

Myogenic differentiation of mesenchymal stem cells co-cultured with primary myoblasts

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Abstract

TE (tissue engineering) of skeletal muscle is a promising method to reconstruct loss of muscle tissue. This study evaluates MSCs (mesenchymal stem cells) as new cell source for this application. As a new approach to differentiate the MSCs towards the myogenic lineage, co-cultivation with primary myoblasts has been developed and the myogenic potential of GFP (green fluorescent protein)-transduced rat MSC co-cultured with primary rat myoblasts was assessed by ICC (immunocytochemistry). Myogenic potential of MSC was analysed by ICC, FACS and qPCR (quantitative PCR). MSC–myoblast fusion phenomena leading to hybrid myotubes were evaluated using a novel method to evaluate myotube fusion ratios based on phase contrast and fluorescence microscopy. Furthermore, MSC constitutively expressed the myogenic markers MEF2 (myogenic enhancer factor 2) and α -sarcomeric actin, and MEF2 expression was up-regulated upon co-cultivation with primary myoblasts and the addition of myogenic medium supplements. Significantly higher numbers of MSC nuclei were involved in myotube formations when bFGF (basic fibroblast growth factor) and dexamethasone were added to co-cultures. In summary, we have determined optimal co-culture conditions for MSC myogenic differentiation up to myotube formations as a promising step towards applicability of MSC as a cell source for skeletal muscle TE as well as other muscle cell-based therapies.

Keywords: muscle tissue engineering; myogenesis; myogenic differentiation

1. Introduction

TE (tissue engineering) of skeletal muscle is a promising method to treat tissue defects caused by trauma, tumour or other diseases that result in a loss of muscle tissue (Hutmacher et al., 2009; Mollmann et al., 2009). Several requirements have to be met to generate functional skeletal muscle tissue, such as cell differentiation, matrix development and *in vivo* biocompatibility. Satellite cells are the most commonly used cell source for skeletal muscle TE because of their muscular origin and ability to restore functional muscle tissue (Bach et al., 2003; Boonen and Post, 2008). Despite these properties, muscle satellite cells lack a sufficient differentiation capacity after several cycles of cell expansion (Carlson and Conboy, 2007), rendering it difficult to generate a sufficient number of well-differentiated cells to create large constructs of skeletal muscle tissue, as needed for a possible clinical application. One reason for this lack of sufficient differentiation capacity is the heterogeneity of the satellite cell population (Rudnicki et al., 2008; Negroni et al., 2009; Pietrangelo et al., 2009). MSCs (mesenchymal stem cells) from adult bone marrow could be a promising alternative as notional progenitors of satellite cells (Bossolasco et al., 2004; Belema Bedada et al., 2005; Satija et al., 2009). Their ability to differentiate into the myogenic lineage has previously been suggested using a variety

of distinct culture conditions (Gang et al., 2004, 2008; Grefte et al., 2007; Liu et al., 2007). Until now, the most efficient differentiation conditions to differentiate MSC towards functional muscle progenitor cells have not been ascertained.

Transplantation in damaged or degenerated muscle as in *mdx* mice for application in Duchenne muscular dystrophy were evaluated in studies before (Gussoni et al., 1999; LaBarge and Blau, 2002; Dezawa et al., 2005; Vieira et al., 2008) and showed possible effects of paracrine influence on myogenic differentiation of MSC. The effect of co-culture with viable myoblast needs to be proved and is one aim of this study. Co-culture experiments with cells from adipose-derived stem cells from mice showed the potential of myogenic differentiation by paracrine effects in a co-culture setting before and underlie our thesis (Di Rocco et al., 2006). Experiments with other cell sources such as human umbilical cord blood or lipoaspirate MSCs from mice showed that myogenic potential of MSC makes them an attractive target as cell source for skeletal muscle TE (Mizuno et al., 2002; Gang et al., 2004; Secco et al., 2008). MSC as a possible cell source for skeletal muscle: TE has distinct advantages compared with primary satellite cells. They can replicate repetitively without losing their differentiation capacities in early passages in contrast with primary myoblasts. Therefore, higher cell numbers may be generated out of a small population. Moreover, they can be transplanted in an autologous fashion and could have

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Abbreviations: bFGF, basic fibroblast growth factor; DAPI, 4', 6-diamidino-2-phenylindole; DHS, donor horse serum; DM1, differentiation medium 1; DM2, differentiation medium 2; GFP, green fluorescent protein; ICC, immunocytochemistry; MEF2, myogenic enhancer factor 2; MFI, mean fluorescence index; MFR, myotube fusion ratio; MHC, myosin heavy chain; MSCs, mesenchymal stem cells; MyoD1, myogenic differentiation factor1; qPCR, quantitative PCR; TE, tissue engineering.

immunomodulatory functions in terms of promoting tissue regeneration rendering them attractive candidates for muscle TE (Pittenger, 2004; García-Castro, 2008). Furthermore, the possibility of allogenic MSC transplantation makes them attractive candidates for cell-based therapies in regenerative medicine (Jung et al., 2009).

An important question has not yet been addressed: does myogenic differentiation of MSCs take place upon cell–cell contact with primary myoblasts? This phenomenon has been evaluated by differentiation of MSC in co-culture with renal or hepatic cells, but not with skeletal muscle cells (Lange et al., 2005b; Singaravelu and Padanilam, 2009). The aim of this study was to evaluate the effect of co-culturing MSC and primary muscle satellite cells or L6 myoblast lineage cells with emphasis on MSC myogenic differentiation. We also measured the effects of dexamethasone and bFGF (basic fibroblast growth factor) on myogenic MSC differentiation compared with common differentiation medium (Eberli et al., 2009). Paracrine effects of the cell–cell contacts are investigated as the most realistic setting for *in vivo* applicability. Our aim was to determine the most promising differentiation conditions to bring MSC towards the myogenic lineage. For this purpose, rat MSC co-cultured with myoblasts were assessed by ICC (immunocytochemistry), FACS (flow cytometry), qPCR (quantitative PCR) and light microscopy regarding the expression of myogenic markers.

2. Materials and methods

2.1. Myoblast cell culture

Satellite cells were isolated from hind limb muscles of male Lewis rats as described previously (Beier et al., 2004). Experiments were approved by the animal care committee of the University of Erlangen and the Government of Mittelfranken, Germany. The medium was changed every second day, and cells were passed when they were subconfluent. Myoblasts of passage 3 were used for all experiments.

2.2. MSC cell culture

MSCs were isolated from Lewis 1WR2 rats. Phenotype was assessed by their ability to differentiate into chondrocytes, adipocytes and osteocytes. Moreover, lack of CD45-positive cells was proved by FACS analysis over 10 passages to avoid contamination with haematopoietic stem cells (Lange et al., 2005c). MSCs were stably transduced with GFP (green fluorescent protein) for cell labelling and GFP-positive clones were

expanded as described previously (Lange et al., 2005a, 2005b). MSCs were cultured at the same conditions as myoblasts (see below) and were used at passages 11 and 12 for all experiments.

2.3. L6 cell culture

Cells of the myogenic cell line L6 (ATCC) were cultured as described below and used at passage 3 for all experiments.

