After coronary ligation in immune-compromised NOD/scid mice, hMSCmyoc (n = 10), hMSC (n = 10) or Medium only (n = 12) were injected into the infarct area. Sham operated mice (n = 12) were used to determine baseline characteristics. LV volumes and ejection fraction (EF) were serially (days 2 and 14) assessed using 9.4T MR imaging. LV pressure-volume measurements were performed at day 15 followed by histological evaluation.

Results  At day 2, no differences in infarct size LV volumes and EF were observed between the MI groups. At day 14, LVEF in both cell-treated groups was preserved compared to the non-treated group; in addition hMSCmyoc injection also reduced LV volumes as compared to Medium injection (P < 0.05). Furthermore, pressure-volume measurements revealed a significantly better LV function after hMSCmyoc injection as compared to hMSC treatment. Immunohistochemistry at day 15 demonstrated that the engraftment rate was higher in the hMSCmyoc group compared to the hMSC group (p < 0.05). Furthermore, these cells expressed a number of cardiomyocyte specific markers not observed in the hMSC group.

Conclusions  After myocardial infarction, injection of hMSCmyoc improved LV function and limited LV remodeling, effects not observed after injection of hMSC. Furthermore, forced Myocardin expression improved engraftment, and induced a cardiomyocyte-like phenotype hMSC differentiation.
INTRODUCTION

Intramyocardial mononuclear bone marrow cell (BMC) transplantation is a promising new treatment modality in patients after myocardial infarction (MI) [1] or with therapy-refractory myocardial ischemia [2] as BMC injections in ischemic myocardium improved myocardial perfusion as well as left ventricular ejection fraction, possibly mediated by neoangiogenesis [2,3]. The mononuclear BMC fraction contains mesenchymal stem cells (MSCs), which are easily expandable and are able to differentiate into multiple cell lineages [4]. As shown previously, intramyocardial MSC transplantation improves angiogenesis [5] and left ventricular (LV) function in different animal models [6,7,8]. Furthermore, MSCs can differentiate into cardiomyocytes in vitro under appropriate conditions [9]. However, it is unclear whether in vivo myogenic differentiation of bone marrow (BM) stem cells, including MSCs [10], actually occurs; at best the differentiation rate seems to be rather low [11]. Theoretically it is preferable that cell therapy results in the generation of new cardiomyocytes to replace cells lost after an acute ischemic event. Novel approaches to identify signals guiding myogenic differentiation into the cardiac lineage have therefore been proposed [12]. Ex-vivo pretreatment of MSCs with the DNA demethylating agent 5′-azacytidine [3,9] or growth factors including bFGF, IGF-1 and BMP-2 [13] enhanced their in vivo differentiation into cardiomyocytes in animal studies. Furthermore, Sheng et al. demonstrated that ex-vivo pretreatment of CD117 (c-kit)-positive mononuclear bone marrow cells with Transforming Growth Factor-ß1 (TGF-ß1) induced the expression of several cardiomyocyte-specific proteins resulting in functional cardiac regeneration in a mouse model of acute MI (AMI) [14]. So far, no efforts have been made to enhance differentiation of MSCs into the cardiomyogenic cell lineage through genetic modification.

Myocardin is a pivotal cardiomyogenic transcription factor, that transactivates the ubiquitous transcription factor serum response factor (SRF) [15]. Myocardin regulates the expression of many growth-related and muscle-restricted genes [16]. Overexpression of the longest splice variant of the human myocardin gene in human MSCs (hMSCs) [17] and human myocardial scar fibroblasts [18] induced the expression of cardiac and smooth muscle cell (SMC) genes in vitro, without expression of skeletal muscle genes [17]. Therefore it was hypothesized that forced myocardin expression in hMSCs may enhance their propensity to differentiate into cardiomyocytes in vivo, thereby increasing the potential therapeutic effect of mesenchymal stem cell therapy in patients with ischemic heart disease (IHD). In this study the effects of forced myocardin expression in hMSC from IHD patients on the in vivo engraftment rate, differentiation and preservation of LV function were evaluated in an immune-compromised mouse model of AMI.
METHODS

Animals

All experiments were approved by the institutional committee on animal welfare. To avoid rejection of injected human cells, experiments were performed in 8-10 weeks old male NOD/Scid mice (Charles River Laboratories, Maastricht, the Netherlands). The experiments conformed to the principles of Laboratory Animal care formulated by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Purification and Expansion of hMSC

Human cellular material was obtained after written informed consent and experiments were approved by the institutional medical ethics committee. BM samples were obtained from 4 adult IHD patients who were enrolled in ongoing clinical stem cell trials [2]. hMSC were purified as described previously [17], expanded by serial passage, and used from passages 3 to 8 in culture conditions. The hMSC surface antigen profile was characterized as previously described [17]. hMSCs abundantly expressed hyaluronate receptor (CD44), major T-cell antigen (Thy-1; CD90), endoglin (CD105), vascular cell adhesion molecule-1 (CD106), and human leukocyte class I (HLA-ABC) antigens. These cells also expressed low levels of transferrin receptor (CD71), P-selectin (CD62P), ß3 integrin (CD61), neural cell adhesion molecule (CD56), and membrane cofactor protein of the complement system (CD46) at their surface. hMSCs differentiated into adipocytes and osteoblasts after proper stimulation confirming their multipotent nature (data not shown).

