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Human Adult Bone Marrow-Derived Mesenchymal Stem Cells: Factors Influencing Skeletal Muscle Differentiation

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Abstract

Muscle disease leads to significant mortality and morbidity worldwide, and few effective treatment options are available. Bone marrow-derived mesenchymal stem cells (MSCs) offer a highly attractive means of cell therapy, having multiple modes of action, accessible, at low risk of malignant transformation and amenable to autologous transplantation. Engraftment of transplanted MSCs into skeletal muscle has been demonstrated but spontaneous incorporation is low. The factors influencing MSC myogenicity are not fully defined, and if better understood could markedly improve therapeutic prospects.

Here, we identified growth factors and culture conditions that showed significant promise in promoting myogenic differentiation of human MSCs. Culture on collagen; using skeletal muscle media (containing dexamethasone, insulin, EGF, bovine fetuin, bovine serum albumin and gentamicin); and exposure to a combination of IGF1, FGF2 and VEGF all selectively increased myogenic marker transcript expression by human MSCs. Initiating and sustaining myogenic differentiation of MSCs *in vitro* may be a critical step in harnessing these cells for therapy.

We also showed that human MSCs aggregate with myoblasts *in vitro* and appear to form multi-nucleated structures expressing a range of skeletal muscle markers. Our observations provide further evidence that MSCs are a credible candidate for cellular therapy in patients with muscle disease.

Keywords: Stem cells; Skeletal muscle; Bone marrow; Mesenchymal; Myogenic differentiation

Introduction

Primary muscle diseases include muscular dystrophies, inflammatory and metabolic myopathies, mitochondrial cytopathies, channelopathies and other inherited diseases. Additionally, there is increasing recognition that secondary muscle dysfunction – from aging, immobility, critical illness, trauma, denervation, cancer and other metabolic abnormalities – is a major cause of muscle-related morbidity. Collectively, these muscle diseases cause major disability, often with few if any treatment options, and the prospect of stem cell therapies has therefore generated much interest [1].

Bone marrow-derived mesenchymal stem cells (MSCs) are an attractive prospect, being multipotent, relatively accessible, of low malignancy potential, and open to autologous transplantation [2]. Animal studies have shown skeletal muscle engraftment of transplanted bone marrow stem cells, with restoration of muscle marker expression and muscle fibre function [3-5]. Persistence of donor nuclei within muscle has also been reported in a human bone marrow recipient with Duchenne muscular dystrophy [6]. However, spontaneous incorporation of these cells is low. Enhancing their myogenic potential could markedly improve therapeutic prospects, but those factors regulating this differentiation remain incompletely understood.

We studied factors that might influence the myogenic potential of cultured adult human bone-marrow derived MSCs, exploring morphology, immunofluorescence and real-time PCR to show changes in transcript expression of a range of mature muscle proteins and receptors. We assessed the effects of a number of growth factors already known to be involved in skeletal muscle growth and repair, including IGF1, FGF2 (bFGF), FGF6 and VEGF [7,8]. We also plated MSCs onto collagen, a technique employed for selecting and promoting differentiation of myogenic progenitors in primary skeletal muscle cell cultures [9-11]; and we explored the possibility that myoblasts or fully-differentiated skeletal muscle cells promote myogenic differentiation of stem cells. Our findings may have implications for the development of mesenchymal stem cell therapy for patients with muscle disease.

Methods

Human bone marrow donors

Local Research Ethics Committee approval was secured. Bone marrow samples were obtained from consenting adults undergoing elective total hip replacement. Samples derived from both female and male donors (all at third passage) were used for this study.

Extraction of human MSCs from bone marrow & maintenance in culture

MSC cultures were prepared as previously described [12]. Briefly, marrow was homogenized with sterile scalpel blades in Hanks balanced salt solution (HBSS; Sigma-Aldrich). Marrow samples were layered over 25 ml of Ficoll (Lymphoprep, Axis-Shield PoC, Norway) in 50 ml tubes and then centrifuged at 1200g for 30 min. The mononuclear cell layer was aspirated from the marrow/Ficoll interface and made up to 30 ml with HBSS. The resulting suspension was centrifuged at 300 g for

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8 min. The pellet was resuspended in 10 ml of red blood cell lysis buffer (0.83% NH_4Cl, 0.1% $KHCO_3$ and 0.004% EDTA in distilled H_2O) for 10 min at 4°C, followed by centrifugation at 300 g for 10 min. The final cell preparation was seeded at 1×107 cells/25 cm^2 flask (BD Falcon) in MSC growth medium, which comprised Dulbecco's modified eagle's medium (DMEM; Sigma-Aldrich), pH 7.2, containing 10% fetal bovine serum (FBS; Stem Cell Technologies Inc.), 2 mM L-glutamine (Sigma-Aldrich), 1 ng/ml recombinant human basic fibroblast growth factor (bFGF; Sigma-Aldrich) and 50 U/ml penicillin/streptomycin (Gibco BRL). Cells were maintained at 37°C/5% CO_2, with media changed after 24 h to remove non-adherent cells, and subsequently every 3d afterwards. Cultures were normally passaged after 7-10 d when cells were confluent. Old MSC growth medium was removed from flasks and cells rinsed with several ml of phosphate buffered saline (PBS), pH 7.2. 1 ml of Accutase (PPA Laboratories) was added to each culture flask/25 cm^2 and flasks incubated at 37°C for 8 min to harvest adherent cells. The resultant cell suspension was centrifuged at 300 g for 8 min. The pellet was resuspended in MSC growth medium, cell numbers established and MSCs plated at 1×105 cells/25 cm^2 flask, 5×105 cells/75 cm^2 flask or 1.2×10 1206 cells/175 cm^2 flask in MSC growth medium at 37°C/5% CO_2.