2.4. Differentiation conditions

For myogenic differentiation, different media based on DMEM (Dulbecco's modified Eagle's medium)/Ham's F-12 were used. DM1 (differentiation medium 1) contained 2% DHS (donor horse serum, Biochrom AG) and 1% L-glutamine, DM2 (differentiation medium 2) contained 2% DHS, 1% L-glutamine, 1 ng/ml bFGF (Sigma–Aldrich)+0.4 µg/ml dexamethasone (Sigma–Aldrich). All groups were supplied with one of the differentiation media for 2 or 5 days. Groups were subsequently analysed by FACS, ICC or qPCR. The following 10 groups (G1–G10) were evaluated (Table 1).

2.5. Myoblast–MSC co-culture

Cells were detached from culture plates using 4 ml of trypsin–EDTA for 5 min at 37°C. The reaction was stopped by adding 8 ml of expansion medium. Suspension was centrifuged at 1500 rev./min for 5 min, and the supernatant was discarded. The pellet was resuspended in differentiation medium and seeded in 75-cm² culture flasks again. Differentiation medium was changed every 2 days. In co-culture groups with myoblasts and MSCs (G7 and G8), cells were mixed at a ratio of 1:1 and then seeded at 2×10^5 . In groups with L6 and MSC (G9 and G10), we changed the ratio to 1:5 because of the fast growth of the L6 cell lineage and also used a total cell number of 2×10^5 . Control groups with monocultures of the three different cell types (G1–G6) were seeded with the same cell number as co-culture groups (G7–G10). Three independent cell cultures were set up per group and time point.

2.6. Light and fluorescence microscopy

Cells of group G7 and G8 were seeded in a six-well Multiwell™ plate (BD Falcon™) and cultured with differentiation medium for 5 days. At days 2 and 5, the cells were analysed with fluorescence and light microscopy to evaluate the growth and myogenic differentiation of both groups. MSC could be distinguished from myoblasts based on their expression of GFP in vital cocultures (Figure 1). All MSCs per defined region of interest were counted by blinded observers, and a MFR (myotube fusion ratio) was

Table 1 Experimental group design
Groups with different cell types and media (+, contains cells of this type; –, without cells of this type). Mb, myoblasts.

Group nr.	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
MSCs	+	+	–	–	–	–	+	+	+	+
Mb	–	–	+	+	–	–	+	+	–	–
L6	–	–	–	–	+	+	–	–	+	+
Diff. medium	DM1	DM2	DM1	DM2	DM1	DM2	DM1	DM2	DM1	DM2

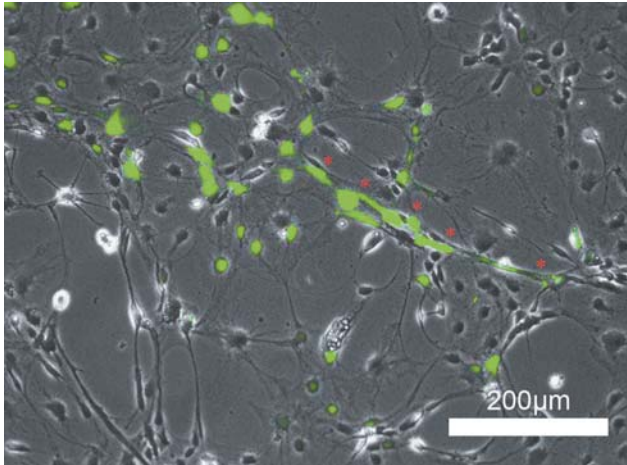


Figure 1 Phase contrast–fluorescence overlay of MSC

Phase contrast and fluorescence overlay image demonstrating GFP-expressing MSC co-cultured with primary myoblasts in medium DM1 at day 5 of co-culture. Differentiation towards myotube formation can be observed here in vital cells. Nuclei that are involved in myotube formation are marked with red asterisks. MSC co-cultured with primary myoblasts in DM1. MSC can be identified by their GFP expression which appears as green fluorescence.

calculated as follows: nuclei of MSC i (involved) and n_i (not-involved) in myotubal formations were counted, and myotube fusion ratio was calculated as $MFR = i/n_i$. MFR of different groups at the same time points were compared statistically using the Student's t test method with significance level of $P < 0.001$.

2.7. FACS

FACS was performed on a FACS Canto II Cytometer with a FACSDiva Software (BD Biosciences) and analysed with FlowJo Software (Tree Star Inc.). Myoblasts and L6 cells were labelled with SNARF[®]-1 at a concentration of 2.5 $\mu\text{M}/\text{ml}$ (Invitrogen Corp.) to avoid horizontal gene transfer. For every group, 5×10^5 cells were seeded in a 75- cm^2 flask and cultured with differentiation medium for 2 or 5 days in DM1. Then, cells were rinsed twice with PBS (Biochrom AG) and harvested using 4 ml of trypsin–EDTA for 5 min at 37°C. Reaction was stopped by adding 8 ml of expansion medium. Cells were centrifuged at 1500 rev./min for 5 min, and the supernatant was discarded. After resuspending the cell pellet in 5 ml PBS with 5% FBS (fetal bovine serum) for a blocking period of 15 min, they were centrifuged and washed with PBS. The pellet was picked up in 100 μl of Cytofix/Cytoperm solution from BD Cytofix/Cytoperm[™] Fixation/Permeabilization Kit (BD Biosciences) and incubated for 20 min at 4°C. Afterwards, cells were washed twice with BD Perm/Wash Buffer from the same kit by centrifugation and resuspending. The supernatant was discarded, the pellet picked up in 100 μl BD Perm/Wash Buffer with the primary antibody at a concentration of 1:50 and incubated for 30 min at 4°C. Anti- α -sarcomeric actin (Abcam), anti-MyoD1 (myogenic differentiation factor1), anti-MEF2 (myogenic enhancer factor 2) and anti-MHC (myosin heavy chain) (all from Labvision Corp.) were used as primary antibodies. All primary antibodies were mouse-anti-rat IgG1 antibodies. Then, cells were washed twice with BD Perm/Wash Buffer as described before. After

discarding the supernatant, cells were resuspended in 100 μl BD Perm/Wash Buffer, which contained 2% PE-anti-mouse IgG1 (BD Biosciences) and incubated for 30 min at 4°C. Cells were centrifuged and the pellet picked up in 0.5 ml of PBS with 2% FBS and 0.1% NaN_3 as FACS buffer for further FACS analysis. Unstained cells were used as negative control. L6 cell line myoblasts were used as positive control. To avoid unspecific binding, an isotype control with mouse-IgG1 (BD Biosciences) at the same concentration as the primary antibodies (1:50) was used with the secondary PE-labelled anti-mouse antibody for each group.

2.8. ICC

Cells of each group were seeded in four-well Permanox[®] Chamber slides (Nunc) and cultivated with 1 ml of differentiation medium per well for the given period. The medium was changed every 2 days. Two or five days after seeding, the medium was discarded, and cells were rinsed twice with PBS. Cells were subsequently fixed with 200 μl of Cytofix/Cytoperm solution from BD Cytofix/Cytoperm[™] Fixation/Permeabilization Kit and incubated for 30 min at 4°C. Then, chambers were washed twice with BD Perm/Wash Buffer and incubated with 100 μl primary antibody solution for 1 h at 24°C. Primary antibodies were diluted 1:50 in BD Perm/Wash Buffer. The same primary antibodies as in FACS analysis were used in ICC (anti- α -sarcomeric actin, anti-MyoD1, anti-MEF2, anti-MHC).