Adenoviruses and Gene Transfer

The generation of the fiber-modified human adenovirus serotype 5 vectors encoding human myocardin (hAd5/F50.CMV.myocL), the enhanced green fluorescent protein (eGFP) gene (hAd5/F50.CMV.eGFP) and the control vector (hAd5/F50.empty) were described elsewhere [17]. Before transduction, hMSC were seeded at a density of 2E4 hMSCs per cm² in 10 cm² dishes, and cultured overnight. Next, the culture medium (DMEM+10% FBS) was replaced by 1 ml fresh culture medium per dish, supplemented with 5 mM Sodium Butyrate, and 100 infectious units (IU) per cell of hAd5/F50.CMV.eGFP and hAd5/F50.CMV.myocL or hAd5/F50.CMV.eGFP and hAd5/F50.empty. After overnight incubation, the cells were washed with PBS and cultured for 24h in normal culture medium.
Myocardial Infarction and Cell Implantation

Animals were preanesthetized with 5% isoflurane in a mixture of oxygen and nitrogen. After endotracheal intubation and ventilation using a Harvard Rodent Ventilator (Model 845) (200 breaths/minute with a stroke volume of 200 ml), animals were kept anesthetized with 0.5-1.5% isoflurane for the remainder of the surgical procedure. After a left thoracotomy, the left anterior descending (LAD) coronary artery was ligated using a 7-0-prolene suture (Johnson and Johnson, New Brunswick, NJ, USA).

After 10-20 minutes after AMI, animals were grouped to receive injections of 2\times10^5 hMSCs transduced with eGFP and myocardin in 20 μl culture medium (MI+hMSC_myoc group, n = 10), hMSCs transfected with eGFP and empty vector (MI+hMSC group, n = 10) or medium only (MI+Medium group, n = 12). Sham operated animals were used to determine baseline characteristics (Sham group, n = 12). The intramyocardial injections were performed at 5 sites within infarcted area and the borderzone using a 20 μl Hamilton syringe with a 33G needle. The chest was closed in layers and animals were allowed to recover.

Cardiac Magnetic Resonance Imaging

Left ventricular anatomy and function were serially assessed 2 and 14 days after surgery by magnetic resonance imaging (MRI). The MRI setup consisted of a vertical 9.4T (400 MHz), 89 mm bore NMR spectrometer equipped with a shielded gradient set (1T/m) and a 30 mm birdcage resonator (Bruker Biospin, Rheinstetten, Germany). Mice were anaesthetised as described above and maintained at 1-2% isoflurane. Biotrig software (Bruker, Rheinstatten, Germany) was used to monitor ECG and respiratory rate. Image reconstruction was performed using Bruker ParaVision 3.02 software.

Infarct Size

To determine the extent of the infarcted area at day 2, contrast enhanced MRI imaging was employed after injection of a 150 μL (0.05 mmol/ml) bolus of gadolinium-DPTA (Gd-DPTA, Dotarem, Guerbet) via the tail vein. A high-resolution ECG- and respiratory-triggered 2D fast gradient echo (FLASH) sequence was used to acquire a set of 18 contiguous 0.5 mm slices in short-axis orientation covering the entire heart. Imaging parameters were: echo time (TE) of 1.9 ms, repetition time (TR) of 90.5 ms, field of view (25.60 mm)^2, matrix size 256x256 (resulting in a resolution of 100 μm) and a flip angle of 60°.
LEFT VENTRICULAR ANATOMY AND DIMENSIONS

At day 2 and day 14, LV dimensions were assessed. A high-resolution 2D FLASH cine sequence was used to acquire a set of contiguous 1 mm slices in short-axis orientation covering the entire long-axis of the heart. Imaging parameters were: echo time (TE) of 1.9 ms, repetition time (TR) of 7 ms, field of view (25.6 mm)², matrix size 256x256 and a flip angle of 15°.

IMAGE ANALYSIS

All MR image data were analysed with the MR Analytical Software System (MASS) for Mice (MEDIS, Leiden, The Netherlands). Endocardial and epicardial borders were traced manually by two independent investigators who were blinded to the treatment group. End-diastolic, end-systolic phases and contrast enhanced area were identified automatically, after which the percentage of infarcted myocardial volume, left ventricular end-diastolic volume (EDV), left ventricular end-systolic volume ( ESV) and ejection fraction (EF) were computed.

Left Ventricular Function by Pressure-Volume Loops

Instrumentation  A 1.4 F pressure-conductance catheter (SPR-719, Millar Instruments, Houston, TX) was introduced via the right carotid artery, positioned into the LV, and connected to a Sigma-SA signal processor (CD Leycom, Zoetermeer, The Netherlands). Calibration was performed as previously described [19]. The abdomen was opened to enable temporary preload reductions by directly compressing the inferior vena cava. All data were acquired using Conduct-NT software (CD Leycom) at a sample rate of 2000 Hz and analyzed off-line with custom-made software.

