Influence of Low Intensity Laser Irradiation on Isolated Human Adipose Derived Stem Cells Over 72 Hours and Their Differentiation Potential into Smooth Muscle Cells Using Retinoic Acid

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SUMMARY

Introduction: Human adipose derived stem cells (hADSCs), with their impressive differentiation potential, may be used in autologous cell therapy or grafting to replace damaged tissues. Low intensity laser irradiation (LILI) has been shown to influence the behaviour of various cells, including stem cells. *Aims*: This study aimed to investigate the effect of LILI on hADSCs 24, 48 or 72 h post-irradiation and their differentiation potential into smooth muscle cells (SMCs). *Methodology*: hADSCs were exposed to a 636 nm diode laser at a fluence of 5 J/cm^2. hADSCs were differentiated into SMCs using retinoic acid (RA). Morphology was assessed by inverted light and differential interference contrast (DIC) microscopy. Proliferation and viability of hADSCs was assessed by optical density (OD), Trypan blue staining and adenosine triphosphate (ATP) luminescence. Expression of stem cell markers, β1-integrin and Thy-1, and SMC markers, smooth muscle alpha actin (SM-αa), desmin, smooth muscle myosin heavy chain (SM-MHC) and smoothelin, was assessed by immunofluorescent staining and real-time reverse transcriptase polymerase chain reaction (RT-PCR). *Results*: Morphologically, hADSCs did not show any differences and there was an increase in viability and proliferation post-irradiation. Immunofluorescent staining showed expression of β1-integrin and Thy-1 72 h post-irradiation. RT-PCR results showed a down regulation of Thy-1 48 h post-irradiation. Differentiated SMCs were confirmed by morphology and expression of SMC markers. Conclusion: LILI at a wavelength of 636 nm and a fluence of 5 J/cm^2 does not induce differentiation of isolated hADSCs over a

72 h period, and increases cellular viability and proliferation. hADSCs can be differentiated into SMCs within 14 days using RA.

INTRODUCTION

Stem cells are regarded as undifferentiated cells that are capable of self-renewal, proliferation, production of a great number of differentiated progeny, and regeneration of tissues [1]. The therapeutic potential of multilineage stem cells for tissue engineering (TE) applications is vast. Two general types of stem cells are potentially useful for this application: embryonic stem cells (ESCs) and adult (autologous) stem cells [2]. Traditionally, ESCs are isolated from the inner cell mass (ICM) of blastocysts, however harvesting of these cells results in the death of the embryo, which has led to ethical, religious and political issues [3]. In contrast, adult stem cells, by virtue of their nature, are immune-compatible and no ethical issues associated with their use are noted [2].

Subcutaneous adipose tissue is an active and highly complex tissue composed of several different cell types, and is derived from the mesodermal germ layer and contains a supportive stromal vascular fraction (SVF) that can be easily isolated. This SVF contains a heterogeneous mixture of cells including mature adipocytes, preadipocytes, fibroblasts, smooth muscle cells (SMCs), endothelial cells and resident monocytes/macrophages and lymphocytes [4-6]. The pre-adipocytes are considered as

the multipotent stem cells termed adipose derived stem cells (ADSCs) that have similar properties to bone marrow mesenchymal stem cells (BM-MSCs) [7]. ADSCs are idyllic for cellular therapy applications due to various factors: they can be harvested, multiplied and handled easily, efficiently and non-invasively, they have a pluripotential and proliferative capacity comparable to BM-MSCs, and morbidity to donors is considerably less, requiring only local anaesthesia and a short wound healing time. hADSCs can be expanded in an undifferentiated state and have multipotential differentiation capacity along the classical mesenchymal lineages of adipogenesis, osteogenesis, chondrogenesis and myogenesis [8]. Non-mesenchymal lineages have also been investigated and the trans-differentiation abilities of ADSCs confirmed, demonstrating that these cells can differentiate into bone, cartilage, fat, heart, nerve, liver and smooth muscle [5,7,9,10].

Smooth muscle is an active component of the cardiovascular, reproductive, urinary and intestinal systems, and has been the subject of intense research in the field of regenerative medicine [11]. They are known to play a key role in a variety of major human diseases, such as arteriosclerosis, asthma, hypertension, and cancer [12]. The process of smooth muscle differentiation is thought to result from interactions between mesenchymal cell precursors and epithelial and/or non-epithelial cells. As in any progression that produces heterotypic cell induction, the epithelial and/or non-epithelial cells could produce morphogens that will trigger smooth muscle differentiation by acting on the surrounding mesenchyme [13]. Factors that are shown to modulate smooth muscle differentiation are transforming growth factor (TGF)-β1, retinoic acid (RA),

platelet derived growth factor BB (PDGF BB) [11,13,14], and ascorbic acid (AA) [11,14]. Vitamin A and its derivatives (the retinoids) are vital for both normal embryonic development and maintenance of differentiation in the adult organism. RA induces numerous genes during development and influences vascular SMC differentiation *in vivo* [15].