Cells were subsequently washed twice with BD Perm/Wash Buffer and incubated with 100 μl of secondary antibody solution for 30 min at 24°C. Alexa Fluor 594 goat-anti-mouse IgG1 (Invitrogen Corp.) was used as secondary antibody at a 1:200 dilution. Probes were rinsed twice and then counterstained with DAPI (4', 6-diamidino-2-phenylindole, Applied Science/Roche) for 5 min. After washing, slides were covered using Fluoprep (Biomérieux) as mounting medium. Skeletal muscle from Lewis rats served as positive control for all ICC stainings, and in each group, an isotype control was performed using mouse-IgG1 (BD Biosciences) instead of the primary antibody. All probes were analysed and digitally photographed with a fluorescence microscope and camera (Leitz DMRBE, Leica Microsystems). No further digital image processing was performed apart from contrast enhancement.

2.9. RNA extraction and qPCR analysis

In each group, the expression rate of desmin, MyoD1, myogenin, MEF2, MHC with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as endogenous control was analysed. Cells from L6 myoblast lineage were used as positive control based on their myogenic potential and constant expression of the various myogenic marker mRNAs. Cells of each group were harvested, and pellets were frozen in liquid nitrogen immediately. RNA of all probes was extracted using the Micro-RNeasy-kit (Qiagen GmbH) with corresponding QiaShredder according to the manufacturer's protocols. After confirming sufficient RNA concentration and assessment of purity with an Eppendorf Biophotometer (Eppendorf AG), the probes were reverse-transcribed into cDNA with Omniscript[®]-RT-kit, oligo-dT primers for cDNA synthesis and RNase Inhibitor (Qiagen GmbH). For qPCR, ABsolute[™] QPCR

SYBR[®] Green kit was used (Thermo Fisher Scientific) with a Light Cycler (Bio-Rad iCycler iQ5, Bio-Rad Inc.). Samples were tested as triplicates, and only variations of less than 1.5 threshold cycles were tolerated. Threshold cycles after cycle 35 were defined as invalid. Data evaluation was performed using the $\Delta\Delta C_T$ method.

Primers were as follows:

Desmin: fwd 5'-ATACCGACACCAGATCCAGTCC-3', rev 5'-TCCCTCATCTGCCTCATCAAGG-3';

Myogenin: fwd 5'-TGAGAGAGAAGGGAGGGAAC-3', rev 5'-ACAATACACAAAGCACTGGAA-3';

MyoD1: fwd 5'-AGAGGGAAGGGAAGAGCAGAAG-3', rev 5'-GCAGCAGCAACAACAACCAG-3';

MEF2: fwd 5'-TGCTGCTCTCACTGTCACTAC-3', rev 5'-TTC-ACGACTTGGGGACACTG-3';

MHC: fwd 5'-TGACTTCTGGCAAATGCAG-3', rev 5'-CCA-AAGCGAGAGGAGTTGTC-3';

GAPDH: fwd 5'-CAACGACCCCTTCATTGACC-3', rev 5'-CA-ACGACCCCTTCATTGACC-3'

3. Results

3.1. MSC expression of myogenic marker proteins and myotube formation upon co-culture with primary myoblasts

Using FACS, we detected a positive staining for MEF2 and α -sarcomeric actin in MSC, L6 lineage myoblasts and primary isolated myoblasts. Those results demonstrate that MSC constitutively express those two myogenic markers at protein level. Compared with the isotype control with purified mouse IgG1, a fluorescence shift was observed (Figures 2a and 2b). Positive staining can also be verified using the MFI (mean fluorescence index), which is based on the quotient of the geometric mean of the probe compared with the geometric mean of the isotype

control. The MFI for MEF2 of MSC cultured 5 days in DM1 was 6.74. Compared with primary myoblasts (2.03) and L6 cells (2.27), MSC showed a high expression of this myogenic marker protein. This effect was enhanced by co-cultivation with primary myoblasts for 5 days under the same conditions. Here, the MSC subpopulation showed a higher MFI (8.18) for MEF2 compared with monocultivation (Figure 3). MSC also showed an expression of α -sarcomeric actin, but there was no verifiable correlation with several groups or culture conditions. The MFI of the α -sarcomeric actin staining was very stable and showed no verifiable differences in the several groups. Staining for MyoD1 and MHC were negative. The geometric means of MSC subpopulations were measured by gating the subpopulations by their different fluorescence. This subpopulation was detected by their green fluorescence, according to their GFP expression and the lack of SNARF-1[®] labelling, which accounted for the myoblast subpopulation.

MSC showed positive staining for α -sarcomeric actin (Figure 4) and MEF2 (Figure 5) in ICC. Highest differentiation levels were observed in group G8, i.e. MSC plus myoblasts cultured under stimulation with bFGF and dexamethasone. Here, a fusion of MSC and myoblasts to multinucleated myotubes was detected (Figure 6). However, since cultivation of L6 myoblasts, which are derived from an immortalized cell line, constitutively results in myotube fusion after less than 5 days under differentiation conditions, this cell population could not be selectively analysed by means of FACS.

3.2. Altered MSC myogenic marker mRNA expression under co-culture conditions and bFGF/dexamethasone influence

After 2 days in monoculture, MEF2 expression on mRNA level was 2.96 (± 0.15 cycles) higher in MSC of group G1 compared with L6 myogenic lineage cells, which were used as a positive control and baseline. Using DM2 instead in group G2 induced a 13.93

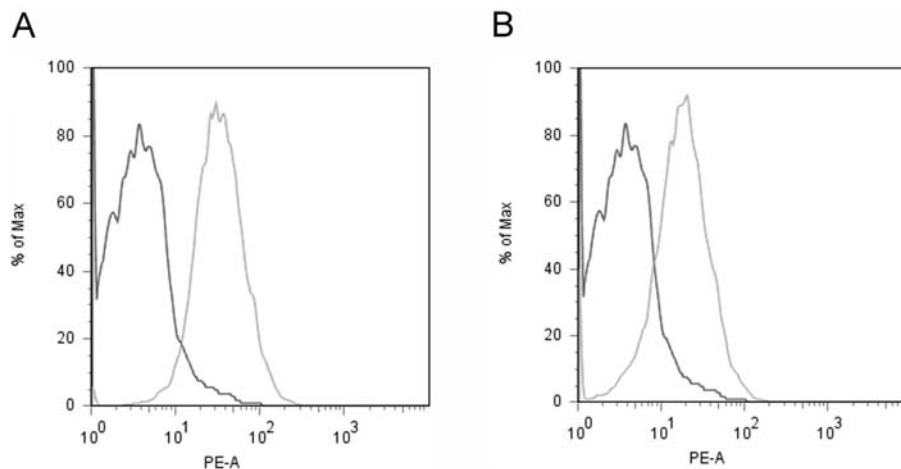


Figure 2a FACS staining for alpha-sarcomeric actin and MEF2 of monocultured MSC and MEF2 of

(A) Green graph, α -sarcomeric actin; red graph, isotype. Shift between green and red graph shows enlarged fluorescence intensity compared with isotype control and demonstrates expression of α -sarcomeric actin. MSC were monocultured 5 days with DM1. (B) Green graph, MEF2; red graph, isotype. Shift between green and red graph shows enlarged fluorescence intensity compared with isotype control and demonstrates expression of MEF2. MSC were monocultured 5 days with DM1.