Confirmation of the mesenchymal stem cell nature of cultures was performed in conventional ways by flow cytometry for CD marker expression, and differentiation into adipogenic, osteogenic or chondrogenic cells using routine methods [13] (data not shown here but as per our previous papers [12,14].

Rat myoblast culture

Rat skeletal muscle myoblasts L6.C11 (European Collection of Cell Cultures [ECCC]; rat thigh muscle) were cultured in DMEM (glucose 4500mg/l, Sigma) with 10% fetal calf serum (Gibco) and 2mM glutamine at 37°C/5% CO_2. Cells were plated at a seeding density of 2,000cells/cm^2. Cells were passaged at >90% confluence and/or when myotubes formed.

Human skeletal muscle culture

Human (female) skeletal muscle cells SkMC (Cambrex) were grown in skeletal muscle basal media (Cambrex). To each 50ml of media was added: 50µl recombinant human EGF, 50µl gentamicin, 50µl dexamethasone, 500µl insulin, 500µl bovine fetuin and 500µl of bovine serum albumin (Cambrex). Cells were plated at a seeding density of 3,500cells/cm^2 and cultured at 37°C/5% CO_2. Cells were passaged at 80-90% confluence.

Human lymphoblast HL60 culture

Human lymphoblast cell line HL60 (ECCC; a pro-myelocytic leukaemia cell line), was used as a negative control, having previously been shown not to express myogenic markers [15]. Cells were cultured in RPMI (Sigma) with 2mM glutamine (Sigma) and FCS (Gibco) at 37°C /5% CO_2 at a seeding density of 5 x 100,000cells/ml. 20% FCS was used initially and reduced to 10% once cells were established.

Human dermal fibroblast culture

Human dermal fibroblasts (Cambrex, NHDF) were cultured in fibroblast basal media (Cambrex) with the following additives: 500µl rh-FGF-B, 500µl insulin and 500µl gentamicin. Cells were plated at a seeding density of 3500cells/cm^2, grown at 37°C /5% CO_2 and passaged when they reached 80-90% confluence.

Rat myoblast & human skeletal muscle-conditioned media

When rat myoblast or human skeletal muscle cells were passaged, media was removed, centrifuged and supernatant filtered and stored.

GFP-labelling of human MSCs by viral transfection

GFP-labelled human MSCs were prepared as previously described [16]. Briefly, recombinant lentiviral construct (NIV-CMV-EGFP-WPRE) was kindly provided by Dr C. Glover and Dr J. Uney (Henry Wellcome Laboratories for Integrative Neuroscience & Endocrinology, The University of Bristol, Bristol, UK). The EGFP reporter gene was driven off the human cytomegalovirus promoter (hCMV), while the woodchuck hepatitis virus post-transcriptional regulatory element was incorporated to enhance EGFP expression [17]. Viral titre used was 4.872 × 109 plaque forming units (pfu)/ml. MSCs were plated at a density of 30,000 cells/well in a 6-well plate with DMEM and 10% FCS, lentivirus (at a multiplicity of infection of 10) and 8µg/ml hexadimethrine bromide added to each well. Cells were subsequently cultured as above.

Cell sorting of GFP+ MSCs using FACS Vantage SE flow cytometer (Becton-Dickinson)

500,000 cells were rinsed with 10ml propidium iodide (PPI, Sigma), centrifuged and re-suspended in PBS containing EDTA. FACS gates were initially set using non-transfected cells, and PPI used to exclude dead cells. GFP-positive and GFP-negative cells were collected in DMEM/10% FCS.

Indirect co-culture

Human MSCs were grown in indirect co-culture with rat myoblasts, with human skeletal muscle cells and with human skeletal muscle cells that had previously been exposed to soluble TNFα [Sigma] at concentrations of 1 and 10ng/ml for 7d. Poly-l-lysine covered coverslips were placed in 15mm wells and 1000 MSCs in 500µl MSC media added to each. A 2µm filtered well insert was placed in each well and either rat myoblasts or human skeletal muscle cells (1000 cells) added to each well insert in 500µl media. Cells were cultured for 7-10d at 37°C/5% CO_2 and observed daily.

Direct co-culture

GFP-labelled human MSCs were directly co-cultured with rat myoblasts, with human skeletal muscle cells, human skeletal muscle cells previously exposed to TNFα, and with human dermal fibroblasts. Human dermal fibroblasts were also directly co-cultured with rat myoblasts. Cells were added to 12mm wells containing PLL-coated coverslips and 200µl of each media type, in a 1:1 ratio using 1000 cells per well, for 7-10d at 37°C/5% CO_2 and observed daily.

