Differentiation of Human Mesenchymal Stem/Stromal Cells into Myogenic Cells for Urethral Sphincter Muscle Engineering

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Abstract

Stress urinary incontinence (SUI) is a medical condition that requires novel alternative therapies aiming to restore and maintain the integrity and function of the urethral sphincter. This study aimed to develop approaches targeting the myogenic differentiation of Mesenchymal Stem/Stromal Cells (MSCs) for urethral sphincter engineering. To this end, the effects of chemical differentiation inducers 5-aza-2'-deoxycytidine (5-AZAd) and PD98059 were tested. MSC differentiation was assessed by the detection of skeletal and smooth muscle lineage-specific markers by flow cytometry and immunofluorescence techniques. Additionally, the myogenic potential of magnetically sorted and unsorted cells from the stromal vascular fraction (SVF), coated on distinct substrates, was investigated.

The results indicate that myogenesis-committed cells - Pax7-expressing cells - exist in uncultured SVF, although no myoblast-like cells were isolated. Still, SVF-derived cells were shown to possess intrinsic myogenic potential that was enhanced when combined with culture substrates (in particular, gelatin coating), thus holding great potential for skeletal muscle engineering applications. Sorted cells in the presence of gelatin coating expressed 34% skeletal myosin heavy chain (MHC) after 8 days in culture with DMEM+2% Horse Serum (HS). Conversely, 5-AZAd supplementation failed to induce myogenesis and triggered severe cytotoxic effects, while PD98059 did not provide enough stimuli to sustain smooth muscle differentiation, which likely requires the use of 3-D culture conditions and/or biomechanical stimulation.

Keywords: Stress Urinary Incontinence; Mesenchymal Stem/Stromal Cells; Stromal Vascular Fraction; Myogenic Differentiation; Smooth Muscle Cells; Skeletal Muscle Cells

Introduction

Urinary incontinence (UI) affects more than 200 million people worldwide.\textsuperscript{1,2} SUI is the most prevalent type of UI, and it is defined as the complaint of involuntary leakage of urine from the urethra on effort or exertion, or on sneezing or coughing.\textsuperscript{1} The urethra plays a key role in the continence mechanism, which is allowed by the action of a circular striated muscle layer that surrounds the urethral smooth muscle, named rhabdosphincter or external urethral sphincter.\textsuperscript{3}

Efficient treatments are currently lacking for this medical condition and alternative therapeutic approaches are needed, namely cellular therapy and tissue engineering strategies using stem/progenitor cells and/or their derived progeny to rescue tissue function. Skeletal progenitors have been studied therapeutically but with no success, as both satellite cells and myoblasts lose their expansion capacity and myogenic potential when cultured in vitro.\textsuperscript{4,5} In this context, MSCs represent an alternative cell source for skeletal muscle engineering approaches.

MSCs are multipotent adult stem cells that belong to the mesodermal lineage and that can give rise to several cell lineages including osteocytes, chondrocytes, adipocytes and muscle cells.\textsuperscript{5,7} MSCs can be isolated from several tissues such as bone marrow (BM), adipose tissue (AT), peripheral blood, cord blood, cord Wharton’s jelly, amniotic fluid, compact bone, peristeum, synovial membrane and synovial fluid, articular cartilage and fetal tissues.\textsuperscript{8} Moreover, MSC expansion in vitro
allows clinically relevant cell numbers to be obtained.\textsuperscript{9}

MSCs have been successfully differentiated into skeletal muscle- and smooth muscle-like cells in vitro. Myogenic induction with the demethylation agent 5-azacytidine (5-AZA) or its deoxy analogue 5-AZAd is one of the most explored strategies. In 1995, Wakitani and colleagues demonstrated that immortalized rat BM-MSCs exposed to 5-AZAd differentiated into myogenic phenotypes.\textsuperscript{7} Since then, several research groups have claimed that 5-AZA-treated MSCs were able to differentiate into skeletal muscle-like cells\textsuperscript{10–12}, cardiomyocyte-like cells\textsuperscript{13} and osteocytes\textsuperscript{14}. Still, the in vitro data regarding the effect of 5-AZA on MSCs are contradictory, and the mechanism through which 5-AZA inflicts cell differentiation is currently unknown.