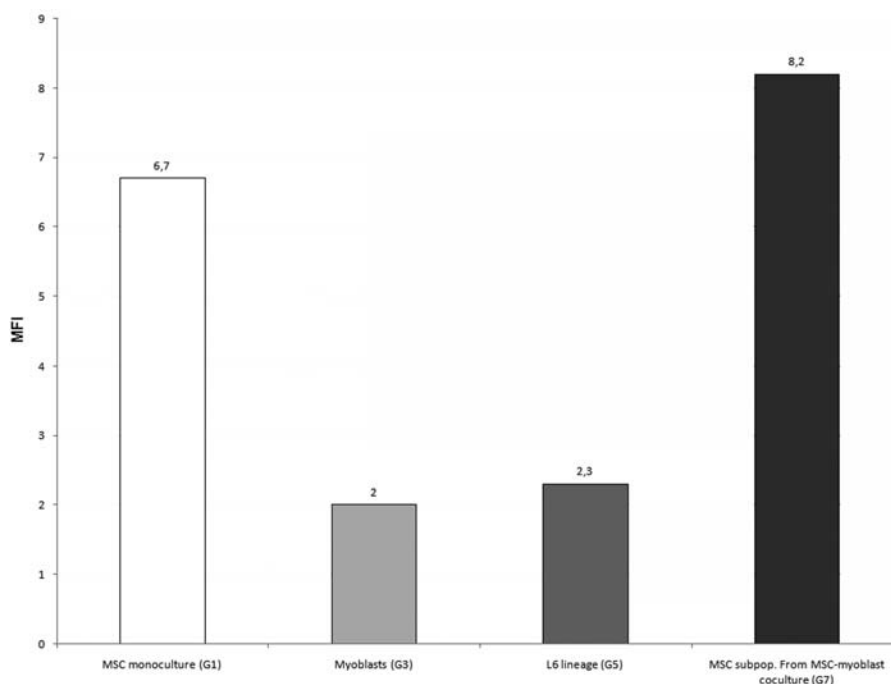


Figure 3 Mean fluorescence index of MEF2 stainings

MFI of MEF2 stainings of monoculture groups compared with MSC subpopulation in co-culture with myoblasts in DM1. Mean fluorescence index shows the geometric mean of fluorescence intensities from the group of interest compared with fluorescence intensities from isotype control.

(± 0.06)-fold expression of MEF2 in MSC at day 2. After 5 days of MSC monocultivation, up-regulation of MEF2 was increased up to 4.00 (± 0.12)-fold with DM1, while it was further increasing up to 17.15 (± 0.28) when cultivated in DM2 (Figure 7a). In co-culture groups G7 and G8, the same but weaker effect of dexamethasone and bFGF could be observed (Figure 7b). Expression increased from 1.48 (± 0.42) to 1.74 (± 0.35) at day 2 and from 0.95 (± 0.32) to 1.74 (± 0.35) at day 5 of cultivation comparing DM1 and DM2.

Desmin was also up-regulated in these groups upon co-cultivation using DM2 in comparison with DM1: desmin expression in DM1 was 10.92 (± 0.78)-fold and 26.91 (± 0.38)-fold in DM2 at day 2 compared with L6 in DM1. Desmin expression was even more up-regulated under stimulation with DM2 medium after 5 days: a 2.76 (± 0.38)-fold up-regulation was noted in DM1, while a 23.70 (± 0.44)-fold increase in expression rate was observed in DM2 medium (Figure 7c). Expression of MyoD1, myogenin and MHC

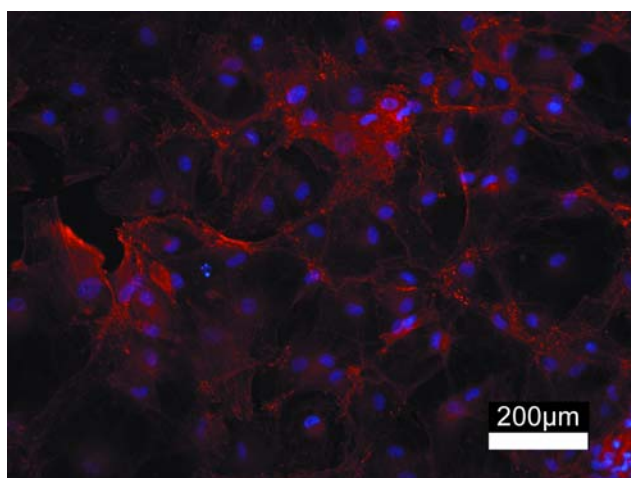


Figure 4 Immunocytochemical staining for α -sarcomeric actin

α -Sarcomeric actin staining (red) of MSC monocultured with DM1 for 5 days. Nuclei were counterstained with DAPI (blue). Red fluorescence shows characteristic appearance of α -sarcomeric actin as muscle-specific cell structure protein

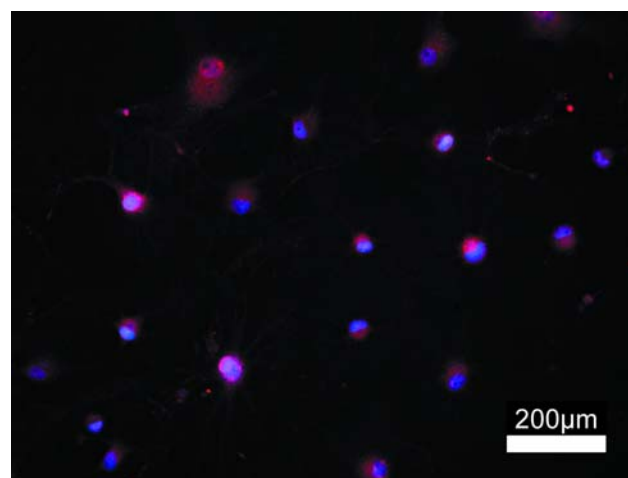


Figure 5 Immunocytochemical staining for MEF2

MEF2 staining (red) of MSC monocultured with DM1 for 5 days. Nuclei were counterstained with DAPI (blue). Red fluorescence shows characteristic archetype of MEF2 near the nuclei where it acts as transcription factor.