Immunocytochemistry

Cells were plated onto PLL-coated coverslips at a density of 1000 cells/coverslip, washed with 5% normal goat serum (NGS, Vector) in PBS and placed on a humidified staining platform. Cells were fixed in 4% paraformaldehyde (PFA), washed twice with 5% NGS and permeabilised with ice-cold methanol at -20°C for 10min. Cells were then washed twice with 5% NGS, blocked for 30min with 5% NGS and incubated with primary antibody overnight at 4°C. Cells were subsequently incubated with secondary antibody for 30min at room temperature, washed twice with 5% NGS and blocked for 30min with

5% NGS. They were then incubated with 1:1000 Hoescht for 5min and washed twice with 5% NGS.

Primary antibodies for the following markers were used: Pax-7 (R & D Systems, 1:100), desmin (Vector, 1:50), Myf-5 (Santa Cruz, 1:100), MyoD (Santa Cruz, 1:200), myogenin (Santa Cruz, 1:100), actin (anti-sarcomeric, 1:100), dystrophin (Sigma, 1:200), acetylcholine receptor α-subunit (Chemicon, 1:100) adiponectin receptor-1 (Affinity Bioreagents, 1:100), sarcoplasmic endoreticulum calcium ATPase 1a (SERCA1a, donated by Professor Elek Molnar, 1:400), heat shock protein-47 (HSP47, AbCam, 1:100), ryanodine receptor (Affinity bioreagents, 1:100), and human nuclear antigen (HuNu, Chemicon).

Experimental differentiation culture conditions

MSCs were cultured in the following conditions for 5-7d: -

1. MSC media alone (Dulbecco's Modified Eagle Medium (glucose 1000mg/l, 4mM glutamine, Sigma) with 10% fetal calf serum (Stem Cell Technologies);
2. 30% rat myoblast media DMEM (glucose 4500mg/l Sigma) with 10% fetal calf serum (Gibco) and 2mM glutamine and 70% MSC media;
3. 30% rat myoblast-conditioned media and 70% MSC media;
4. 30% human skeletal muscle cell media with constituents as above (Cambrex);
5. 30% human skeletal muscle-conditioned media and 70% MSC media;
6. MSC media in a flask coated with collagen I (Cultrex);
7. VEGF: MSC media containing 10ng/ml VEGF (R&D Systems);
8. MSC media containing 10ng/ml VEGF (R&D Systems), 10ng/ml IGF1 (R&D systems) & 10ng/ml FGF2 (Sigma);
9. FGF6: MSC media containing 5ng/ml FGF6 (R&D Systems).

RNA preparation

Cells were lysed with Lysis buffer (Applied Biosystems) and RNA extracted using an Applied Biosystems Prism 6100 nucleic preparation station. RNA concentrations were measured using a ribogreen RNA quantification kit (Invitrogen) and a Fluostar Optima plate reader (BMG Labtech).

Real-time PCR

An Applied Biosystems Prism 7000S machine was used and all probes purchased from Applied Biosystems Taqman gene expression assay inventory: desmin (Hs00157258_m1), α-actinin (Hs00241650_m1), dystrophin (Hs00187805_m1), adiponectin receptor 1 (Hs00360422_m1), SERCA1 (Hs00188877_m1) and 18S (Hs99999901_s1). A rat desmin probe was also used (Rn00574732_m1). Probes were added to supermix (i-Taq supermix with ROX, BioRad Laboratories #170-8855) and DNA/RNAase-free water (Sigma) to a volume of 22.5 microlitres per well in an optical reaction plate (Applied Biosystems) and 2.5 microlitres (200ng) cDNA added. Samples were run in triplicate and compared with a ribosomal RNA probe (18S), non-template (DNA/RNAase-free water) and human skeletal muscle cell line (Cambrex, SkMC, CC2561) controls, also in triplicate. A serial dilution study was performed for each probe, to confirm operation in a linear range for cDNA used. Skeletal muscle transcript expression in MSCs was compared with expression in human skeletal muscle cells and human lymphoblasts. Serial dilution studies were performed for each marker probe confirmed that all probes were operating in a linear range for the cDNA used. Levels of 18S were not influenced by the culture conditions we assessed.

Real-time PCR data analysis

Relative gene expression was determined using the real-time quantitative PCR 2-ΔΔCt method [18]: ΔΔCt = ΔCt – mean cycle count for 18S (where ΔCt is the mean cycle count muscle marker probe). From this, 2-ΔΔCt was calculated to make gene expression comparisons. Statistical analysis was performed using Kruskall Wallis with Dunn's post-testing to compare results from the range of culture conditions used with those derived from MSCs cultured in MSC media.

Results

Differentiation effects of defined factors

We explored the influence on (i) morphology, (ii) antigen expression assessed by immunofluorescence, and (iii) transcript expression assessed by real time PCR of three sets of manipulations – growth in conventional MSC or 'basal' culture medium; the addition of specific growth factors alone or in combination, or of various types of conditioned medium; growth on a collagen substrate; and growth in co-culture conditions with various muscle-related cell types, as described below.

Morphology: We assessed MSC morphology in basal media, and following exposure to a number of different culture conditions.