Importantly, cells from the SVF of the AT are believed to possess intrinsic myogenic potential, without requiring chemical induction with compounds such as 5-AZA. Cells derived from the SVF cultured in DMEM supplemented with 5% HS were reported to spontaneously differentiate into skeletal muscle cells without any external stimuli.\textsuperscript{15} However, these myogenesis-committed cells only account for 0.001% of total plated cells.\textsuperscript{15} Conversely, DMEM supplementation with HS, hydrocortisone and dexamethasone appears to be sufficient to induce SVF-derived cells to express skeletal MHC and fuse at average frequencies around 12\% and 15\%, without any kind of cell separation methodology.\textsuperscript{16,17}

MSC smooth muscle induction strategies often include TGF-β supplementation, a molecule known to play an important part in embryonic smooth muscle differentiation.\textsuperscript{18–20} Additionally, treatment of BM-MSCs with ERK/MEK pathway inhibitor PD98059 in low serum conditions was proven to be a successful approach to induce smooth muscle marker expression.\textsuperscript{21}

In this study, we sought to establish myogenic differentiation protocols for MSCs, with particular focus on exploring the myogenic potential of the SVF.

Materials and Methods

Human-derived samples

AT samples (lipoaspirates) were obtained following informed donor consent, from the abdomen and thigh areas of both female and male healthy donors with ages ranging from 30 to 52 years old, at Clínica de Todos-os-Santos, Lisbon, Portugal. A BM sample was obtained following informed donor consent from a 36-year-old male healthy donor at Instituto Português de Oncologia, Lisbon, Portugal. SVF cells were isolated by an enzymatically-based method and cryopreserved.\textsuperscript{22}

Ex-vivo expansion of ADSCs, BM-MSCs and SVF-derived cells

Cryopreserved SVF cells were recovered by dilution in a proportion of 1:5 in DMEM+20\%FBS supplemented with DNase (0.01mg/mL, Roche Applied Science) while ADSCs and BM MSCs were recovered in DMEM+20\%FBS. All cells were maintained in culture using low glucose (LG) (1g/L) Dulbecco’s modified Eagle’s Medium (DMEM), supplemented with 10\% fetal bovine serum (FBS, Gibco®) and antibiotics (0.025µg/mL penicillin and 0.025U/mL streptomycin). The FBS used to culture ADSCs and BM-MSCs was MSC qualified. The connotation “AT-SVF cells” is referred to cells derived from AT that were isolated and cultured in standard FBS. The medium was replaced every 3 to 4 days. Cell expansion was performed in static culture systems (T-flasks, BD Falcon™ Biosciences®) in an incubator at 37°C with a humidified atmosphere of 5\% CO\textsubscript{2}.

Immunophenotype characterization

Once isolated, ADSCs, BM-MSCs and AT-SVF cells were characterized immunophenotypically according to a panel of extracellular markers.\textsuperscript{23} SVF-derived cells were also tested for satellite cell markers (CD34 and CD56). The cells were treated with 0.05\% trypsin (Gibco®), centrifuged for 7 min at 1250 rpm and resuspended in PBS. The cells were distributed into FACS tubes (BD Falcon™) and incubated for 15 min at RT in the dark with the following mouse anti-human monoclonal antibodies (1:10 dilution).
reached confluence, they were harvested with 0.05% trypsin and the cells were then centrifuged for 5 min at 1500 rpm and resuspended in 1% PFA. Quantitative analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and with FlowJo software (Version 8.8.6, Tree Star Inc., Ashland, Oregon, USA). MFI values were calculated by dividing the geometrical mean fluorescence values of the sample by the respective isotype.  

**Control groups were incubated with FITC-/PE-conjugated mouse IgG1 isotype antibodies (1:10 dilution).** After incubation, 2 mL of PBS was added to each FACS tube, and the cells were then centrifuged for 5 min at 1500 rpm and resuspended in 1% PFA. The effects of gelatin (0.2%, Sigma®), according to the manufacturer’s instructions.  

**Effect of 5-aza-2'-deoxycytidine on cell apoptosis**  
ADSCs were plated at a cell density of 5000 cells/cm² on T75 flasks and 12-well plates (BD Falcon®) in DMEM+10%FBS. Myogenic induction was performed by cell exposure to 2 or 10 µM of 5-azaAd (Sigma®) in DMEM+2%HS (Sigma®). To quantify the percentage of apoptotic and necrotic cells after 2, 7 and 14 days of 5-azaAd exposure, the cells were stained with FITC-conjugated anti-Annexin V and PE-conjugated anti-Pi antibodies (Kit from Life Technologies®). The cells were analysed by flow cytometry within 1 h.  