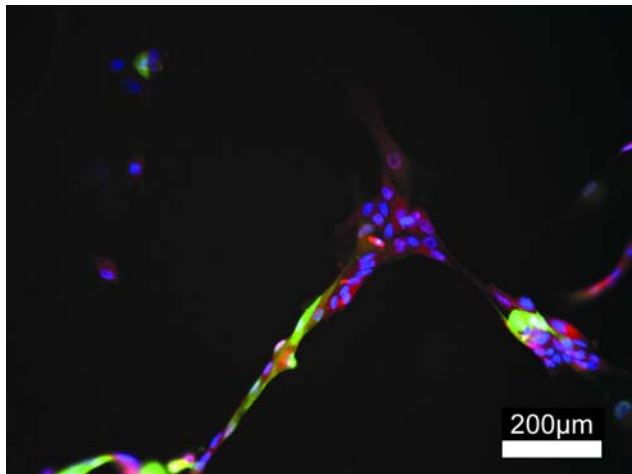


Figure 6 Myotube formation of MSC and myoblasts
Staining for α -sarcomeric actin (red) showing myotube fusion of GFP (green)-expressing MSC co-cultured with myoblasts under myogenic culture conditions (medium containing bFGF and dexamethasone). Nuclei were counterstained with DAPI (blue). The appearance of green fluorescing cells in multinucleated myotube formations and interaction with primary myoblasts, which appear with red fluorescence as staining for α -sarcomeric actin is shown here.

did not show significant differences in most groups. In nearly all groups, mRNA expression of MyoD1, myogenin and MHC was very low. The only groups in which the expression of these markers was strongly increased were the control groups with L6 myoblasts (data not shown). This might be explained by the low amount of MyoD1 and myogenin mRNA expressed in primary cells and the short time period during which these very specific markers are detectable throughout the process of myogenic differentiation. MHC is a marker which is expressed at a very late stage of myogenic differentiation playing an important role in maturation and gaining of functionality in contractile myotubes. Thus, MHC expression might increase after a longer period of stimulation with DM2, which could be an interesting topic for future studies. Since L6 myoblasts display such a high proliferative capacity that after a few days of co-cultivation with MSC the proportion of L6 myoblasts clearly predominate the MSC proportion, mRNA analysis for myogenic marker genes in these groups were not performed. RNA extraction and analysis of a purified MSC subpopulation in the primary myoblast/MSc co-culture groups were attempted. However, due to the strong adherence of MSC and myoblasts, no sufficient RNA extraction could be achieved. Experiments under several different conditions to perform a separation by digestion with trypsin/EDTA and/or collagenase did not result in a high volume single-cell solution. However, even after filtering through a cell strainer and subsequent sorting of the cells with FACS, no sufficient number of differentiated cells could be extracted from the subpopulations to perform valid mRNA analysis.

3.3. Hybrid myotube formation with MSC and primary myoblasts in co-culture

MSC of group G7 showed a fibroblast-like growth with no orientation after 2 days in culture. Cells of group G8 began to align and build myotubal formations with more than one nucleus.

MSC of G8 showed significantly higher alignment and nuclei contributions to these syncytia compared with MSC in G7, where no myotubes could be detected, and no signs of differentiation appeared. These results were similar to those after 5 days of culture, where MSC of G7 also showed a significantly higher grade of myogenic differentiation by alignment and myotubal formations (Figure 8).

4. Discussion

TE holds promise for numerous applications in medicine such as cancer therapy (Hutmacher et al., 2009), plastic and reconstructive surgery or treatment of inherited diseases (Helenius et al., 2004). TE of skeletal muscle is a promising tool to prefabricate muscle tissue for clinical applications (Beier et al., 2006). This tissue could either be used in regenerative medicine to reconstruct functional muscle defects or to cover defects caused by trauma, cancer or other acquired diseases. The most common cell sources for TE of skeletal muscle are primary satellite cells isolated from autogenous muscle by various digestion protocols. Another very promising application of *in vitro* cultivated (and genetically modified) myoblasts is the delivery of insufficiently produced blood clotting factors in patients' certain bleeding disorders (Thorrez et al., 2006). This cell source comes along with certain limitations and problems, which hampers the use of primary muscle cells as an only source for future applications of this technology. Primary muscle cells lose their differentiation capacity after extensive expansion and, therefore, lack the possibility to generate high cell numbers, which are needed for TE of three-dimensional constructs, as well as for gene therapy. Other cells may also be used for TE of skeletal muscle (Stern-Straeter et al., 2007). One promising cell source based on their good availability and their high differentiation capacities are MSCs (Mollmann et al., 2009; Satija et al., 2009). These cells, which are able to transdifferentiate into different tissues, such as cartilage, bone or fat, may have the potential to differentiate into the myogenic lineage (Pittenger, 2008). One way of inducing this differentiation is to culture them with certain medium supplements, such as growth factors or platelet lysate (Gang et al., 2004, 2008; Grefte et al., 2007; Lange et al., 2007; Liu et al., 2007). Our aim was to induce myogenic differentiation by cell-cell contact with primary myoblasts or L6 lineage cells to evaluate paracrine effects on MSC. For this purpose only, experiments with differentiation media (DM1 or DM2) were performed as growth medium does not effectively support myogenic differentiation (Gawlitta et al., 2008; Stern-Straeter et al., 2008).

Paracrine effects on MSC in co-culture with differentiated cells, such as hepatocytes or renal tubular cells had been previously shown in a few studies (Lange et al., 2005a, 2005b; Singaravelu and Padanilam, 2009). The use of myoblast-conditioned medium or of cell culture inserts (for separation chamber co-cultures) was not intended in our study, since we did not expect MSC differentiation based on factor secretion solely, according to previous works on MSC differentiation through co-cultivation with differentiation compared with conditioned media (Lange, 2005b;