These included: IGF1, FGF2 & VEGF; VEGF alone, FGF6; culture on a collagen surface; and the effects of skeletal muscle basal media or skeletal muscle-conditioned media obtained using either human or rat cells.

MSCs cultured in basal media had spindle-shaped or broad, flat fibroblast-like morphology (Figure 1a,c) and adhered to plastic culture flasks. No consistent changes were seen in the presence of individual additional growth factors IGF1, FGF2 or VEGF (not shown).

Striking morphological changes were seen, however, in MSCs cultured in media containing a combination of IGF1, FGF2 and VEGF. Cells adopted a relatively uniform elongated, bipolar or tubular morphology within 48 hours (Figure 1b) and by 5-7 days assumed a series of linear adjacent positions, forming bands of cells across the flask (Figure 1d). This arrangement was similar to that of myotubes formed by fusion of myoblasts (Figure 1e).

Immunofluorescence: We investigated MSC expression of a range of skeletal muscle markers, including desmin, Myf-5, MyoD, myogenin, Pax-7, actin, dystrophin, acetylcholine receptor α-subunit, adiponectin receptor-1, SERCA1 and the ryanodine receptor, in basal and experimental culture conditions using immunocytochemistry. We found no consistent alterations in the expression of these markers following exposure to various growth factors alone or in combination (VEGF; FGF6; IGF1; or IGF1 + FGF2 + VEGF); neither did the culture of MSCs on collagen, or in human skeletal muscle basal media, in human skeletal muscle-conditioned media, in rat myoblast basal media, or in rat myoblast-conditioned media alter staining.

Immunocytochemical staining patterns, intensities and percentage of cells expressing markers were indistinguishable from MSCs cultured in basal media [not shown].

Real-time PCR: Real-time PCR was used quantitatively to assess skeletal muscle transcript expression under the same experimental conditions. First, however, levels of skeletal muscle marker transcripts in human MSCs were compared with those in human skeletal muscle cells, and were found to be markedly lower in MSCs from all four marrow samples. Levels of dystrophin expression showed the greatest difference (mean cycle count 14 in MSCs corrected for 18S and 11.2 in skeletal muscle cells), followed by desmin (mean cycle count 19.2 in MSCs corrected for 18S and 17.5 in skeletal muscle cells). SERCA1 (mean cycle count 21.7 in MSCs corrected for 18S and 21.6 in skeletal muscle cells) was expressed at the lowest level and α-actinin (mean cycle count 9.1 in MSCs corrected for 18S and 8.6 in skeletal muscle cells) at the highest level in both MSCs and skeletal muscle cells, followed by adiponectin receptor 1 (mean cycle count 11.9 in MSCs corrected for 18S and 11.6 in skeletal muscle cells).

Substrate effects (collagen): Human MSCs cultured on a collagen layer in MSC basal media demonstrated a dramatic increase in desmin transcript expression (mean > three-fold) compared with MSCs grown in MSC media in the absence of collagen (Table 1 and Figure 2). A mean 80% and 30% increase in alpha-actinin and SERCA1 expression respectively was also seen. Collagen marginally reduced adiponectin receptor expression in all four marrows. Effects on dystrophin expression were less clear (although mean levels fell slightly: MSCs from one marrow showed a 20% increase, two remained unchanged and one showed a 25% decrease in dystrophin expression).

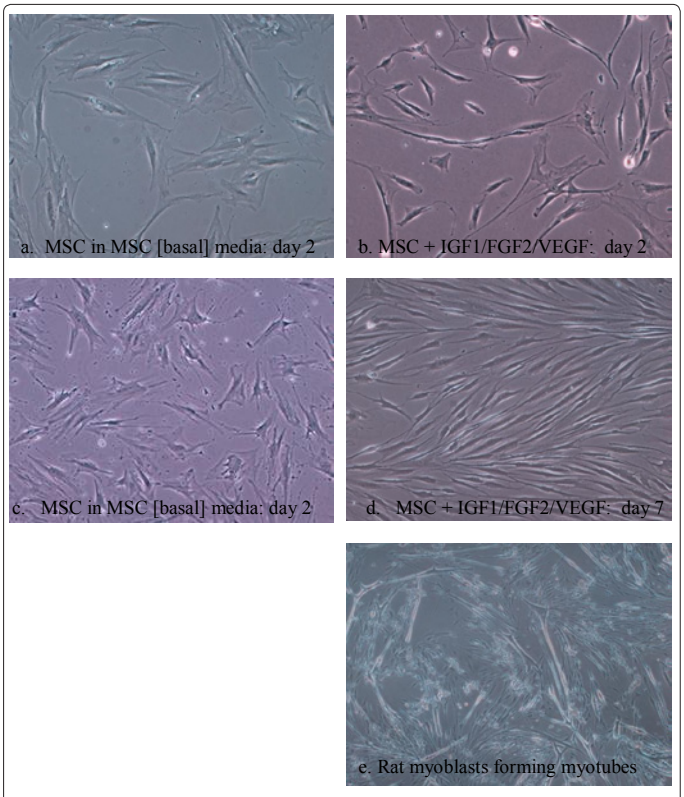


Figure 1: Morphological characteristics of human adult MSCs cultured in MSC media (a&c) & MSC media supplemented with combined IGF1, FGF2 & VEGF (b&d); (e) rat myoblasts forming myotubes.

