

Human Adipose Tissue Derived Stem Cells as a Source of Smooth Muscle Cells in the Regeneration of Muscular Layer of Urinary Bladder Wall

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Abstract

Background: Adipose tissue provides an abundant source of multipotent cells, which represent a source of cell-based regeneration strategies for urinary bladder smooth muscle repair. Our objective was to confirm that adipose-derived stem cells (ADSCs) can be differentiated into smooth muscle cells.

Methods: In this study, adipose tissue samples were digested with 0.075% collagenase, and the resulting ADSCs were cultured and expanded in vitro. ADSCs at passage two were differentiated by incubation in smooth muscle inductive media (SMIM) consisting of MCDB I31 medium, 1% FBS, and 100 U/mL heparin for three and six weeks. ADSCs in non-inductive media were used as controls. Characterisation was performed by cell morphology and gene and protein expression.

Result: The differentiated cells became elongated and spindle shaped, and towards the end of six weeks, sporadic cell aggregation appeared that is typical of smooth muscle cell culture. Smooth muscle markers (i.e. alpha smooth muscle actin (ASMA), calponin, and myosin heavy chain (MHC)) were used to study gene expression. Expression of these genes was detected by PCR after three and six weeks of differentiation. At the protein expression level, ASMA, MHC, and smoothelin were expressed after six weeks of differentiation. However, only ASMA and smoothelin were expressed after three weeks of differentiation.

Conclusion: Adipose tissue provides a possible source of smooth muscle precursor cells that possess the potential capability of smooth muscle differentiation. This represents a promising alternative for urinary bladder smooth muscle repair.

Keywords: adipose, stem cells, muscle cells, regeneration, bladder wall

Introduction

Smooth muscle is a major component of human tissues and is essential for the normal function of a multitude of organs, including the intestine, urinary tract, and vascular system. The

multidisciplinary science of regenerative medicine and tissue engineering (TE), which combines key elements such as biomaterials, stem cells, and bioactive agents (e.g. growth factors), has evolved in parallel with recent biotechnological advances. These future cell-based therapies will benefit

from a source of autologous stem cells that are pluripotent and easily accessible (1,2). Recent findings suggest that pluripotent stem cells might reside in adult tissues in a dormant state (3). Over the past 25 years, bone marrow stem cells (BMSCs) have been the focus of extensive research; however, the clinical use of these cells has presented various challenges, such as low cell number upon harvest and pain and morbidity to the donor. Like bone marrow, adipose tissue is derived from the mesenchyme, and it contains an easily isolated supportive stroma containing stem cells. Human stromal (mesenchymal) stem cells (hMSCs) are multipotent stem cells with high proliferation potential (4,5). Adipose-derived stem cells (ADSCs) possess multilineage differentiation potential and can be used from an autologous origin; therefore, they are attractive candidates for clinical applications to repair or regenerate damaged tissues and organs (6).

The use of stem cells for cell-based tissue engineering and regeneration strategies represents a promising alternative for smooth muscle repair (7). Scientific interest in stem cells is centred on their ability to divide or self-renew indefinitely, and to generate all the cell types of the organ from which they originate, potentially regenerating the entire organ from a few cells. This ability can be induced by modifying the growth medium when stem cells are cultured in vitro or by transplanting them to an organ of the body different from the one from which they were originally isolated.

Adipose tissue contains a large number of multipotent stromal stem cells and is relatively easy to obtain in large quantities; thus, it constitutes a very convenient source of stromal stem cells (7,8). Adipose tissue represents a potential alternative reservoir of cells with stem cell properties (9).

In this study, the capacity of ADSCs to differentiate into smooth muscle cells was evaluated. Smooth muscle, an active component of the cardiovascular, gastrointestinal, reproductive, and urinary systems, has been the subject of intense research in the field of cellular therapeutics involving these tissues. However, one major limitation to this approach has been finding a reliable source of smooth muscle cells, as smooth muscle biopsies of these systems can be impractical and morbid. In addition, biopsies usually lead to limited amounts of cells that need to be extensively expanded before they can be used for therapeutic applications. Furthermore, it has been shown that smooth muscle cells derived from diseased organs can lead to abnormal cells

that are different from healthy smooth muscle cells, thus limiting their use (10,11). As such, there is a great need for alternative sources of healthy smooth muscle cells. The recent discovery and characterisation of multilineage cells from adipose tissue has been met with a great deal of excitement in the fields of tissue engineering and regenerative medicine (12).