**CD34⁺ SVF-derived cell sorting and myogenesis induction**  
Human SVF-derived CD34⁺ cells were isolated using CD34 MicroBeads (kit from Miltenyi Biotec®), according to the manufacturer’s instructions. Crude SVF cells and magnetically sorted cells from the CD34-enriched and CD34-depleted fractions were analysed by flow cytometry and plated on T25 flasks at 10000 cells/cm² in myogenic expansion medium, composed by DMEM+ 20%FBS + 10⁻⁶M dexamethasone (Sigma®) + 2.5 ng/mL bFGF (Sigma®). The cells were incubated with the respective secondary antibody (both from Zen-Bio, Inc.). The expression of smooth muscle markers α-SMA, calponin, SM-MHC and desmin (when stated) was evaluated by immunofluorescence after 21 days of culture in P6 SMCs.

<table>
<thead>
<tr>
<th>Surface Markers</th>
<th>Brand</th>
<th>Conjugated Fluorophore</th>
<th>Isotype</th>
</tr>
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<tbody>
<tr>
<td>CD14</td>
<td>BioLegend©</td>
<td>PE</td>
<td>IgG1</td>
</tr>
<tr>
<td>CD31</td>
<td>BioLegend©</td>
<td>PE</td>
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</tr>
<tr>
<td>CD34</td>
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<td>IgG1</td>
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<tr>
<td>CD45</td>
<td>BioLegend©</td>
<td>PE</td>
<td>IgG1</td>
</tr>
<tr>
<td>CD56</td>
<td>BD Biosciences©</td>
<td>PE</td>
<td>IgG1</td>
</tr>
<tr>
<td>CD73</td>
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<td>IgG1</td>
</tr>
<tr>
<td>CD90</td>
<td>BioLegend©</td>
<td>FITC</td>
<td>IgG1</td>
</tr>
<tr>
<td>CD105</td>
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<td>PE</td>
<td>IgG1</td>
</tr>
<tr>
<td>HLADR</td>
<td>BioLegend©</td>
<td>PE</td>
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<td></td>
</tr>
<tr>
<td>IgG1/IgG1</td>
<td>BD Biosciences©</td>
<td>FITC/PE</td>
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**Smooth muscle differentiation induction**  
After isolating and expanding AT-SVF cells and BM-MSCs in DMEM+10%FBS (normal and MSC-qualified FBS, respectively), the cells were plated at 5000 cells/cm² on T25 flasks and 12-well plates in the same conditions. When confluence was reached, the cells were exposed to smooth muscle induction medium, composed by DMEM (low glucose) supplemented with 3%FBS (standard FBS) and 1, 10 and 30 µM of PD98059 (25 mM stock solution in DMSO, Merck Millipore®) for 7 days. Cells plated in DMEM+3%FBS, DMEM+10%FBS and DMEM-HG+10%FBS alone were tested as control groups. The expression of smooth muscle markers α-SMA, calponin, SM-MHC and desmin (when stated) was evaluated after 2, 4 and 7 days of medium replacement by intracellular flow cytometry and immunofluorescence. Bladder smooth muscle cells (SMCs) plated in bladder SMC growth medium were used as positive control (both from Zen-Bio, Inc.). The expression of these markers was evaluated by immunofluorescence after 21 days of culture in P6 SMCs.

**Immunofluorescence staining**  
Cells cultured on 12-well plates were washed with PBS and fixed with 4% PFA for 20-30 minutes at RT, followed by rehydration with PBS. Cell permeabilization was performed with Blocking Solution (10% NGS+ 0.1%Triton X100 (Gibco®)+PBS) for 15 min at RT. The cells were washed with PBS and incubated with the primary antibody in Staining Solution (5%NGS+0.1%Triton X100+PBS) at a concentration of 1µL/mL for 2 h at RT. After being washed with PBS, the cells were incubated with the respective secondary antibody (1:500 dilution) for 1 h at RT (Table 2). The cells were washed and incubated in a 1.5 µL/mL solution of DAPI (Sigma®) in PBS for 2 min. The stained cells were washed with PBS and viewed with a CK40 Microscope equipped with DAPI, TRITC and FITC filters. Images were edited with Image-J (Version 1.48, NIH, Maryland, USA).
Intracellular staining for flow cytometry

Intracellular staining was performed in order to assess the expression of skeletal muscle markers (Pax7, MyoD, myogenin and skeletal MHC), smooth muscle markers (α-SMA, calponin and SM-MHC) and desmin. Cells cultured on T25 flasks were harvested with 0.05% trypsin for 7 min, centrifuged for 7 minutes at 1250 rpm and resuspended in PBS. The cells were permeabilized and fixed in fixed FACS tubes using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences©) according to manufacturer’s instructions. After the fixation/permeabilization steps, the cells were incubated with a primary monoclonal antibody, washed with PBS and permeabilization buffer, and then labelled with the respective secondary antibody.