	Collagen	MBM	MBC	SkMM	SkMC \	VEGF	I/F/V	FGF6
Desmin	↑↑	↓	↓	↓				↓
Dystrophin		↓	↓	↑↑	↑	↑	↑	↓
SERCA1	↑	↓	↓		↓	↓	↑↑	↓
AdipoR1			↓	↓	↓	↑	↑↑	
α-actinin	↑	↑	↓	↑	↑↑		↑	

MBM: Myoblast media; MBC: Myoblast-conditioned media; SkMM: Skeletal muscle media; SkMC: Skeletal muscle-conditioned media; I/F/V: IGF1/FGF2/VEGF

Table 1: Influence of culture conditions on skeletal muscle marker transcript expression by human adult MSCs.

Conditioned media: MSCs cultured in skeletal muscle media showed a marked increase in mean dystrophin transcript expression (> two-fold; Figure 2) and a two-fold increase in alpha-actinin expression. A small increase in mean SERCA1 expression was also seen (though this varied markedly between marrow samples). However, mean levels of desmin and adiponectin-1 expression consistently fell slightly.

Skeletal muscle-conditioned media resulted in increased dystrophin and α-actinin expression but not increased expression of other markers assessed. Dystrophin transcript expression was >80% greater in MSCs cultured in skeletal muscle-conditioned media compared with those in MSC media, but was less than for MSCs cultured in basal (non-conditioned) skeletal muscle media.

Culturing human MSCs in myoblast media resulted in reduced expression of dystrophin and desmin. Expression of α-actinin was increased in all four marrow samples, with a mean increase of 40% in MSCs cultured in myoblast media compared with those cultured in MSC media. The effect on adiponectin expression was less clear - modestly reduced in three marrow samples and increased in one (Figure 2).

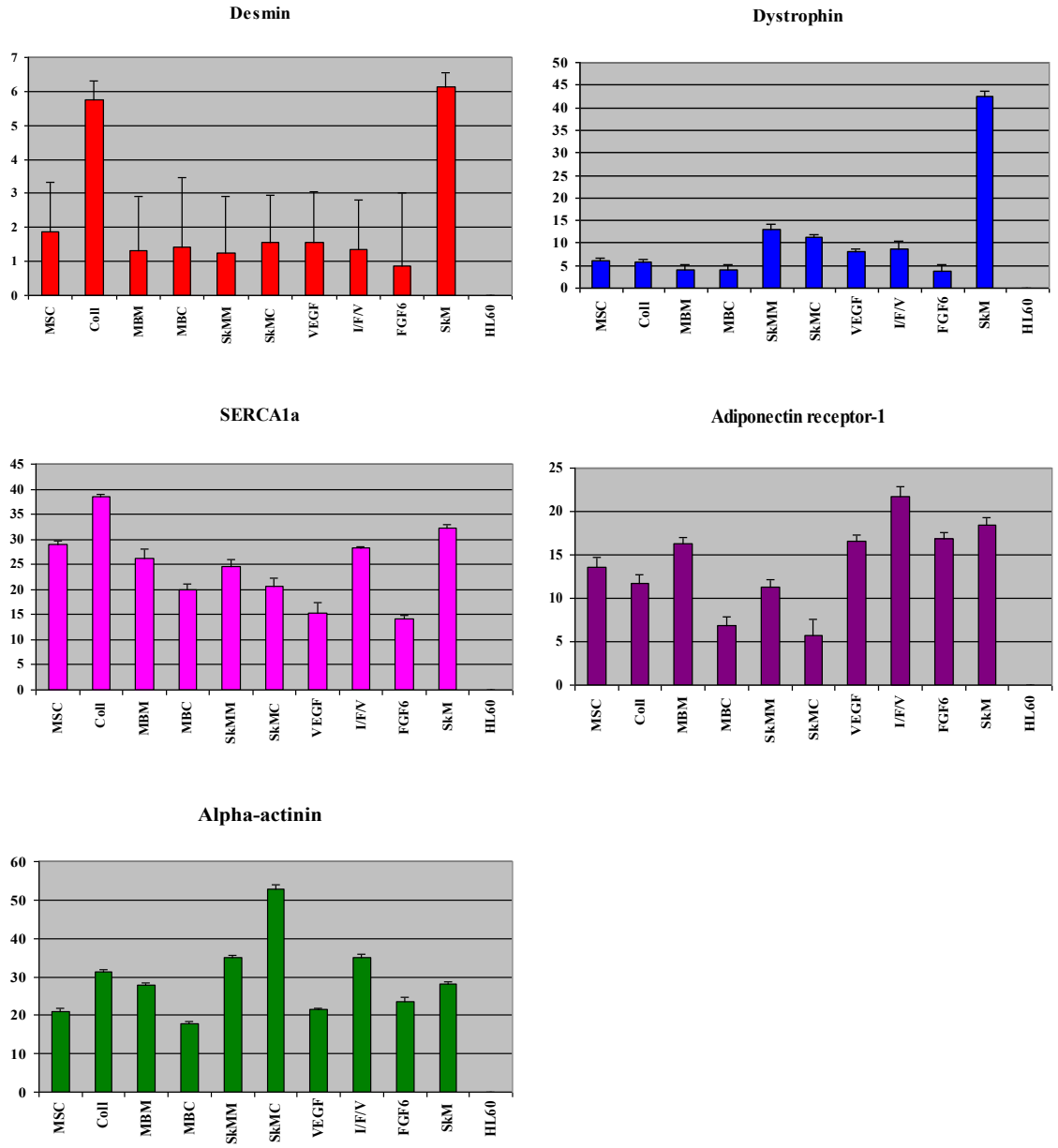
MSCs cultured in myoblast-conditioned media showed reduced expression of all muscle markers compared with MSCs maintained in MSC media.