Pressure-volume relations  LV pressure-volume signals were acquired in steady state to quantify general hemodynamic conditions: Heart rate (HR), stroke volume (SV), cardiac output (CO), EDV, ESV, EF, end-diastolic pressure (EDP), and end-systolic pressure (ESP) were determined. Stroke work (SW) was determined as the area of the pressure-volume loop, and the maximal and minimal rate of LV pressure change, dp/dtm and dp/dtmin, and the isovolumic relaxation time constant Tau were calculated. Load-independent indices of systolic and diastolic LV function were determined from pressure-volume relations obtained during preload reductions. To quantify systolic function, we used the end-systolic pressure-volume relation (ESPVR), the relation between dp/dtMAX and EDV, and the preload recruitable stroke work relation (PRSWR: SW versus EDV). The slopes of these relations, EES (end-systolic elastance), S-dP, and S-PR, respectively, are sensitive measures of intrinsic systolic LV function. In addition, the positions of the pressure-volume relations were quantified by their intercepts at, respectively ESP = 82 mmHg, μL and dp/dtMAX = 6090 (These levels were selected retrospectively as the overall mean
values of ESP, SW and dP/dtMAX for all groups). For diastolic function, the chamber stiffness $E_{ED}$ was determined from a linear fit to the end-diastolic pressure-volume points.

**Histological Examination**

At day 15 mice were sacrificed and hearts and lungs removed. The body weight and wet lung weight were measured from all animals and lungs were then freeze-dried. The difference between wet and dry lung-weight was used as a measure of pulmonary congestion.

From each group, four hearts were immersion-fixed in 4% paraformaldehyde, dehydrated in graded ethanol and xylene and subsequently embedded in paraffin. Serial sections of 5 μm were cut along the entire long-axis of the LV for immunohistochemical analysis. Sections were deparaffinated and dehydrated in xylene and graded alcohol. Antigen retrieval was performed by heating in a microwave oven (97 °C) in 0.01 M citric buffer of pH 6.0 for 10 minutes. Sections were incubated overnight at room temperature with the primary antibodies diluted in PBS with 1% BSA and 0.05% Tween.

**Engraftment Rate of Transplanted hMSCs**

hMSC engraftment was detected by immunostaining with a rabbit anti-GFP antibody (A11122, Molecular Probes Inc.) followed by a biotinylated goat anti-rabbit IgG (BA-1000, Vector Labs, Burlingame, CA, USA). For visualisation of the eGFP specific anti-body, the signal was amplified with the ABC staining kit (PK-6100, Vector Labs). 3,3’-diamino-benzidine tetrahydrochloride (D5637, Sigma-Aldrich) was used as substrate for horseradish peroxidise. Sections were counterstained with Mayer’s hematoxillin.

The number of engrafted hMSCs was determined by counting the GFP positive cells with a 20x magnification in every 10th serial section of the entire long axis of the heart. Only elongated cells with a length >20 μm were counted. The number of GFP-positive cells was multiplied by 10 to estimate the total number of engrafted cells in the heart. The hMSC engraftment rate was calculated by dividing this number by the number of injected hMSCs ($2 \times 10^5$ cells).

**Assessment of Cell Differentiation**

Double immunostainings were performed with mouse monoclonal antibodies against α-sarcomeric actin (αSA, clone 5C5, A2172, Sigma-Aldrich), Atrial Natriuretic Factor (ANF, CBL66, Chemicon), anti-cardiac troponin T (cTnT, AB33589, Abcam), troponin I (cTnI; clone 19C7, HyTest) and a polyclonal rabbit anti-myosin light chain 2a (MLC2a, gift from S.W. Kubalek).

Primary Abs were visualized with appropriate secondary biotinylated Abs followed by Qdot 655 streptavidin-conjugated (Q10121MP, Invitrogen) Abs. The eGFP labelled cells were detected by immunostaining with monoclonal rabbit anti-GFP or polyclonal goat anti-GFP (AB6673, Abcam).
antibodies followed by an appropriate donkey Alexa Fluor 488 antiserum (A21206 and A11055). Colocalization of eGFP and differentiation markers was examined using dedicated Q-dot compatible filter sets.

**DIFFERENTIATION IN VITRO**

To compare the protein expression of *in vitro* cultured and *in vivo* injected hMSCs, a small fraction of hMSCs and hMSCs<sub>myoc</sub> from the same cell batch that was injected in the mice was propagated *ex vivo*. The cells were maintained *in vitro* for 15 days and evaluated for cardiomyocyte-specific protein expression by immunofluorescence staining using the same aforementioned antibodies.

**ANALYSIS OF VASCULAR DENSITY**

To determine the effect of hMSC transplantation on vascular density, vascular endothelial cells were stained with a PECAM-1 antibody (CD31, clone MEC13.3, Pharmingen, San Diego, CA, USA) followed by a biotinylated goat anti-rat IgG (Pharmingen). Visualisation was enhanced by the CSA system (K1500, DAKO). 3,3'-diamino-benzidine tetrahydrochloride was used as substrate for horseradish peroxidise. Sections were counterstained with Mayer’s hematoxillin. Morphometric measurements were performed on three equidistant slices (at the midpoint between LAD ligation and the apex, between the midpoint and the LAD ligation and between the midpoint and the apex) from 4 animals per group. Per slice, 4 areas of interest equally distributed in the infarcted antero-lateral wall of the LV were photographed at a 20x magnification. Vascular density was then determined by the cumulative area of PECAM-1 stained vessel lining per total left ventricular area. Endocardial endothelium staining was excluded from the quantitative analysis. The percentage of PECAM-1 staining was measured using the Image-Pro Plus software package (Media Cybernetics Inc., Carlsbad, CA, USA). The value was expressed as the ratio of the sum of all areas containing PECAM divided by the total area of the image. The measurements were performed by two independent examiners who were blinded to the treatment assignment.