Adipose-derived stem cells are ideal for cellular therapy applications, due to various factors: they can be harvested, multiplied, and handled easily, efficiently, and non-invasively; they have a multipotential and proliferative capacity comparable to bone marrow mesenchymal cells; and morbidity to donors is considerably less, requiring only local anaesthesia and a short wound-healing time (13,14).

Materials and Methods

Adipose-derived stem cells: isolation, expansion, and differentiation

Adipose stem cells were isolated from subcutaneous adipose tissue (redundant tissue after surgery and by consent of the patients). The adipose samples were kept in isotonic solution (normal saline) and sent to a tissue centre for processing or kept at room temperature for no more than 24 h prior to use. Adipose stem cells were isolated from the adipose tissue by first washing the tissue sample extensively with phosphate-buffered saline (PBS) containing an antibiotic/antifungal three times to remove debris and clots. The adipose tissue samples were placed in sterile Petri dishes and minced into small pieces of 1 mm or smaller. They were then digested with 0.075% collagenase Type I solution, followed by incubation in the incubator shaker for 30–60 min at 37 °C and 5% CO₂, with periodic observation to avoid over-digestion. The stromal vascular fraction (SVF), containing the ADSCs, was obtained by centrifuging the sample at 6500 rpm for 5 min, discarding the solution, re-suspending the cell pellet in 10 mL PBS, filtering through 100-µm nylon mesh, and centrifuging at 6500 rpm for 5 min. The supernatant was aspirated and the cell pellet re-suspended in 12 mL of control medium (Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum [FBS]). The media containing the cells was aspirated into a six-well culture plate, and the cells were maintained in a humidified tissue culture incubator at 37 °C and 5% CO₂. The medium was changed every third day until the cells reached 80–90% confluence, followed by trypsinization and re-plating in a 75 mL flask until

passage two. Undifferentiated ADSCs at passage two were differentiated into smooth muscle cells (SM-ADSCs) by incubation in smooth muscle inductive media (SMIM) consisting of MCDB 131 medium, 1% FBS, and 100 U/mL heparin for three to six weeks at 37 °C and 5% CO₂, as previously described. The media was changed every five days. Cell splitting was not required. ADSCs in non-inductive media were used as controls.

Assessment of SM-ADSC differentiation by gene expression

Four groups of cells were prepared for the evaluation of SM-ADSC differentiation by gene expression. Differentiated and undifferentiated cells at three and six weeks were evaluated. Total ribonucleic acid (RNA) was extracted using the TRI reagent method. After genomic deoxyribonucleic acid (DNA) elimination, the RNA sample was ready for reverse transcription (RT) using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The analysis of the polymerase chain reaction (PCR) products depends on the type of PCR applied. In this study, conventional PCR was used, so the PCR product was detected using agarose gel electrophoresis and ethidium bromide. The following primers were used:

ASMA (595 bp)

F: ACCACAATGTCCCCATCTA
R: TGATCCACATCTGCTGGAAG

MHC (656 bp)

F: GGACGACCTGGTGTGTTGATT
R: TAGCTGCTTGATGGCTTCC

Calponin (453 bp)

F: ATGTCCTCTGCTCACTTCA
R: TTTCCGCTCCTGCTTCTCT

GAPDH (218 bp)

F: TCCCTGAGCTGAACGGAAG
R: GGAGGAGTGGGTGTCGCTGT

Expression of SM-ADSC differentiation at protein level (immunocytochemistry)

Immunocytochemistry is an immunological technique used to visualise the presence of a specific protein or antigen in cells. In this study, the cells were trypsinised and seeded on a chamber slide at an average of about (1~1.5x10⁴) thousand cells per chamber, and then incubated for one to two days for cell attachment before processing. To ensure access of the antibody to its antigen, cells must be fixed and permeabilised. The procedure was initiated by observing the attached cells under a microscope, followed by cell fixation on the culture surface with 4% formaldehyde in PBS for

10 minutes. The slides were incubated with 0.1% Triton X-100 in PBS for 5 minutes to permeabilise the membranes, then rinsed twice for 5 minutes in PBS. As a secondary antibody was used, masking non-specific protein with blocking solution was performed using 10% serum for 30 minutes.