Growth factors: *VEGF* had variable effects (Figure 2). There were no consistent changes in the expression of desmin or α-actinin. Dystrophin and adiponectin receptor 1 transcript expression were increased by 40% and 15% respectively; SERCA1 expression decreased by 25%.

FGF6 decreased expression of desmin, dystrophin and SERCA1 expression in all four marrow samples by means of 53%, 37% and 55% respectively (Figure 2). Three marrow samples showed decreased expression of adiponectin receptor 1 and α-actinin when cultured with FGF6.

Finally, MSCs were exposed to a combination of *VEGF+FGF2+IGF1*. This cocktail consistently increased muscle transcript expression (Figure 2). Dystrophin, adiponectin receptor 1 and α-actinin expression were increased by 43%, 57% and 62% respectively. SERCA1 expression was not consistently changed, and there was very little influence on desmin transcript expression.

In control experiments, the human lymphoblast cell line HL60 did not express any of the skeletal muscle markers as assessed by real-



MSC: Human BMDMSCs in MSC media; Coll: Collagen; MBM: Myoblast media; MBC: Myoblast-conditioned media; SkMM: Skeletal muscle media; SkMC: Skeletal muscle-conditioned media; VEGF: MSC media with VEGF; I/F/V: MSC media with IGF1, FGF2, VEGF; FGF6: MSC media with FGF6.

Figure 2: Skeletal muscle transcript expression by human BMDMSCs cultured in potential differentiating culture conditions & by human skeletal muscle cells & human lymphoblasts.

time PCR, and these cells were not induced to express these markers when cultured in the same range of culture conditions that to which we exposed MSCs. HL60 cells expressed similar levels of 18S to human MSCs, human skeletal muscle cells and rat myoblasts.

Effects of co-culture on morphology and behaviour of human MSCs

We also studied the effects on MSC behaviour and morphology of direct and indirect co-culture (using a filter membrane to separate cell types).

Indirect co-culture of MSCs with rat myoblasts or with human skeletal muscle cells had no effect on either MSC morphology or immunocytochemical staining patterns for muscle markers.

However, when human MSCs (GFP-labelled for identification) were directly co-cultured with rat myoblasts, the latter appeared over a period of days to migrate towards, cluster around and adhere to human MSCs. Indeed there appeared to be some fusion of rat myoblasts with MSCs to form multi-nucleated myofibres which expressed mature skeletal muscle markers, including actin, desmin and dystrophin (Figures 3 & 4) – by 5 days, very few MSCs in close proximity to rat

myoblasts appeared separate. We did not have the opportunity to confirm cell fusion using confocal or (preferably) electron microscopy, but this will be examined in further studies. Adhesion (or fusion) between MSCs and rat myoblasts occurred at the cell body and poles of MSCs. Occasionally, myoblasts derived from several individual clones were seen simultaneously to adhere to or fuse with a single MSC (Figure 3d).

By contrast, GFP-labelled human MSCs did not adhere to or fuse with each other or with skeletal muscle cells when cultured directly with normal skeletal muscle cells, or with skeletal muscle cells exposed to an inflammatory insult in the form of TNFα (Figure 3e).

Discussion

We explored skeletal muscle marker expression by human adult MSCs in a range of culture conditions. Various environmental changes lead to increased expression of skeletal muscle marker transcripts – culture on a collagen substrate; using skeletal muscle media (containing EGF, insulin, dexamethasone, BSA, bovine fetuin and gentamicin); or adding combined IGF1, FGF2, and VEGF. This is the first quantitative study to consider the effect of culture conditions on skeletal muscle transcript expression by human MSCs using real-time PCR. Additionally, direct co-culture with rat myoblasts led to MSC adhesion to or possibly fusion with myoblasts to form multi-nucleated myotube-like structures which expressed mature skeletal muscle markers, including actin, desmin and dystrophin. We also observed apparent migration of myoblasts towards MSCs.