Results and Discussion

Cell isolation and characterization

Lipoaspirates removed from healthy donors were processed and SVF cells were isolated according to the enzymatic isolation protocol from Shah and co-workers. The SVF cells were cryopreserved in vials and were subsequently used for the isolation of MSCs (when the plating medium included MSC-qualified FBS) or AT-SVF cells (when standard FBS was used). A bone marrow sample removed from a 36-year-old male healthy donor was isolated in DMEM+10%FBS, in order to obtain a BM-MSC population. The culture medium was changed every 3 to 4 days, allowing the non-adherent cells to be removed, until 80-90% confluence was reached. After reaching confluence, the cells isolated from the SVF and bone marrow were harvested and characterized immunophenotypically using the surface antigens indicated by Bourin and co-workers and Dominici and colleagues. The flow cytometry results suggested that the isolated populations expressed MSC markers at proportions indicated by the ISCT that allow MSC classification (Table 3).

The isolated ADSCs, AT-SVF cells and BM-MSCs were characterized in terms of multilineage differentiation ability, i.e. the ability of MSCs to differentiate into adipocytes, chondrocytes and osteocytes under specific in

Table 2. Panel of anti-human primary monoclonal antibodies and respective fluorophore-conjugated secondary antibodies used to stain cells for markers relevant for myogenic differentiation assessment, dilution used, commercial brand and isotype.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Brand</th>
<th>Secondary Antibody</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
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<td>Pax7</td>
<td>1:100</td>
<td>SCBT®</td>
<td>Goat anti-mouse FITC</td>
<td>IgG2a</td>
<td>1:500</td>
<td>Abcam®</td>
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<td>MyoD</td>
<td>1:100</td>
<td>Abcam®</td>
<td>Goat anti-rabbit Alexa 488/PE</td>
<td>IgG</td>
<td>1:500</td>
<td>Invitrogen™</td>
</tr>
<tr>
<td>Myogenin</td>
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<td>BD Biosciences©</td>
<td>Goat anti-mouse Alexa 488/PE</td>
<td>IgG</td>
<td>1:500</td>
<td>Invitrogen™</td>
</tr>
<tr>
<td>α-SMA</td>
<td>1:40</td>
<td>SCBT®</td>
<td>Goat anti-mouse Alexa 488/PE</td>
<td>IgG</td>
<td>1:500</td>
<td>Invitrogen™</td>
</tr>
<tr>
<td>Calponin</td>
<td>1:100</td>
<td>Abcam®</td>
<td>Goat anti-mouse Alexa 488/PE</td>
<td>IgG</td>
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<td>Desmin</td>
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<td>Abcam®</td>
<td>Goat anti-rabbit Alexa 488/546</td>
<td>IgG</td>
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<td>Invitrogen™</td>
</tr>
<tr>
<td>SM-MHC</td>
<td>1:40</td>
<td>SCBT®</td>
<td>Goat anti-mouse Alexa 488/PE</td>
<td>IgG</td>
<td>1:500</td>
<td>Invitrogen™</td>
</tr>
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Table 3. Expression of the specific markers used in the identification of SVF-derived cells and MSCs, according to Bourin et al. and Dominici et al., by several P0 SFV-derived plate-adherent populations cultured in distinct media, and by P0 BM-MSCs. For AT-SVF cells in expansion medium n=3 (except CD14 and HLA-DR, for which n=1). FBS*= MSC-qualified FBS

<table>
<thead>
<tr>
<th>Expression (%)</th>
<th>ADSCs</th>
<th>AT-SVF cells</th>
<th>AT-SVF cells</th>
<th>AT-SVF cells</th>
<th>BM-MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMEM+10%FBS*</td>
<td>Expansion Medium</td>
<td>DMEM+3%FBS 15 day culture</td>
<td>DMEM+3%FBS 25 day culture</td>
<td>DMEM+10%FBS*</td>
</tr>
</tbody>
</table>