**Statistical Analysis**

Numerical values were expressed as means ± SEM. Comparisons of parameters between the Sham, Medium, hMSC and hMSC<sub>myoc</sub> groups were made using one-way analysis of variance (ANOVA). If the omnibus tests among groups were significantly different, post-hoc tests between groups using unpaired t-tests were used. A p-value < 0.05 was considered significant.
RESULTS

LV Infarct Size, Volumes and Function

Two days after coronary artery ligation, infarct size was determined in all mice in the Medium (n = 12), hMSC (n = 10) and hMSC_{myoc} (n = 10) groups by contrast–enhanced MRI with Gd-DPTA (Fig. 1A and B). Infarct size did not differ between the three groups (36 ± 2 % vs. 32 ± 2 vs. 31 %, resp.; Fig. 1C) indicating that cell injection had no early positive effect on the infarct size.

Cardiac volumes and ejection fraction were evaluated 2 and 14 days after myocardial infarction by MRI. Representative examples of systolic and diastolic 3D reconstructions of MRI short axis views for all four experimental groups at day 14 are shown in Figure 2A.

Compared to Sham animals (EDV 48 ± 2 μl; ESV 24 ± 1 μl; LVEF 51 ± 1 %, P < 0.05), 2 days after AMI, all MI groups developed typical changes indicative of LV remodeling as reflected by an increase in EDV (Medium: 61 ± 2 μl, hMSC: 59 ± 3 μl, hMSC_{myoc}: 57 ± 3 μl), and ESV (Medium, 42 ± 2 μl; hMSC, 40 ± 3 μl; hMSC_{myoc} 38 ± 3 μl), and a decrease in LVEF (Medium 30 ± 2 %; hMSC 33 ± 3 %; hMSC_{myoc} 34 ± 2 %) (Fig. 2B-D). There were no significant differences in LV parameters between the 3 MI groups at day 2 post MI.

At day 14 post MI, there was a further decrease of LVEF and a progression of LV remodeling in all MI groups (Fig. 2B-D). However, LVEF deterioration in the hMSC and the hMSC_{myoc} group (25 ± 3 % vs. 27 ± 3 %) was significantly attenuated as compared to the Medium group (15 ± 1 %, P < 0.05). Interestingly, LV remodeling in the hMSC_{myoc} group was significantly reduced (EDV: 104 ± 7 μl and ESV: 78 ± 8 μl) as compared to the Medium group (133 ± 8 μl and 114 ± 9 μl, resp., both P < 0.05), whereas for hMSC group no difference was observed compared to the medium group (115 ± 13 μl and 90 ± 14 μl, resp.; both p = ns).

Figure 1. Infarct size as assessed by delayed enhancement MRI. (A) representative Gd-DPTA-enhanced MR image 2 days after MI and (B) after tracing endo- and epicardial borders and automatic quantification of infarct size. (C) No difference in infarct size was found 2 days after MI between the MI+Medium, MI+hMSC and MI+hMSC_{myoc} groups (P = ns).
Figure 2. Assessment of left ventricular function and volumes with the 9.4T small animal MRI. Representative 3D reconstructions at day 14 of EDV and ESV for all four groups (A). Quantification of EDV (B), ESV (C) and EF (D) two and fourteen days after acute myocardial infarction for all groups. Data are expressed as mean ± SEM. * = p < 0.05 versus time-matched sham group, # = p < 0.05 versus time-matched MI+Medium group.

Hemodynamic Measurements

The functional pressure-volume-derived data for all 4 groups are presented in table 1. Summarized schematic pressure-volume loops (based on mean end-systolic and end-diastolic pressures and volumes) and mean end-systolic pressure-volume relations for all four groups are shown in Figure 3.

Figure 3. Typical examples of pressure volume relations in the sham, MI+Medium, MI+hMSC and MI+hMSCmyoc groups (based on mean ESV, EDV, ESP, EDP). The oblique lines represent the end-systolic pressure-volume relations.
CHAPTER 6: FORCED MYOCARDIN EXPRESSION ENHANCES THE THERAPEUTIC EFFECT OF HUMAN MESENCHYMAL STEM CELLS AFTER TRANSPLANTATION IN ISCHEMIC MOUSE HEARTS