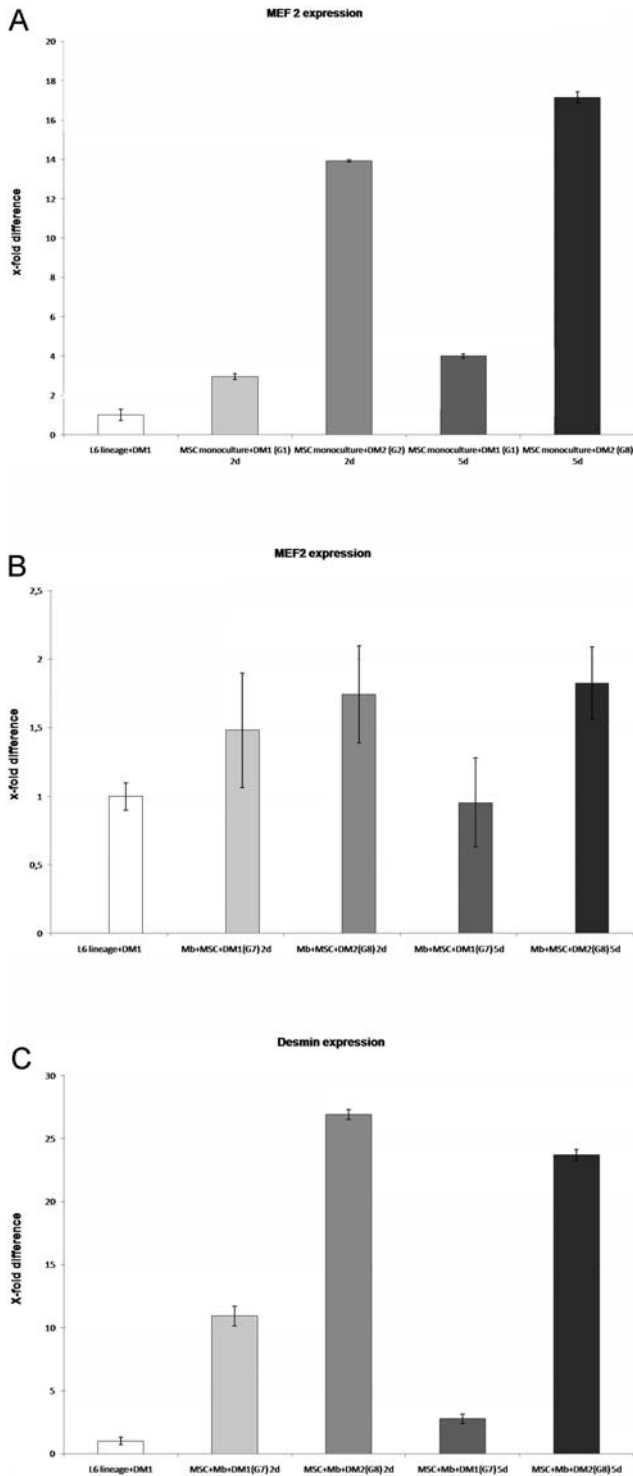


Figure 7 Expression of myogenic marker genes

(A) PCR analysis of MEF2 expression in monoculture groups. Quantitative RNA analysis of MEF2 expression in monocultures of MSC using medium without (DM1) and with dexamethasone+bFGF (DM2) after 2 days (2d) and 5 days (5d). Expressions are demonstrated in *x*-fold difference compared with the positive control baseline cells (L6 lineage in DM1). (B) PCR analysis of MEF2 expression in co-culture groups. Quantitative RNA analysis of MEF2 expression of co-culture groups with myoblasts (Mb) and MSC cultivated in medium without (DM1) and with dexamethasone and bFGF (DM2) after 2 days (2d) and 5 days (5d). Expressions are demonstrated in *x*-fold difference compared with the positive control baseline cells (L6 lineage in DM1). (C)

PCR analysis of desmin expression in co-culture groups. Quantitative RNA analysis of desmin expression of co-culture groups with myoblasts (Mb) and MSC cultivated in medium without (DM1) and with dexamethasone and bFGF (DM2) after 2 days (2d) and 5 days (5d). Expressions are demonstrated in *x*-fold difference compared with the positive control baseline cells (L6 lineage in DM1).

Singaravelu and Padanilam, 2009). Moreover, differentiation with conditioned medium would not be as useful in a setting for later *in vivo* experiments or clinical applicability because of the impossibility to control strict dissociation of myoblasts and MSC. However, further investigations will be necessary to elucidate the underlying mechanisms of action leading to myogenic MSC differentiation. Blocking of cell–cell contacts responsible for myoblast-fusion processes might be one promising approach for future studies. Until now, it remains speculative upon which cell–cell contact-mediated process and which downstream signal transduction pathway constitute the basis for the observations presented in this study.

We demonstrate MSC constitutive expression of some muscle-specific markers in the absence of any co-cultured myoblasts and without specific differentiation conditions. The expression of MEF2 is a new finding. Expression of α -sarcomeric actin underlines the myogenic potential of MSC according to previously published results (Rose et al., 2008; Quevedo et al., 2009). We could demonstrate the expression of these markers at the mRNA level, as well as on protein level. Furthermore, co-culture with two different media and assessed myogenic differentiation phenomena after 2 and 5 days was performed. Here, a differentiation of MSC towards the myogenic lineage in one co-culture group, where MSCs were cultured with primary myoblasts under the influence of bFGF and dexamethasone, was observed. The latter growth factors/hormones are known to support myogenic differentiation (Khezri et al., 2007; Eberli et al., 2009). Myogenic differentiation resulting in alignment of the cells with directed growth patterns and fusion towards multinucleated myotubes could be observed and may be regarded as a sign of

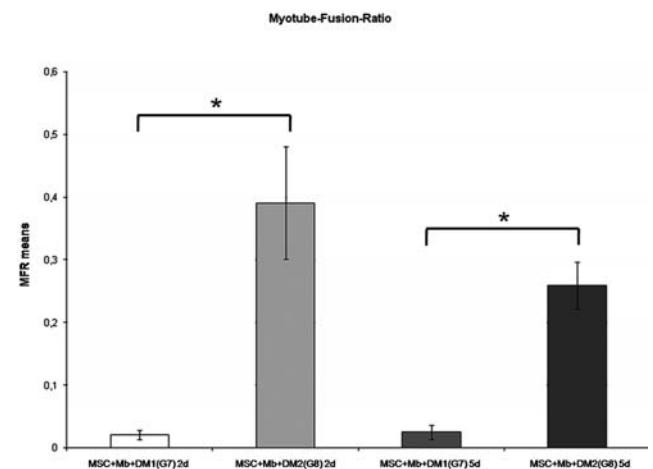


Figure 8 Myotube fusion ratios in co-culture groups

Significant difference ($*P < 0.001$) of MFR based on MSC contribution to myotube formation, compared with MSC not involved in myotube formation in co-culture groups of MSC and myoblasts, cultivated without (DM1) or with dexamethasone and bFGF (DM2) after 2 days (2d) and 5 days (5d).

advanced myogenic differentiation. qPCR analysis revealed an expression of muscle-specific desmin and MEF2 in MSC with an up-regulation of this key initial transcription factor in this most differentiated co-culture group.

Desmin is plotted because of its characteristic appearance in myoblasts and myocytes as muscle-specific marker gene and its common use to detect cells with myogenic potential. The increased expression of desmin shows the myogenic potential in the group with myoblasts and MSCs with DM2. An up-regulation of myogenin, MyoD1 or MHC could not be shown in this study. Maybe longer cultivation periods under differentiation conditions could demonstrate and increase the expression of MHC. For detection of MyoD1 and myogenin expression, an earlier time point of mRNA extraction might be promising because of their restriction of expression to a very short time gap during myogenic differentiation. Another problem in PCR analysis came up with the co-culture itself. The primary myoblasts seem to express less MEF2 mRNA than the monocultured MSC. Therefore, MSC seemed to express more MEF2 in monocultures than in the isolated mRNA of co-cultures. One might speculate that this phenomenon is related to dilution of whole mRNA with mRNA from primary myoblasts leading to a relatively lower expression of MEF2 in these myoblast-containing groups. However, these findings did not correlate with data obtained by ICC and FACS and our experiences in morphological analysis of cell cultures. Probably, the expression of the very early transcription factor MEF2 could have been detected earlier in the co-culture group with DM2 culture medium. In further studies, assessment of MEF2 expression during an earlier period, the first 12 or 24 h, e.g. might be a promising investigation.

A limitation of this work was the impossibility of isolating and purifying the MSC subpopulation after co-culturing, hindering specific mRNA analysis of this distinct cell fraction. The strong adherence of MSC and myoblasts in this group could not be dissolved by digestion with trypsin/EDTA or collagenase. Unfortunately, only very limited numbers of single cells could be isolated for FACS. Hence, not enough pure mRNA could be isolated for quantitative RNA analysis. Other digestion methods may solve this problem in the future and could render mRNA analysis of this subpopulation possible. Still, even digestion methods that may overcome the strong cell adherence would still not permit evaluation of MSC involved in myotube formation. Desired fusion to multinucleated cell syncytia itself appears to make retrospective analysis of the already fused MSC subpopulation extremely difficult.