CD14 | 5.5 | 0 | 11 | - | - | 24 |
CD31 | - | - | 0.10±0.2 | 0.7 | 0 | - |
CD34 | 3.8 | 3.6±2.1 | 32 | 20 | 1.0 |
CD45 | 0.9 | 0±0 | 0.50 | 0 | 2.0 |
CD73 | 99 | 99±0.4 | - | 98 | 99 |
CD90 | 99 | 97±2.3 | 97 | 99 | 98 |
CD105 | 99 | 98±0.5 | - | 93 | 99 |
HLA-DR | 0.30 | 0 | - | - | 0 | 0 |
vitro tissue culture differentiating conditions, and were confirmed to possess trilineage differentiation potential (data not shown).

5-aza-2’-deoxycytidine effects on cell viability, morphology and phenotype

5-AZA and its deoxy analogue, 5-AZAd, are described in the literature as myogenic inducers in MSCs; however, these compounds indirectly induce alterations in the chromatin conformation of DNA, potentially leading to mutations or toxicity. A cell viability assay was thus performed to assess if 5-AZAd at concentrations used in the literature evoke cytotoxic effects. ADSCs were plated on 12-well plates, exposed to 2 and 10 µM of 5-AZAd for 24 h and subjected to a propidium iodide (PI)/Annexin V flow cytometry analysis. No 5-AZAd was added to control cells. After day 2 of 5-AZAd treatment (24 h in the presence plus 24 h in the absence of the agent) the cells exposed to 10 µM were necrotic (92.0%), while most of the cells exposed to 2 µM were viable (89%), as well as the control cells (94%) – Figure 1A.

By contrast, the 50% inhibitory concentration of 5-AZA (less toxic analogue of cytidine) after 24 h of induction was reported to be around 40 µM, with little decrease in viability at concentrations below 10 µM.  

ADSCs treated with 10 µM of 5-AZAd developed extremely thin and elongated morphologies, as opposed to control ADSCs, which maintained their fibroblastic shape. It has been described that 5-AZA-treated BM-MSCs show more heterogeneous morphologies and alterations in size and growth properties. 5-AZAd at 10 µM was confirmed to induce morphology alterations by staining with Phalloidin-TRITC and DAPI (data not shown).

Finally, by assessing the expression of surface markers through flow cytometry at days 2, 7 and 14 after 5-AZAd treatment (Figure 1B), it was concluded that 5-AZAd did not induce myogenesis, as no CD56 expression was detected (neural adhesion molecule, expressed in skeletal muscle). These results indicate that after 48 h of 10 µM 5-AZAd treatment, severe cytotoxic effects and cell morphology alterations were induced, and that the cells did not undergo myogenesis. Based on these results, it is reasonable to conclude that research in the field of muscle engineering using MSCs as a source of muscle-like cells should focus on the establishment of novel protocols adequate for clinical applications.

Myogenic induction of CD34+ cells from the Stromal Vascular Fraction

In order to assess if myogenesis-committed cells exist in the human white AT, intracellular flow cytometry staining for Pax7 was performed in a fresh SVF sample, for the purpose of identifying Pax7-expressing cells. The flow cytometry data indicated that approximately 1.05x10^5 cells, in a total of 49.6x10^5, were Pax7+.

Figure 1. Alterations in cell viability and marker expression of ADSCs cultured in DMEM+2%HS after 5-AZAd treatment. (A) Effect of 5-AZAd on cell viability. PI/Annexin V flow cytometry analysis for at days 2, 7 and 14 after treatment. The expression of annexin and PI served as a measure of cell viability, apoptosis and necrosis. (B) 5-AZAd effect on surface marker expression after 2, 7 and 14 days of treatment.
Hence, MACS for CD34 (expressed in quiescent and activated satellite cells) was performed in crude SVF samples with the aim of developing a strategy to isolate these cells. Cells from the CD34-enriched and CD34-depleted fractions were plated in the following conditions: a) non-coated dishes, b) gelatin-coated dishes, c) fibronectin-coated dishes and d) fibronectin-coated dishes in the presence of DMEM-HG medium, to test if increased glucose concentrations promote cell differentiation. Unsorted SVF cells were plated onto non-coated dishes. An expansion step was performed in the presence of myogenic expansion medium (DMEM supplemented with FBS, dexamethasone and bFGF) and the levels of CD34 and CD56 were determined after the first cell passage. No cells derived from the CD34-depleted fraction adhered to tissue culture plates after 7 days in culture, even in the presence of a gelatin or a fibronectin coating, so the cells were discarded. Plated cells from the CD34-enriched fraction displayed similar sizes and morphologies when compared to unsorted SVF cells.