Consistent with the MRI data, the PV loops revealed substantial cardiac dilatation and a decreased stroke volume post MI, of interest these effects are less pronounced after cell-treatment, particularly in the hMSC\textsubscript{myoc} group. As expected, MI caused a marked deterioration in LV function demonstrated by significant differences for almost all conductance catheter-derived indices between the Sham group and the MI+Medium group (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>MI +</th>
<th>P-value: hMSC vs hMSC\textsubscript{myoc}</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Medium</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>429 ± 50</td>
<td>465 ± 52</td>
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<tr>
<td>CO, mL/min</td>
<td>8.6 ± 4.1 **</td>
<td>4.5 ± 1.7</td>
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<tr>
<td>ESP, mmHg</td>
<td>89 ± 19 **</td>
<td>72 ± 13</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>6.9 ± 3.1 **</td>
<td>16.7 ± 6.7</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>14.5 ± 3.9 *</td>
<td>19.9 ± 5.5</td>
</tr>
<tr>
<td>dP/dt\textsubscript{MAX}, mmHg/s</td>
<td>8202 ± 1680 **</td>
<td>3946 ± 1432</td>
</tr>
<tr>
<td>-dP/dt\textsubscript{MIN}, mmHg/s</td>
<td>5865 ± 1199 **</td>
<td>3085 ± 949</td>
</tr>
<tr>
<td>SW, mmHg.μL</td>
<td>1880 ± 766 **</td>
<td>481 ± 297</td>
</tr>
<tr>
<td>ESPVR</td>
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<tr>
<td>slope: E\textsubscript{ES}, mmHg/μL</td>
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<td>1.86 ± 0.39</td>
</tr>
<tr>
<td>intercept: ESV\textsubscript{INT}, μL</td>
<td>31 ± 11 **</td>
<td>84 ± 22</td>
</tr>
<tr>
<td>PRSW</td>
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<tr>
<td>slope: M\textsubscript{W}, mmHg</td>
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<td>31 ± 16</td>
</tr>
<tr>
<td>intercept: EDV\textsubscript{INT}, μL</td>
<td>38 ± 8 **</td>
<td>107 ± 24</td>
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<tr>
<td>dP/dt\textsubscript{MAX}-EDV</td>
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<tr>
<td>slope: S\textsubscript{dpH}, mmHg/s/μL</td>
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<td>119 ± 48</td>
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<tr>
<td>intercept: EDV\textsubscript{INT}, μL</td>
<td>39 ± 14 **</td>
<td>108 ± 35</td>
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<td>EDPVR</td>
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<tr>
<td>slope: E\textsubscript{ED}, mmHg/μL</td>
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<tr>
<td>intercept: EDV\textsubscript{INT}, μL</td>
<td>69 ± 22 *</td>
<td>91 ± 22</td>
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Table 1: Pressure-volume loops-derived LV function indices. R indicates heart rate; CO, cardiac output; ESP, end-systolic pressure; EDP, end-diastolic pressure; Tau, relaxation time constant; dP/dt\textsubscript{MAX}, maximum rate of pressure increase; dP/dt\textsubscript{MIN}, maximum rate of pressure decrease; SW, stroke work; ESPVR, end-systolic pressure-volume relationship; E\textsubscript{ES}, end-systolic elastance; ESV\textsubscript{INT}, end-systolic volume intercept; PRSW, preload recruitable stroke work relation (SW vs. EDV); EDV\textsubscript{INT}, end-diastolic volume intercept; EDPVR, end-diastolic pressure-volume relationship; E\textsubscript{ED}, end-diastolic stiffness. * p < 0.05 vs Medium; ** p < 0.01 vs Medium.
In both hMSC and hMSC_myoc groups an improvement in dP/dt\textsubscript{MAX}, SW, ESV\textsubscript{INT} and E\textsubscript{ED} was observed as compared to the MI+Medium group. In addition, in the hMSC\textsubscript{myoc} group but not in the hMSC group ESP, Tau, -dP/dt\textsubscript{MIN}, EES, EDV\textsubscript{INT} and the slope and intercept of the dP/dt\textsubscript{MAX}-EDV relation were also improved. In comparison to the hMSC vs. the hMSC\textsubscript{myoc} group, a significant difference was observed for Tau and ESV\textsubscript{INT}, whereas the intercepts of PRSW and the dP/dt\textsubscript{MAX}-EDV relation displayed a non-significant trend.

**BODY AND LUNG WEIGHT**

Body weight prior to the operation was similar between the 4 groups (sham group: 26.5 ± 0.6g, MI+Medium group: 27.3 ± 0.6g, MI+hMSC group: 25.9 ± 0.7g and MI+hMSC\textsubscript{myoc} group: 27.0 ± 0.6g; p = ns). After 15 days, the body weight decrease in the Medium group (-3.8 ± 1.1g, -14 ± 4 %) was attenuated in the hMSC\textsubscript{myoc} group (-0.8 ± 0.7g, -3 ± 3 %; p < 0.05), but not in the hMSC group (-1.2 ± 1.2g, -4 ± 5 %; p = ns). Body weight in the Sham group did not change over time. Furthermore, compared to the sham group there was a significant increase in lung fluid in the Medium group (0.15 ± 0.01g vs. 0.19 ± 0.02g; p < 0.05), this difference was not observed in the hMSC and hMSC\textsubscript{myoc} groups (0.16 ± 0.03g and 0.16 ± 0.01 respectively; p = ns).