The primary antibody was diluted to the recommended concentration in 1% normal serum in PBS. The concentration was 1/100 for alpha smooth muscle actin (ASMA) and myosin heavy chain (MHC), and 1/200 for smoothelin. 1:100 fluorescein (FITC) conjugated goat anti-mouse was used as a secondary antibody (Kerkegaard Perry Laboratories). The blocking buffer was then removed from the slides. The primary antibodies used were ASMA: mouse monoclonal Abcam, 1A4, ab7817, UK; MHC: mouse monoclonal Abcam, 3F8, ab682, UK; and smoothelin: mouse monoclonal, 4A83, ab21108, UK. The primary antibody was added to each well and incubated overnight at 4 °C. The fluid was removed from the slide and the secondary antibody solution (goat anti-mouse) was added to the well. After incubating for one hour at room temperature, the antibody was discarded and washed with Tris buffered saline (TBS) and PBS. The nuclei were counterstained with 4'-6 - Diamidino-2-phenylindole (DAPI) and incubated at room temperature for 30 minutes, followed by washing with PBS. Visualisation was conducted under a confocal microscope.

Results

Characterisations of adipose stem cell differentiation were performed by cell morphology, induced genetic expression, and phenotypic antigenic protein expression of smooth muscle markers. Four groups of cells were cultured and proliferated until passage two (P₂), where two of them were differentiated by adding smooth muscle inductive medium and followed for three and six weeks, respectively. The other two groups were cultured in control media (CM). All the groups grew to sub-confluence proliferation before their incorporation into the comparative evaluation of morphological change. At three and six weeks, the non-differentiated control groups formed a monolayer of fibroblast-like ADSCs presenting a flat morphology and stress fibre pattern. In the presence of SMIM, the differentiated cells acquired typical smooth muscle cell (SMC) morphology with spindle-like, elongated, fibroblast-shaped cells, and towards the end of six weeks, a cell aggregation arrangement typical of smooth muscle culture.

They proliferated at a lower rate compared to those cultured in CM (Figure 1).

In this study, smooth muscle cell induction of adipose stem cells from passage two were cloned for up to three and six weeks. Twelve clones evaluated for smooth muscle differentiation capacity in SMIM resulted in cell differentiation and expression of multiple myogenic factors confirmed by RT-PCR. Smooth muscle markers—ASMA, calponin, and MHC—were used to study gene expression. ASMA is an early marker of developing smooth muscle cells, whereas the other markers, especially MHC, are highly restricted to differentiated smooth muscle cells. Gene expression of these markers was expressed by PCR in three weeks as well as in six weeks differentiated cells, while there was no gene expression detected in the control group. Expression of these myogenic genes was also observed in human bladder smooth

muscle cells (control). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalise gene expression reading (Figure 2).

Four groups of cells were prepared for phenotypic expression of ADSC differentiation—two groups of differentiated cells at three and six weeks and the other two control groups growing in control medium for three and six weeks. Protein expression was established by cell staining. 1:100 dilutions were used for ASMA and MHC primary antibodies while 1:200 dilutions was used for Smoothelin primary antibodies and 1:100 dilution was used for fluorescein (FITC) conjugated goat anti-mouse secondary antibodies. The nuclei were counterstained with DAPI. At the protein expression level, the cells exhibited smooth muscle marker positive cells for ASMA, MHC, and smoothelin after six weeks of differentiation. However, only ASMA and smoothelin was expressed after three weeks of differentiation. The