Myogenic potential of MSC may already be postulated based on their constitutive expression of MEF2 and α -sarcomeric actin at the protein level, as demonstrated here. This hypothesis is further underlined by up-regulation of MEF2 expression in MSC upon co-cultivation with myoblasts at the mRNA level. Signs of increased MEF-2 expression were also observed in ICC, but this effect was not as robust as on mRNA level, thus possibly lying below quantitative detection limit in ICC technique. A next step to check on the regulation of MEF-2 protein expression could be a Western blotting analysis. Expression of α -sarcomeric actin and MEF2 was also detected by FACS. Compared with isotype controls and positive controls of L6 lineage cells, we could demonstrate that

there were no unspecific bindings. To avoid horizontal gene transfer, we labelled the partner cells with SNARF[®]-1 and analysed only the subpopulation that was positive for GFP expression and negative for SNARF[®]-1 labelling.

To analyse the quantitative contribution of MSC to multinucleated myotube formation, a new method was developed for this study: overlay images were generated after successive image acquisition using phase contrast microscopy and fluorescence microscopy for GFP of the same region of interest. Thus, the ratio of green fluorescing nuclei involved in myotube formations and of green fluorescing nuclei not involved in myotube formations could be calculated. As a result, we observed a significant difference between MSC contributing to myotube formation while being co-cultured with myoblast and stimulated by bFGF and dexamethasone compared with myoblast–MSC co-cultures without myogenic growth factors. The stimulation resulted in a significantly higher fusion ratio ($P < 0.001$) of the group stimulated by these medium supplements. For future studies, a standardized, fully automatic image analysis to determine the ratio of GFP nuclei involved/not involved in myotube formations would be desirable and is the subject of ongoing studies.

Our study demonstrates that MSC may be a promising cell source for skeletal muscle TE or other myoblast-based therapies. *In vivo* application using a three dimensional matrix (Beier et al., 2009) could be the next step to evaluate the potential of MSC to prefabricate vascularized muscle tissue, e.g. in an animal model for axial vascularization (Arkudas et al., 2009; Beier et al., 2009, 2010). Thus, the advantages of axial vascularization for prefabrication of transplantable tissue constructs could be applied in a rodent and subsequently large animal model for skeletal muscle TE in future studies. Also, questions concerning myogenic differentiation *in vivo* as related to matrix components, matrix assembling or other matrix properties, e.g. mechanical attributes, can be evaluated (Zhang et al., 2009). Further attempts to induce myogenic differentiation *in vitro* and *in vivo* could be based on the injection of microRNAs (Nakasa et al., 2009) or on neurotization of these neo-muscle constructs *in vivo* that may once lead to generation of functional muscle tissue (Charge et al., 2008).

5. Conclusion

Since myoblasts suffer from limitations because of their lack of differentiation in higher passages, MSC have some clear advantages for their use in skeletal muscle TE. The ability to differentiate into the myogenic lineage and their expression of muscle-specific markers underline their myogenic potential. Moreover, based on their immunomodulatory properties, they could provide a most promising cell source for skeletal muscle TE. MSCs show an expression of myogenic markers at the mRNA and protein level. We could show that MSC can be differentiated towards the myogenic lineage up to multinucleated myotubes by co-cultivation with primary myoblasts and stimulation with bFGF and dexamethasone. Myogenic differentiation induction in MSC may represent a promising approach for

TE of skeletal muscle and could bring new advantages and possibilities towards reconstruction of functional skeletal muscle tissue.

Author contribution

Justus Beier and Franz Bitto carried out the cell culture, FACS studies and RT-PCR studies. Claudia Lange carried out the MSC isolation, characterization and transduction. Dorothee Klumpp, Oliver Bleiziffer and Anja Boos participated in the immunohistochemistry and FACS studies. Andreas Arkudas participated in the statistical analysis. Justus Beier, Raymund Horch and Ulrich Kneser participated in the design of the study and co-ordination and helped to draft the manuscript. All authors read and approved the final manuscript