![Engraftment of hMSCs and hMSC\textsubscript{myoc}](image)

Figure 4. Engraftment of hMSCs and hMSC\textsubscript{myoc} (relative to the total number of injected cells) 15 days after transplantation in the acutely infarcted NOD/scid mouse heart. Upper panel shows representative examples of DAB stained eGFP labeled hMSCs (A) and hMSC\textsubscript{myoc} (B) in the infarcted area. After quantification, a significantly higher engraftment rate was observed after hMSC\textsubscript{myoc} transplantation (C). * = p < 0.05 vs MI+hMSC.
**hMSC Engraftment and Differentiation**

Fifteen days after cell transplantation, substantial engraftment was observed in both cell transplant groups (Figure 4A and B). However, quantitative assessment revealed a significant higher engraftment rate in the hMSCs\textsubscript{myoc} group (5.8 ± 0.5 \%) as compared to the hMSC group (4.2 ± 0.3 \%, \( p < 0.05 \)) (Fig. 4C). Sections were examined to assess differentiation of hMSC and hMSC\textsubscript{myoc} into a cardiomyocyte-like phenotype. None of the injected hMSCs expressed αSA, MLC2a, ANF, cTnT and cTnI (Fig. 5, left panel). In contrast, over expression of myocardin in hMSCs resulted in expression of sarcomeric α-actin, MLC2a, ANF, and cTnT (Fig. 5, right panel). The differentiation rate of the engrafted cells was approximately 90-100 \% for the various differentiation markers. The staining pattern for these proteins was however diffuse, and we did not observe cross striations in the hMSC\textsubscript{myoc}. Furthermore none of the hMSC\textsubscript{myoc} expressed cardiac Troponin I (Fig. 5, right panel).

![Figure 5](image.png)

*Figure 5. Immunofluorescent double staining of hMSCs transduced with or without myocardin. Left panel, hMSCs, and right panel myocardin transduced hMSC\textsubscript{myoc}. Blue, nuclear staining with Hoechst 33342; green, eGFP labeled hMSCs; red, cardiac markers as depicted in the figure. Abbreviations: αsa, α-sarcomeric actin; MLC2a, atrial form of myosin light chain; ANF, atrial natriuretic factor; cTnT, cardiac troponin T; cTnI; cardiac troponin.*
Figure 6. Cardiac markers 15 days after in vitro culture in hMSCs and hMSCs_{myoc} from the same batches that were used for the in vivo experiments. Nuclei are stained blue; cardiac markers as depicted in the figure are stained red. Abbreviations: αSA, α-sarcomeric actin; MLC2a, atrial form of myosin light chain; ANF, atrial natriuretic factor; cTnT, cardiac troponin T; cTnI; cardiac troponin I.
After 15 days, of *in vitro* culture of cells from the same cell batches that were injected in the mice, immunohistochemistry revealed that hMSC transduced with hAd5/F50.CMV.eGFP and hAd5/F50.empty did not express αSA, Mlc2a, ANF, cTnT and cTnI (Fig. 6A-E). Transduction of hMSCs with both hAd5/F50.CMV.myoLC and hAd5/F50.CMV.eGFP, however, resulted in the expression of all these proteins (Fig. 6F-I) with the exception of cTnI (Fig. 6J). However, the hMSCs did not exhibit sarcomeric organization or spontaneous beating within the 15 day period of observation.

**Vascular Density**

Vascular density in the infarcted scar area was compared between the 3 MI groups 15 days after cell administration (Fig. 7A). Total blood vessel density (as determined by PECAM-1 staining) in the scar area was significantly higher in the hMSC and hMSCmyoc groups (11.8 ± 2.1 %, and 11.3 ± 1.3 %; p = ns) as compared to the Medium group (5.9 ± 0.9 %; p < 0.05; Fig. 7B).

![Figure 7. A: Representative photographs of PECAM-1 staining of the infarct scar 15 days after acute myocardial infarction followed by Medium injection (left panel), hMSC injection (middle panel) and hMSCmyoc injection (right panel). B: Infarct scar vascularity was significantly increased in the hMSC and hMSCmyoc groups as compared to the medium group. * = p < 0.05 versus medium-treated mice.](image-url)
DISCUSSION

Key findings of this study are: (1) Injection of hMSC and hMSC\textsubscript{myoc} into infarcted myocardium results in a relatively preserved LV function compared to injection of medium alone; (2) Forced myocardin expression in hMSCs of IHD patients increases engraftment 15 days post AMI, attenuates ventricular remodeling and improves systolic and diastolic function as compared to untreated hMSCs; and (3) Forced myocardin expression in hMSCs results in expression of several cardiomyogenic markers, but not in full cardiomyocyte differentiation.

MSCs are able to differentiate into a number of organ specific cell types [4]. Therefore, the MSC population is potentially an attractive therapeutic cell type for patients with IHD with reported beneficial effects on LV function in animal studies [7,20,6] and a initial clinical trial [21]. However, the underlying mechanism is still not fully understood as the ability of BM derived stem cells, including MSCs, to differentiate into cardiomyocytes has been questioned [10,22]. This lack of cardiomyogenic differentiation potential prompted the development of techniques to stimulate cardiomyogenic differentiation [12] after \textit{in vivo} transplantation. Stimulation of MSCs with 5'azacytidine [9,3], or a combination of several growth factors including fibroblast growth factor (FGF), insulin growth factor 1 (IGF-1) and bone morphogenetic protein-2 (BMP-2) [13], or TGF-\beta1 [14] resulted in increased cardiomyocyte differentiation and improved functional recovery after myocardial infarction in animal models [3,14,13]. However, pharmacological stimulation did either not result in cardiomyogenic differentiation of all treated cells [13,14,3], or resulted in incomplete differentiation [14,3]. To achieve effective cardiomyogenic differentiation, it is therefore mandatory to find a more physiological way of improving the \textit{in vivo} differentiation of transplanted MSCs.