Based on both these observations, it was hypothesized that only the CD34+ cells within the unsorted SVF were able to adhere and proliferate in culture. This hypothesis was confirmed through a marker decay assay (data not shown). It was concluded that in future studies, CD56 should be the chosen marker for sorting myogenic cells from the SVF, as AT-derived MSCs do not express this marker.

The cells were cultured for 10 days (SVF and no coating conditions) and 11 days (coated cells) before being harvested. The results suggest that the presence of coating had great influence on CD56 expression, since cells cultured on gelatin and fibronectin-coated plates presented CD56 expression levels around 80%, while SVF (unsorted cells) and non-coated cells presented negligible levels of CD56 expression (Figure 2A). Conversely, CD34 expression was more variable between the tested conditions.

The expression of myogenic intracellular markers was assessed only in cells cultured on gelatin- and fibronectin-coated (HG) plates. Surprisingly, the cells expressed MyoD and myogenin (over 98% and 84%, respectively) before the medium replacement (Figure 2B). These results suggest that the expansion medium in which the cells were isolated might have positively influenced the cells’ commitment to the myogenic lineage. Moreover, it can be proposed that AT-SVF cells possess some myogenic potential per se and begin expressing myogenesis-related proteins in the presence of adequate biochemical stimuli (e.g. dexamethasone and bFGF). In fact, dexamethasone supplementation is known to promote myogenic differentiation in BM-MSC, leading to the formation of desmin-positive myotubes in a dose-dependent manner, and enhance the proliferation and differentiation of...
myoblasts. Concordantly, a study showed that significantly higher numbers of MSC nuclei were involved in myotube formation when bFGF and dexamethasone were added to co-cultures with myoblasts when compared to DMEM medium plus HS alone, thus suggesting a possible role of dexamethasone and bFGF as pro-myogenic factors.

Myogenic induction was performed by replacing the plating medium by DMEM-HG supplemented with 2% HS. After 2, 4 and 8 days of medium replacement, the expression of Pax7, MyoD, myogenin and skeletal MHC (only at day 8, in order to identify terminally differentiated myoblasts) was evaluated through intracellular flow cytometry and immunostaining methodologies (Figure 3).

At day 2, although MyoD expression was maintained, myogenin expression decreased drastically for cells cultured on gelatin and fibronectin-coated (HG) plates (Figure 3B), when compared to the expression values before induction. These results were confirmed by immunofluorescence, in which MyoD expression was detected in all tested conditions (Figure 3A, top), while myogenin and Pax7 were not. At day 4 after medium replacement, the results regarding marker expression were similar to day 2. However, the flow cytometry results obtained for day 8 show a strong reduction in MyoD percentage levels. A reduction in MyoD-expressing cells was also observed in the immunofluorescence results. At day 8 skeletal MHC was introduced in the panel. The results indicate that gelatin coating was the condition that promoted myogenesis the most, since almost 34% of cells expressed skeletal MHC. In accordance, phalloidin staining allowed the visualization of aligned nuclei and some fused cells (Figure 3A, bottom). Still, it is possible that important factors might have been lacking in the medium formulation used.
Common myogenic differentiation medium formulations include DMEM supplemented with insulin and transferrin, dexamethasone and FBS, apart from HS, which is usually used in ranges from 2 to 5%. Importantly, DMEM-HG medium has been reported to not only promote adipogenic differentiation in skeletal muscle and ADSCs but also inhibit myogenesis in C2C12 myoblasts, while DMEM-HG and high insulin activate myoblast differentiation. To further enhance myotube formation, specific biomaterials or substrates with different stiffnesses could be tested. ADSCs were shown to undergo stiffness-induced lineage commitment and fuse in the absence of any biochemical stimuli (albeit at low percentages – 2%) when plated in a muscle-mimicking extracellular matrix (10 kPa) surpassing BM-MSCs, which never underwent stiffness-mediated fusion.