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References

- Arkudas A, Prymachuk G, Hoereth T, Beier JP, Polykandriotis E, Bleiziffer O et al. Dose-finding study of fibrin gel-immobilized vascular endothelial growth factor 165 and basic fibroblast growth factor in the arteriovenous loop rat model. *Tissue Eng Part A* 2009;15:2501–11.
- Bach AD, Stern-Straeter J, Beier JP, Bannasch H, Stark GB. Engineering of muscle tissue. *Clin Plast Surg*. 2003;30:589–99.
- Beier JP, Horch RE, Arkudas A, Polykandriotis E, Bleiziffer O, Adamek E et al. *De novo* generation of axially vascularized tissue in a large animal model. *Microsurgery* 2009a;29:42–51.
- Beier JP, Klumpp D, Rudisile M, Dersch R, Wendorff JH, Bleiziffer O et al. Collagen matrices from sponge to nano: new perspectives for tissue engineering of skeletal muscle. *BMC Biotechnol* 2009 Apr 15;9:34.
- Beier JP, Horch RE, Hess A, Arkudas A, Heinrich J, Loew J et al. Axial vascularization of a large volume calcium phosphate ceramic bone substitute in the sheep AV loop model. *J Tissue Eng Regen Med* 2010 Mar;4(3):216–23.
- Beier JP, Kneser U, Stern-Straeter J, Stark GB, Bach AD. Y chromosome detection of three-dimensional tissue-engineered skeletal muscle constructs in a syngeneic rat animal model. *Cell Transplant* 2004;13:45–53.
- Beier JP, Stern-Straeter J, Foerster VT, Kneser U, Stark GB, Bach AD. Tissue engineering of injectable muscle: three-dimensional myoblast–fibrin injection in the syngeneic rat animal model. *Plast Reconstr Surg* 2006;118:1113–21.
- Belema Bedada F, Technau A, Ebelt H, Schulze M, Braun T. Activation of myogenic differentiation pathways in adult bone marrow-derived stem cells. *Mol Cell Biol* 2005;25:9509–19.
- Boonen KJ, Post MJ. The muscle stem cell niche: regulation of satellite cells during regeneration. *Tissue Eng Part B Rev* 2008;14:419–31.
- Bossolasco P, Corti S, Strazzer S, Borsotti C, Del Bo R, Fortunato F et al. Skeletal muscle differentiation potential of human adult bone marrow cells. *Exp Cell Res* 2004;295:66–78.
- Carlson ME, Conboy IM. Loss of stem cell regenerative capacity within aged niches. *Aging Cell* 2007;6:371–82.
- Charge SB, Brack AS, Bayol SA, Hughes SM. MyoD- and nerve-dependent maintenance of MyoD expression in mature muscle fibres acts through the DRR/PRR element. *BMC Dev Biol* 2008;8:5.
- Dezawa M, Ishikawa H, Itokazu Y, Yoshihara T, Hoshino M, Takeda S et al. Bone marrow stromal cells generate muscle cells and repair muscle degeneration. *Science* 2005;309:314–7.
- Di Rocco G, Iachininoto MG, Tritarelli A, Straino S, Zacheo A, Germani A et al. Myogenic potential of adipose-tissue-derived cells. *J Cell Sci* 2006;119:2945–52.
- Eberli D, Soker S, Atala A, Yoo JJ. Optimization of human skeletal muscle precursor cell culture and myofiber formation *in vitro*. *Methods* 2009;47:98–103.
- Gang EJ, Bosnakovski D, Simsek T, To K, Perlingeiro RC. Pax3 activation promotes the differentiation of mesenchymal stem cells toward the myogenic lineage. *Exp Cell Res* 2008;314:1721–33.
- Gang EJ, Jeong JA, Hong SH, Hwang SH, Kim SW, Yang IH et al. Skeletal myogenic differentiation of mesenchymal stem cells isolated from human umbilical cord blood. *Stem Cells* 2004;22:617–24.
- García-Castro J TC, Madrenas J, Pérez-Simón JA, Rodríguez R, Menendez P. Mesenchymal stem cells and their use as cell replacement therapy and disease modelling tool. *J Cell Mol Med* 2008;12:2552–65.
- Gawlitta D, Boonen KJ, Oomens CW, Baaijens FP, Bouten CV. The influence of serum-free culture conditions on skeletal muscle differentiation in a tissue-engineered model. *Tissue Eng Part A* 2008;14:161–71.
- Grefte S, Kuijpers-Jagtman AM, Torensma R, Von den Hoff JW. Skeletal muscle development and regeneration. *Stem Cells Dev* 2007;16:857–68.
- Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 1999;401:390–4.
- Helenius G, Heydarkhan-Hagvall S, Siegbahn A, Risberg B. Expression of fibrinolytic and coagulation factors in cocultured human endothelial and smooth muscle cells. *Tissue Eng* 2004;10:353–60.
- Hutmacher DW, Horch RE, Loessner D, Rizzi S, Sieh S, Reichert JC et al. Translating tissue engineering technology platforms into cancer research. *J Cell Mol Med* 2009;13:1417–27.
- Jung DI, Ha J, Kang BT, Kim JW, Quan FS, Lee JH et al. A comparison of autologous and allogenic bone marrow-derived mesenchymal stem cell transplantation in canine spinal cord injury. *J Neurol Sci* 2009;285:67–77.
- Khezri S, Valojerdi MR, Sepelri H, Baharvand H. Effect of basic fibroblast growth factor on cardiomyocyte differentiation from mouse embryonic stem cells. *Saudi Med J* 2007;28:181–6.
- LaBarge MA, Blau HM. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* 2002;111:589–601.
- Lange C, Bassler P, Lioznov MV, Bruns H, Kluth D, Zander AR et al. Hepatocytic gene expression in cultured rat mesenchymal stem cells. *Transplant Proc* 2005a;37:276–9.
- Lange C, Bassler P, Lioznov MV, Bruns H, Kluth D, Zander AR et al. Liver-specific gene expression in mesenchymal stem cells is induced by liver cells. *World J Gastroenterol* 2005b;11:4497–504.
- Lange C, Togel F, Ilttrich H, Clayton F, Nolte-Ernsting C, Zander AR et al. Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. *Kidney Int* 2005c;68:1613–7.
- Lange C, Cakiroglu F, Spiess AN, Cappallo-Obermann H, Dierlamm J, Zander AR. Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. *J Cell Physiol* 2007;213:18–26.
- Liu Y, Yan X, Sun Z, Chen B, Han Q, Li J et al. Flk-1+ adipose-derived mesenchymal stem cells differentiate into skeletal muscle satellite cells and ameliorate muscular dystrophy in mdx mice. *Stem Cells Dev* 2007;16:695–706.
- Mizuno H, Zuk PA, Zhu M, Lorenz HP, Benhaim P, Hedrick MH. Myogenic differentiation by human processed lipoaspirate cells. *Plast Reconstr Surg* 2002;109:199–209.

- Mollmann H, Nef HM, Voss S, Troidl C, Willmer M, Szardien S et al. Stem cell-mediated natural tissue engineering. *J Cell Mol Med* 2011;15:52–62.
- Nakasa T, Ishikawa M, Shi M, Shibuya H, Adachi N, Ochi M. Acceleration of muscle regeneration by local injection of muscle-specific microRNAs in rat skeletal muscle injury model. *J Cell Mol Med* 2009;14(10):2495–505.
- Negrone E, Riederer I, Chaouch S, Belicchi M, Razini P, Di Santo J et al. *In vivo* myogenic potential of human CD133+ muscle-derived stem cells: a quantitative study. *Mol Ther* 2009;17:1771–8.
- Pietrangolo T, Puglielli C, Mancinelli R, Beccafico S, Fano G, Fulle S. Molecular basis of the myogenic profile of aged human skeletal muscle satellite cells during differentiation. *Exp Gerontol* 2009;44:523–31.
- Pittenger MF. Mesenchymal stem cells from adult bone marrow. *Methods Mol Biol* 2008;449:27–44.
- Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* 2004;95:9–20.
- Quevedo HC, Hatzistergos KE, Oskoue BN, Feigenbaum GS, Rodriguez JE, Valdes D et al. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proc Natl Acad Sci USA* 2009;106:14022–7.
- Rose RA, Jiang H, Wang X, Helke S, Tsoporis JN, Gong N et al. Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes *in vitro*. *Stem Cells* 2008;26:2884–92.
- Rudnicki MA, Le Grand F, McKinnell I, Kuang S. The molecular regulation of muscle stem cell function. *Cold Spring Harbor Symp Quant Biol* 2008;73:323–31.
- Satija NK, Singh VK, Verma YK, Gupta P, Sharma S, Afrin F et al. Mesenchymal stem cell-based therapy: a new paradigm in regenerative medicine. *J Cell Mol Med* 2009;13(11–12):4385–402.
- Secco M, Zucconi E, Vieira NM, Fogaca LL, Cerqueira A, Carvalho MD et al. Multipotent stem cells from umbilical cord: cord is richer than blood! *Stem Cells* 2008;26:146–50.
- Singaravelu K, Padanilam BJ. *In vitro* differentiation of MSC into cells with a renal tubular epithelial-like phenotype. *Ren Fail* 2009;31:492–502.
- Stern-Straeter J, Bran G, Riedel F, Sauter A, Hormann K, Goessler UR. Characterization of human myoblast cultures for tissue engineering. *Int J Mol Med* 2008;21:49–56.
- Stern-Straeter J, Riedel F, Bran G, Hormann K, Goessler UR. Advances in skeletal muscle tissue engineering. *In Vivo* 2007;21:435–44.
- Thorrez L, Vandenberg H, Callewaert N, Mertens N, Shansky J, Wang L et al. Angiogenesis enhances factor IX delivery and persistence from retrievable human bioengineered muscle implants. *Mol Ther* 2006;14:442–51.
- Vieira NM, Bueno CR, Jr., Brandalise V, Moraes LV, Zucconi E, Secco M et al. SJL dystrophic mice express a significant amount of human muscle proteins following systemic delivery of human adipose-derived stromal cells without immunosuppression. *Stem Cells* 2008;26:2391–8.
- Zhang S, Sun A, Liang Y, Chen Q, Zhang C, Wang K et al. A role of myocardial stiffness in cell-based cardiac repair: a hypothesis. *J Cell Mol Med* 2009;13:660–3.

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