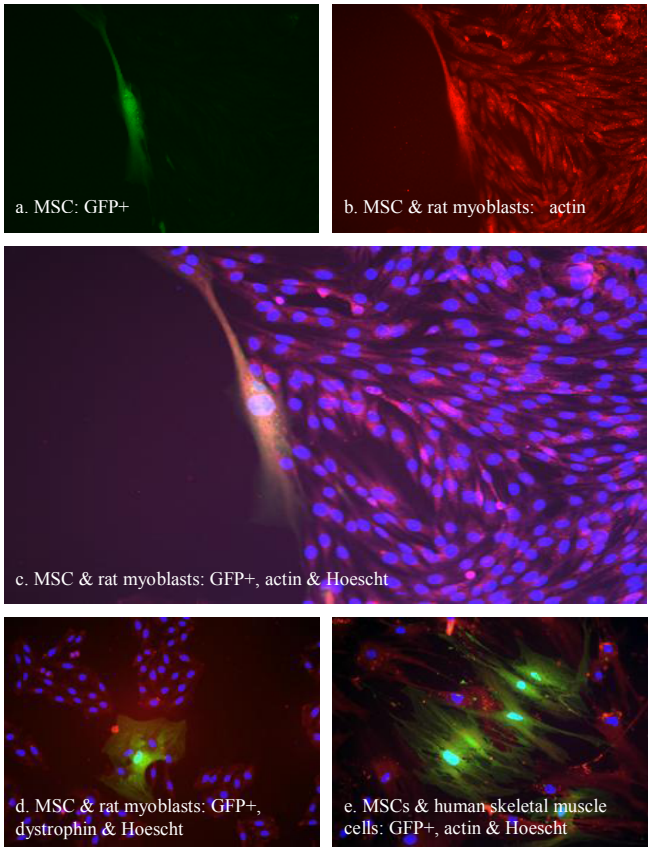


Figure 3: Direct co-culture of GFP-labelled human adult MSCs with rat myoblasts, demonstrating adhesion and possible fusion (a-d); & GPF-labelled human adult MSCs with human skeletal muscle cells (e. no evidence of fusion).

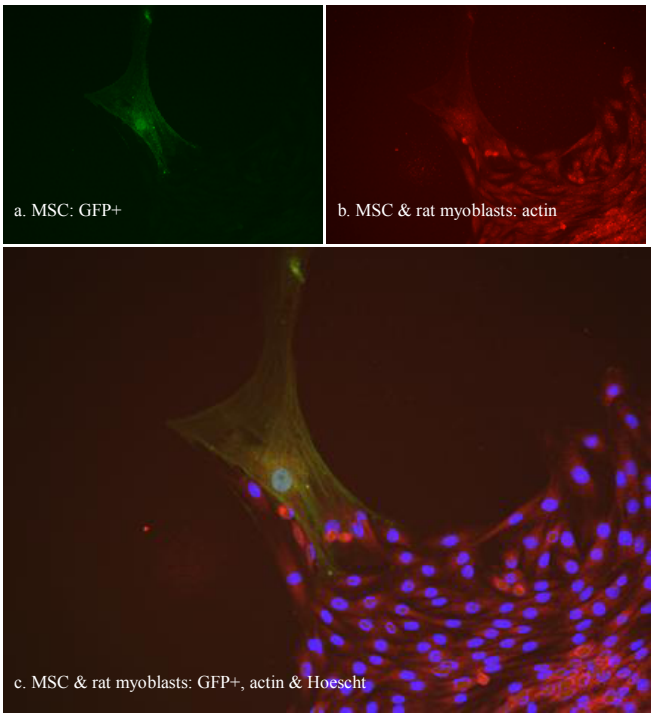


Figure 4: Direct co-culture of GFP-labelled human adult MSCs & rat myoblasts suggesting migration of myoblasts towards the MSC, and apparent fusion.

Culturing hMSCs on a collagen substrate caused a dramatic (over three-fold) increase in desmin transcript expression, and significant increases in alpha-actinin and SERCA1. Collagen may exert its influence by interacting with growth factors: acting as a growth factor reservoir, as is the case for FGF2 [19]; stimulating growth factor production by MSCs; or influencing MSC responses to growth factors.

MSCs cultured in skeletal muscle-conditioned media showed a doubling of dystrophin and of alpha-actinin transcript expression. One previous study assessed the influence of (murine) skeletal muscle-conditioned media (prepared with and without muscle injury) on myogenic differentiation of rat MSCs [20]. In this study, exposing rat MSCs to media conditioned with damaged skeletal muscle increased the number of cells expressing muscle markers, and levels of marker expression; it also precipitated formation of multi-nucleated, muscle marker expressing structures that spontaneously twitched in culture. Media conditioned with undamaged skeletal muscle induced no such changes, but this experimental paradigm is clearly very different from, and difficult to compare with, that which we report.

MSC media supplemented with combined VEGF, FGF2 and IGF1 caused striking and consistent increases in mature skeletal muscle transcript expression by MSCs. Morphological changes were also observed in MSCs in these conditions, cells adopting a uniform elongated, bipolar morphology and forming bands of cells across the flask – morphology similar to myotubes formed by fusion of myoblasts. This combination (and concentration) of growth factors was used on the basis of previously reported success in one study precipitating myogenic differentiation of another population of human bone

marrow derived stem cells, *multi-potent adult progenitor cells* (MAPCs) [8]. In addition to changes in gene expression and morphology, this study also reported enhanced MAPC incorporation into skeletal muscle following transplantation in mice. We and others have found difficulty in establishing human MAPC cultures; and we are not aware of any other studies on human MSC populations of this growth factor combination. Our results may be of promise in helping to define growth factor combinations of therapeutic potential in promoting myogenic differentiation of MSCs.