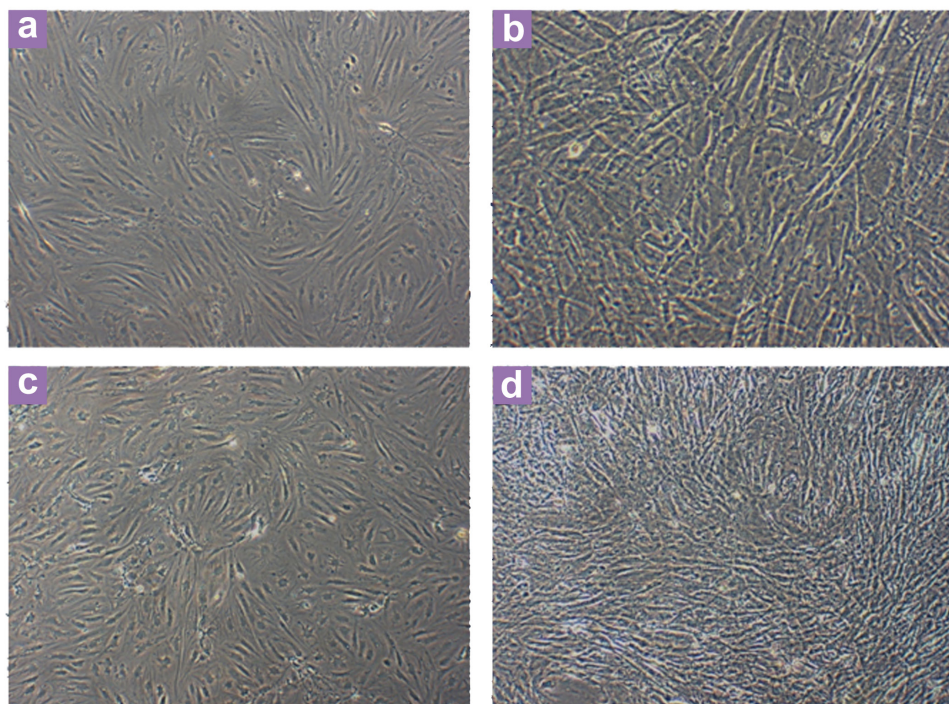


Figure 1: Phase contrast microscopy image represent morphological changes in induced compared with uninduced human adipose derived stem cells. Non-differentiated control groups at three and six weeks formed a monolayer of fibroblast-like adipose derived stem cells presenting a flat morphology and stress-fiber pattern. In the presence of SMIM the differentiated cells acquired typical SMC morphology with spindle-like, elongated, fibroblast shape cells, and towards the end of 6 weeks cell aggregation arrangement which is typical for smooth muscle culture. A&B represents three weeks induced and control cells respectively. C&D represent six weeks induced and control cells respectively.