Differentiation of MSCs into Smooth Muscle Cells using PD98059

With the aim of achieving smooth muscle differentiation of MSCs from two different sources (SVF and bone marrow), PD98059 (a compound already successfully used in another study for this purpose) was tested. PD98059 is a potent and selective inhibitor of MAPKK, a key protein in the ERK/MAPK pathway. This pathway is believed to play an important part in inhibiting the activity of SRF, a critical regulator of vascular SMC differentiation. A commercially available line of human bladder SMCs was used as positive control. These cells were initially spindle-like but became larger and more rectangular in shape as time in culture increased.

The effects of PD98059 in the expression of smooth muscle markers α-SMA, calponin, SM-MHC and desmin were tested in the abovementioned cells after 2, 4 and 7 days of induction. Different conditions were tested in the two assays. For BM-MSCs, two controls (DMEM+10%FBS and DMEM+3%FBS) were implemented, and 1 μM, 10 μM and 30 μM of PD98059 were tested, based on the work of Tamama and co-workers. For AT-SVF cells, DMEM-HG+3%FBS plus 10 μM of PD98059 was added as a test condition, and DMEM-HG+3%FBS was the only control used. A medium change was performed at day 4.

Cell morphology and viability did not suffer any significant changes from PD98059 supplementation. Calponin- and desmin-expressing cells could be observed by immunofluorescence in all conditions (Figure 4). Thus, serum alone (3% and 10% FBS) induced the expression of these markers, possibly because of proteins and factors existing in the serum that might stimulate smooth muscle-lineage commitment. All isoforms of calponin and desmin were shown to be expressed in undifferentiated BM-MSCs, which is consistent with the results obtained herein. Importantly, fluorescent cells were only observed near the edges of the wells, which suggests that 3D-like spatial stimuli might induce myogenic differentiation. Calponin-expressing cells presented broad polygonal morphologies and increased sizes, as opposed to desmin-expressing cells, which tended to be smaller and more spindle-like. In general, higher fluorescence intensities were observed for calponin in cells exposed to PD98059, however, the same correlation was not valid for desmin. In BM-MSCs, very low intensity fluorescence could be observed for α-SMA and SM-MHC at day 2 in cells near the edges of the wells for all conditions (data now shown). Conversely, no fluorescence was detected for α-SMA and SM-MHC in any condition for AT-SVF cells, which ultimately
indicates that smooth muscle differentiation was not achieved. Analogously, the flow cytometry results obtained for all tested conditions indicated that PD98059 did not provide enough stimuli to significantly and consistently enhance the expression of smooth muscle markers (data now shown). It is possible that several PD98059 pulses might be necessary to fully induce smooth muscle differentiation (e.g. medium replacement every day for 5 days). Alternatively, TGF-β might be a promising candidate to test in future studies.

Conclusions

Skeletal muscle engineering has suffered from several setbacks that have precluded clinical translation of research in this field. These setbacks arise from the inability to expand and maintain skeletal muscle progenitors (satellite cells and myoblasts) in an undifferentiated state in vitro. The results obtained here contribute to the development of this field of research, as they indicate that (i) 5-AzAinduces cytotoxic effects, which excludes its application in Regenerative Medicine approaches, and did not lead to myogenic differentiation; (ii) myogenesis-committed cells are present in uncultured SVF and (iii) even if this subpopulation is not efficiently selected, plated AT-SVF cells show intrinsic myogenic potential in the presence of adequate stimuli (e.g. dexamethasone and bFGF), that can be enhanced in the presence of a gelatin coating, as indicated by the higher skeletal MHC expression levels obtained.

![Image of Figure 4](image-url)

**Figure 4.** Smooth muscle marker expression in P1 AT-SVF cells and P3 BM-MSCs, after 7 days of differentiation induction with PD98059, and P6 SMCs, after 21 days in culture, assessed by immunofluorescence. A) Calponin (top) and desmin (bottom) detection in AT-SVF cells. C) Calponin detection in BM-MSCs. C) α-SMA, SM-MHC, calponin and desmin detection in SMCs. Scale bar = 50 µm.
Chemical induction with PD98059 was not sufficient to promote a consistent increase in smooth muscle marker expression in AT-SVF cells and BM-MSCs. The observations made in these assays suggest that 3D-like spatial organization might have promoted desmin and calponin expression even in non-chemically stimulated cells. Therefore, 3-D stimuli in the form of patterned substrates and ECM mimicking through the presence of coating should be explored, as well as biomechanical cues.

References