Genetic modification of stem cells can be another approach to improve the cardiomyogenic differentiation potential of these cells. Transduction of mouse MSCs with a hypoxia-regulated heme oxygenase-1 vector improved cell survival in the ischemic mouse myocardium [23]. Also, transplantation of rat MSCs overexpressing the prosurvival gene Akt-1 in a rat MI model resulted in the prevention of remodeling and improvement of cardiac function [24]. In addition, rat MSC co-overexpressing angiopoietin, a mediator of angiogenesis, and Akt-1 restored cardiac function after permanent coronary artery occlusion [25]. However, to our knowledge, genetic modification to stimulate cardiomyogenic differentiation of MSCs has not been employed.

We recently demonstrated that transduction of hMSCs and human myocardial scar fibroblasts with human adenovirus vectors expressing the longest splice variant of the human myocardin gene \textit{in vitro}, induced the expression of a panel of genes involved in the development of cardiomyocytes as well as smooth muscle cells, but not skeletal muscle cells [18,17]. Forced myocardin expression in hMSCs before transplantation in a mouse model of AMI may therefore enhance their propensity to differentiate into cardiomyocyte-like cells \textit{in vivo}. 
EFFECTS OF hMSC AND hMSCmyoc TRANSPLANTATION ON INFARCT SIZE AND LEFT VENTRICULAR FUNCTION

Delayed enhancement 9.4T MR imaging and LV conductance measurements in small animals are relatively new techniques allowing detailed assessment of LV anatomy and function in different pathophysiological states [26]. MR imaging revealed no differences in infarct size at day 2 between the different groups, and all three AMI groups showed typical changes indicative of left ventricular failure, as reflected by an increase in LV volume and a decrease in LVEF. This indicates that hMSC or hMSC_{myoc} transplantation had no protective effect on early LV remodeling and function. However, 14 days after AMI, progressive deterioration of LVEF was observed in the Medium group only, whereas deterioration of LVEF was significantly attenuated in the hMSC and hMSC_{myoc} groups. Despite the preservation of LVEF, hMSC transplantation did not prevent LV remodeling at 2 weeks. In contrast, hMSC_{myoc} transplantation resulted in attenuation of LV remodeling, indicating an additional beneficial effect of forced myocardin expression. Pressure volume loop measurements at day 15 were consistent with the MRI data at day 14. In addition, LV systolic function parameters, like maximum rate of LV pressure (dP/dt_{max}) and total amount of external work performed by the LV (stroke work) also improved after hMSC and hMSC_{myoc} transplantation compared to the medium only group. Furthermore, after hMSC_{myoc} and to a lesser extent after hMSC transplantation, the end-systolic pressure-volume relation (ESPVR, a load-independent parameter of LV systolic function), shifted towards smaller volumes, indicating improved systolic function. Moreover, hMSC_{myoc} transplantation improved the other intrinsic LV function parameters preload recruitable stroke work relation (PRSW), and the relation between dP/dt_{max} and EDV (dP/dt_{max} - EDV), effects not observed after hMSC transplantation alone. Regarding the LV diastolic function, the slope (E_{ED}) of the end-diastolic pressure-volume relation significantly decreased after both hMSC and hMSC_{myoc} transplantation indicating a decreased diastolic stiffness (i.e. improved diastolic function). Importantly, transplantation of hMSC_{myoc} but not transplantation of hMSC resulted in a significant increase in the peak rate of pressure decline (dP/dt_{min}), and decrease of the relaxation time constant Tau, indicating a faster isovolumic relaxation.

From these results it can be concluded that transplantation of hMSC_{myoc} and to a lesser extent of hMSC had beneficial effects on infarct size and systolic and diastolic LV function 15 days after myocardial infarction as compared to the Medium only group. The decline in cardiac function in the MI+Medium group was associated with a significant weight loss (qualifying as cardiac cachexia [27]). This weight loss was significantly attenuated after hMSC_{myoc} but not after hMSC transplantation. Furthermore, in the Medium group, MI resulted in an increase in lung fluid, indicative of pulmonary congestion, which was not observed after hMSC and hMSC_{myoc} transplantation.
POSSIBLE MECHANISMS OF BENEFIT AFTER HMSC AND HMSCmyoc TRANSPLANTATION

In this study it is demonstrated that forced expression of myocardin in hMSCs confers an additional beneficial effect on LV function after injection into the acutely infarcted myocardium of NOD/scid mice as compared to non myocardin transfected hMSCs. Although the improved preservation of LV function in the hMSCsmyoc group could be caused by differentiation of hMSCmyoc into a cardiomyogenic phenotype, no fully developed cardiomyocytes with sarcomeric striations were observed, making a contractile contribution of transplanted cells unlikely. Although, hMSCmyoc transplantation in the immune compromised NOD/scid mouse model of acute MI resulted in the expression of α-sarcomeric actin, ANF, atrial myosin light chain 2 (Mlc2a) and cardiac troponin T, the staining patterns for muscle-specific proteins remained diffuse. This was consistent with the hMSC that were kept in \textit{in vitro} culture, indicating that the ischemic environment did not result in a further differentiation towards a complete cardiomyogenic phenotype.