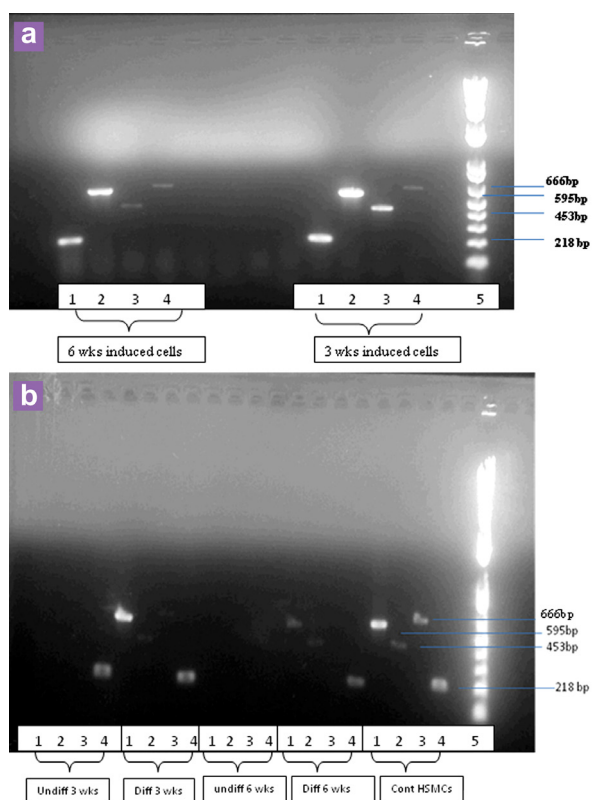


Figure 2: (a) DNA gel electrophoresis using ethidium bromide for visualisation of gene expression by RT-PCR in induced human adipose stem cells for 3 and 6 weeks. GAPDH lane (1), ASMA lane (2), Calponin lane (3), MHC lane (4), DNA marker lane (5). (b) DNA gel electrophoresis using ethidium bromide for visualisation of gene expression by RT-PCR in induced human adipose stem cells for three and six weeks and uninduced human adipose stem cells for three and six weeks. Human smooth muscle cells (HSMCs) used as a control, ASMA lane (1), Calponine lane (2), MHC lane (3), GAPDH lane (4), and DNA marker lane (5). Gene expression of these markers was expressed by PCR in three weeks and as well as in six weeks differentiated cells, while there were no gene expressions detected in the control group. Expression of these myogenic genes was also observed in human bladder smooth muscle cells (control). GAPDH was used to normalise gene expression reading.

control cells were positive for ASMA at three and six weeks (Figure 3).

Discussion

Smooth muscle cells, which represent the bulk of the urinary bladder wall, can be affected by a variety of diseases, leading to dramatic changes in bladder capacity and compliance. There are various available treatment methods, none of which leads to a satisfactory return of the native urinary bladder wall structure and function. Cell therapy-based regeneration of damaged smooth muscle cells of the urinary bladder represents a newly developed therapeutic method for the repair of damaged urinary bladder wall.

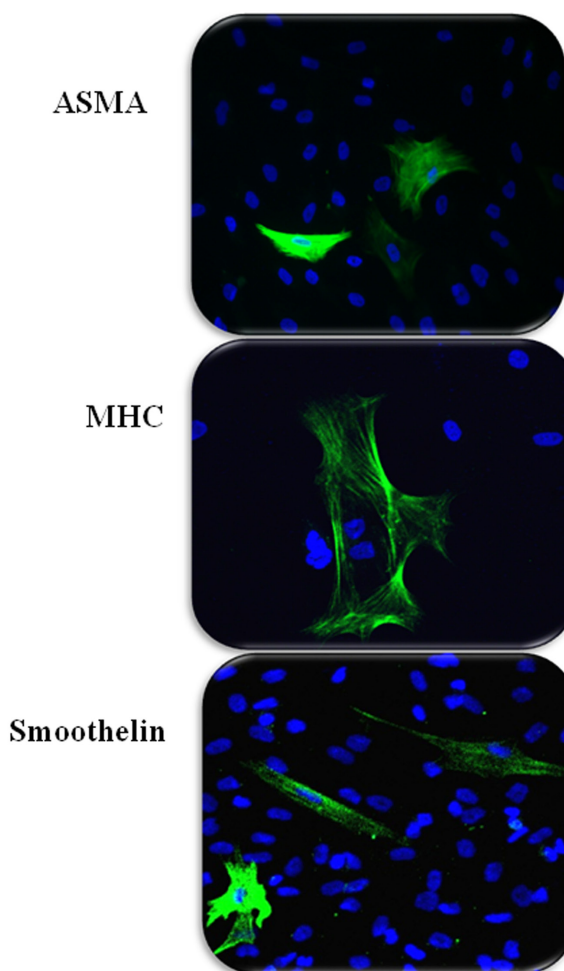


Figure 3: Immunocytochemistry of induced human adipose derived stem cells-smooth muscle cells, the cells exhibit smooth muscle marker positive for alpha smooth muscle actin (ASMA), myosin heavy chain (MHC) and smoothelin.

Smooth muscle cells, which are the major component of the urinary bladder wall and other hollow organs, have been involved in several cell therapy research studies involving the tissues of these organs. The main limitation is finding a reliable source of smooth muscle cells, as the affected organ cannot provide suitable specimens for cell therapy, due to either organ tissue shortage or damaged cells, which limits their use (10,11). Adipose tissue is abundant and accessible, and it represents a potential alternative source of adult stem cells that can be isolated. ADSCs have potential applications for the repair and regeneration of acute and chronically damaged tissues. A large population of cells isolated from the stromal vascular fraction of human adipose tissue, called processed lipoaspirate (PLA) cells, possesses multipotent, multilineage potential (15,16). In a previous study, performed in our laboratory ADSCs derived from the mesenchyme were positive for CD90, CD73, CD44, CD9, and HLA-ABC, and negative (or minimally expressed) for CD45, CD117, CD31, and CD34 (17).