Real-time PCR is a highly sensitive means of detecting and quantifying changes in transcript expression. It has been employed in only one other study of human MSC differentiation – showing that 5-azacytidine increased desmin transcript [21].

Interestingly, our immunocytochemical studies revealed no changes in muscle-related antigen expression by MSCs, as assessed by immunofluorescence staining, in the same range of culture conditions. One interpretation might be that the environmental stimuli we tested can, in cell culture conditions, only partially stimulate myogenic differentiation pathways: other signals are needed to activate and coordinate the full necessary spectrum of translational and other pathways. Further research is required to determine the functional significance of the transcript changes we found, particularly in relation to the immunocytochemical data, and also to explore gene transcript expression alterations *in vivo* after transplantation. Real-time PCR is likely to represent an important component of the armamentarium needed to understand therapeutically beneficial myogenic differentiation.

Our observations provide further evidence of the myogenic potential of human MSCs, showing that expression of skeletal muscle genes can be enhanced in culture without the introduction of mitogens (such as 5-azacytidine). MSCs are an increasingly credible candidate as a cell therapy for patients with muscle disease.

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References

1. Bhagavati S (2008) Stem cell based therapy for skeletal muscle diseases. Curr Stem Cell Res Ther 3: 219-228.
2. Rice CM, Scolding NJ (2008) Autologous bone marrow stem cells--properties and advantages. J Neurol Sci 15: 59-62.
3. Bittner RE, Schöfer C, Weipoltshammer K, Ivanova S, Streubel B, et al. (1999) Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. Anat Embryol (Berl) 199: 391-396.
4. Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, et al. (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279: 1528-1530.
5. Sherwood RI, Christensen JL, Weissman IL, Wagers AJ (2004) Determinants of skeletal muscle contributions from circulating cells, bone marrow cells, and hematopoietic stem cells. Stem Cells 22: 1292-1304.
6. Gussoni E, Bennett RR, Muskiewicz KR, Meyerrose T, Nolta JA, et al. (2002) Long-term persistence of donor nuclei in a Duchenne muscular dystrophy patient receiving bone marrow transplantation. J Clin Invest 110: 807-814.
7. Chargé SB, Rudnicki MA (2004) Cellular and molecular regulation of muscle regeneration. Physiol Rev 84: 209-238.
8. Muguruma Y, Reyes M, Nakamura Y, Sato T, Matsuzawa H, et al. (2003) *In vivo* and *in vitro* differentiation of myocytes from human bone marrow-derived multipotent progenitor cells. Exp Hematol 31: 1323-1330.
9. Alessandri G, Pagano S, Bez A, Benetti A, Pozzi S, et al. (2004) Isolation and culture of human muscle-derived stem cells able to differentiate into myogenic and neurogenic cell lineages. Lancet 364: 1872-1883.
10. Foulstone EJ, Huser C, Crown AL, Holly JM, Stewart CE (2004) Differential signalling mechanisms predisposing primary human skeletal muscle cells to altered proliferation and differentiation: roles of IGF-I and TNFalpha. Exp Cell Res 294: 223-235.
11. Yaffe D (1968) Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc Natl Acad Sci USA 61: 477-483.
12. Gordon D, Scolding NJ (2009) Human mesenchymal stem cell culture for neural transplantation. Methods Mol Biol 549: 103-118.
13. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, et al. (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147.
14. Gordon D, Pavlovska G, Uney JB, Wraith DC, Scolding NJ (2010) Human mesenchymal stem cells infiltrate the spinal cord, reduce demyelination, and localize to white matter lesions in experimental autoimmune encephalomyelitis. J Neuropathol Exp Neurol 69: 1087-1095.
15. Bossolasco P, Corti S, Strazzer S, Borsotti C, Del Bo R, et al. (2004) Skeletal muscle differentiation potential of human adult bone marrow cells. Exp Cell Res 295: 66-78.
16. Gordon D, Pavlovska G, Glover CP, Uney JB, Wraith D, et al. (2008) Human mesenchymal stem cells abrogate experimental allergic encephalomyelitis after intraperitoneal injection, and with sparse CNS infiltration. Neurosci Lett 448: 71-73.
17. Glover CP, Bienemann AS, Heywood DJ, Cosgrave AS, Uney JB (2002) Adenoviral-mediated, high-level, cell-specific transgene expression: a SYN1-WPRE cassette mediates increased transgene expression with no loss of neuron specificity. Mol Ther 5: 509-516.
18. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
19. Kanematsu A, Yamamoto S, Ozeki M, Noguchi T, Kanatani I, et al. (2004) Collagenous matrices as release carriers of exogenous growth factors. Biomaterials 25: 4513-4520.
20. Santa María L, Rojas CV, Minguell JJ (2004) Signals from damaged but not undamaged skeletal muscle induce myogenic differentiation of rat bone-marrow-derived mesenchymal stem cells. Exp Cell Res 300: 418-426.
21. Xu W, Zhang X, Qian H, Zhu W, Sun X, et al. (2004) Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype *in vitro*. Exp Biol Med (Maywood) 229: 623-631.

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