Furthermore, none of the myocardin transduced hMSCs expressed cTnI which is in accordance with our previous RT-PCR results in myocardin transduced hMSC, where also no cTnI but only conversely slow-twitch skeletal troponin I (ssTnI) specific transcripts were found [17]. ssTnI is active not only in skeletal muscle but also in the embryonic, foetal and neonatal myocardium [28]. These results therefore suggest that Myocardin expression activates an early developmental gene program, but does not result into complete cardiomyocyte differentiation of hMSCs.

The relative preservation of LV function observed in the hMSC group and the amelioration of remodeling and contractility observed in the hMSCsmyoc group can be explained by paracrine effects mediated by cytokines and/or other signaling molecules secreted by engrafted hMSCs and hMSCmyoc. Nagaya et al. demonstrated that rat MSCs increased capillary density in a rat model of dilated cardiomyopathy by secretion of the angiogenic factors vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [8]. Furthermore, Tang et al. demonstrated that rat MSCs engrafted in ischemic myocardium secrete angiogenic factors including stromal cell-derived factor-1α (SDF-1α), VEGF and basic fibroblast growth factor (bFGF) [5]. In line with these results we observed that hMSC and hMSCmyoc injections resulted in a higher vessel density in the infarcted area as compared to medium-treated animals. As no eGFP positive cells were found incorporated into the vascular structures, this indicates that the vessels were derived from the host tissue. However, although LV function was better in the hMSCmyoc treated group, vessel density in the scar area was the same in both groups. This is comparable to results from Li et al., showing that pretreatment of mouse MSCs with transforming growth factor-ß1 (TGF- ß) to enhance myogenic differentiation after AMI did not result in a higher microvessel density as compared to non treated MSCs [14]. Also in \textit{in vitro} pre-treatment of rat BMC with 5-aza did not result in an increased capillary density compared to normal BMCs in a rat model of chronic ischemia [3].
Therefore the additional beneficial effects of hMSC\textsubscript{myoc} transplantation can not be fully attributed to possible paracrine angiogenic effects. Substantial engraftment of both hMSCs and hMSC\textsubscript{myoc} after injection in the ischemic myocardium was observed. However quantitative assessment of engrafted cells revealed a significant higher engraftment rate in the hMSC\textsubscript{myoc} group. This suggests improved survival of hMSC\textsubscript{myoc} compared to hMSC, resulting in a more robust therapeutic effect. Which is comparable to the results from Mangi et al., who demonstrated that transduction of rat MSCs with Akt, a serine-threonine kinase and powerful survival signal, protects MSCs against apoptosis and as a result prevented remodeling and preserved LV function [24]. Additionally, this beneficial effect was also dose (cell number) dependent, where a 20 fold increase in the number of transplanted cells resulted in a significant increase in LV function [24]. Interestingly, Berry et al. demonstrated by atomic force microscopy that hMSC engraftment attenuates post-infarction remodeling by softening of the border zone area, thereby improving the elastic moduli resulting in a more compliant infarct scar [29]. Furthermore, our group recently demonstrated that myocardin transduction also leads to the functional expression of cellular components involved in electrical conduction [18]. Therefore transplantation of forced myocardin expressing hMSCs might result in improved electrical coupling between areas of surviving myocardium, leading to a more efficient contraction of the scarred myocardium.

Other beneficial effects of MSCs have been attributed to the secretion of substances acting on spared host cardiomyocytes to reduce apoptosis [30], or reduce scarring [29]. In addition, MSCs also secrete insulin-like growth factor-1 (IGF-1), which plays an important role in myocardial muscle growth and can exert positive inotropic effects [8]. However, the exact mechanisms responsible for the observed additional beneficial effects of forced myocardin expression in injected hMSCs remain at present unclear and needs to be defined in future studies.

LIMITATIONS

Despite the clinical relevance (as most patients will be IHD patients), one of the limitations of the present study is the lack of a control group with hMSCs derived from healthy subjects, as the differentiation potential of IHD derived hMSCs may be less than the differentiation potential of healthy subjects. Secondly, we used a model of acute MI with permanent ligation of the LAD, which does not reflect contemporary medical practice where patients with a MI undergo early reperfusion of the culprit artery. Furthermore human adenovirus serotype 5 vectors were used which do not integrate into the genome, resulting in transient expression of eGFP and myocardin genes, thereby allowing only short term experiments. Although the effects of forced myocardin expression were studied over a 15 day period, the stability of the observed beneficial effects should be studied over a longer period.
CONCLUSION

Forced expression of myocardin in hMSCs results in the expression of cardiomyogenic proteins but not in a true cardiomyocyte phenotype in vitro or in vivo. However compared to treatment with untreated hMSCs, injection of hMSC$_{myoc}$ resulted in a higher engraftment and further preservation of LV function.

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