The aim of this study was to confirm that ADSCs could be differentiated into smooth muscle cells and to prove their differentiation in order to use them in the regeneration of damaged smooth muscle. ADSC differentiation into smooth muscle cells represents part of our main project of urinary bladder reconstruction via a tissue engineering technique.

As has been shown in the literature, smooth muscle displays fully phenotypic differentiation and achievement of functional contractile properties, depending on the induction media, the effects of its surrounding components, and the degree of the proliferation state (15,18). Previous studies have reported that differentiation occurred when the cells were not in the proliferative state. ADSC differentiation induction was performed in a controlled proliferation state using SMIM composed of CMDB 131 supplemented with 100 units/mL heparin and a low concentration of foetal bovine serum to control cell differentiation and proliferation. Heparin is known to prevent proliferation and induce ASMA expression (19–21).

The aim of this study was to obtain differentiated smooth muscle cells derived from adipose tissue cells and to prove their differentiation in order to use them in the regeneration of damaged smooth muscle. A combined morphological, genetic expression, and cell phenotype diversion assessment was performed to evaluate the successful

differentiation of ADSCs into smooth muscle cells. Two groups of cells were prepared to make a differentiation comparison. The first group, the control group, consisted of cells cultured in non-inductive media. The second group consisted of cells cultured in smooth muscle inductive media for three and six weeks to evaluate the suitable differentiation culturing period.

The final differentiation assessment concept depends on our combined results evaluation. Cell morphology and the acquired change on a monolayer culture plate were observed carefully; observation monitoring began when the cell culture reached sub-confluent proliferation, which was associated with the induction of differentiation in the differentiated groups. Differentiated cells become spindle shaped with prominent nuclei, they are arranged in a uniform direction, and at the end of the planned period for differentiation, differentiated cell aggregation take place and exhibits a hill and valley appearance, which is one of the visual characteristics of smooth muscle culture. The proliferation rate of the differentiated cells in our study was low. There was no difference in cell morphology between the three- and six-week cell groups, which indicates early differentiation into smooth muscle cells. The undifferentiated control cells kept their fibroblast-like morphology, with a marked proliferation rate.

Smooth muscle markers (ASMA, calponin, and MHC) were used to study gene expression. These markers represent the early, mid, and late expression markers of cell differentiation. Expression of these genes was detected by PCR in the three-week and six-week induced cells.

To confirm cell differentiation and its transfer to mature smooth muscle cells at the protein level, certain phenotypic protein expression should be manifested, especially specific cytoskeletal protein markers that occur in fully differentiated contractile smooth muscle cells. In this study, ASMA, MHC, and smoothelin were used. The expression of ASMA, which is an early marker of smooth muscle differentiation, was not enough to prove full maturation of smooth muscle cells (3). To confirm the full differentiation of smooth muscle cells, two of the late and specific markers were used, MHC and smoothelin, which are highly specific markers detected only in fully differentiated contractile smooth muscle cells (22). ASMA and smoothelin were expressed after three weeks of differentiation; ASMA, MHC, and smoothelin were expressed after six weeks of differentiation. However, only ASMA was expressed in the controls.

Conclusion

This study demonstrates the ability of adipose stem cells to be differentiated into smooth muscle cells upon induction; adipose stem cells assumed typical smooth muscle morphology and expressed characteristic leiomyogenic markers at both the genetic and protein levels. Adipose-derived stem cells represent a promising alternative source of smooth muscle precursor cells that possess the potential capability of smooth muscle differentiation for urinary bladder smooth muscle repair.

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Conflict of Interest

None.

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Analysis and interpretation of the data: SAS, ZMD, IS, MRY

Drafting of the article: SAS

Critical revision of the article for the important intellectual content and final approval of the article: RHI, ANMH

Provision of study materials or patient: ZMD, CCKH, RS

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Obtaining of funding: AS, CCKH, RS

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