Low intensity laser irradiation (LILI) refers to the use of photons, which are absorbed by chromophores (photoacceptors) in the cell, to alter biological activity. Non-thermal laser light, from the red to the near-infrared region of the spectrum, is used in this type of therapy. Photonic energy which is absorbed by the chromophores stimulates a variety of biochemical pathways and since the effects of LILI are not thermal, there is no damage caused to living tissue at a cellular level [16,17]. Laser irradiation at various intensities has been found to be able to stimulate or inhibit an assortment of cellular processes. LILI of a particular wavelength can initiate cascades, such as proliferation at a cellular level [18]. Studies on LILI and stem cells have shown that LILI can alter the metabolism and migration of stem cells [19]. It has been shown that 5 J/cm^2 of laser irradiation at a wavelength of 635 nm positively affects hADSCs by increasing cellular proliferation, viability, and protein expression [20] and LILI in combination with epidermal growth factor (EGF) enhances their proliferation [21].

Due to the fact that stem cells grow and proliferate at such slow rates, and their harvest yields are low, a therapy which can increase their proliferation and still maintain their stem cell character is warranted, especially if the cells are to be used in stem cell therapies. This study aimed to determine if LILI, at a wavelength of 636 nm, could

stimulate isolated hADSC proliferation and maintain stem cell character over a 72 h period. The study further aimed to investigate the differential potential of these cells into SMCs using RA.

MATERIALS AND METHODS

hADSCs

Adipose tissue excised from consenting adult donors undergoing abdominoplasty was used for the isolation of hADSCs. Ethical approval in accordance with the Human Tissue Act 65, 1983, was obtained from the Academic Ethics Committee of the Faculty of Health Sciences, University of Johannesburg (09/06).

hADSCs were isolated from adipose tissue as previously described [13]. Briefly, separated adipose tissue was stored overnight in Hanks Balanced Salt Solution (HBSS; Invitrogen, INV/14170088) containing 10,000 units Penicillin-Streptomysin (PAA Laboratories GmbH, P11-010) and 250 µg/ml Amphotericin B (PAA Laboratories GmbH, P11-001). The adipose tissue was minced, added (1:1) to 300 U/ml collagenase type-1 (Invitrogen, INV/17100017) and incubated in a shaking incubator (Labcon, Instrulab, SA) at 70 rpm for 80 min at 37°C. Following incubation, equal volumes of complete media, consisting of Dulbecco's Modified Eagle Medium F12 (DMEM-F12; Invitrogen, INV/21331020) supplemented with 10% Fetal Bovine Serum (FBS; PAA Laboratories GmbH, A15-101), 0.1% Penicillin-Streptomysin and 1 µg/ml Amphotericin B was added. The suspensions were then centrifuged at 1,000 rpm (Orto Acresa, Mod. Digicen-R) for 5 min at 20°C, the infranatant under the oil layer was removed and filtered (Beckson

Dickinson, BD/352340). This filtrate was then centrifuged at 2,000 rpm for 5 min and the resultant cell pellet pooled with complete media, and re-pelleted. The supernatant was removed and re-suspended in 8 ml Erythrocyte Lysis Buffer (ELB; NH_4Cl, $KHCO_3$, ethylenediaminetetraacetic acid (EDTA)), incubated for 10 min at room temperature to lyse the red blood cells, then centrifuged at 2,200 rpm (Orto Acresa, Mod. Digicen-R) for 5 min. The cell pellet was re-suspended and seeded into 75 cm^2 culture flasks and incubated at 37°C in 85% humidity and 5% CO_2.

A commercial hADSC line (StemPro® Human Adipose Derived Stem cells) was purchased from Invitrogen (R7788-110) and used as a positive control for the isolated hADSCs. Cells were grown to semi-confluence (60 to 80%) in MesenPro RS™ Medium (Invitrogen, INV/12746-012) supplemented with 2 mM L-glutamine (Invitrogen, 25030), 0.1% Penicillin-Streptomysin and 1 µg/ml Amphotericin B.

For experiments semi-confluent cells were detached by trypsinization (1 ml/25 cm^2, 0.25% trypsin–0.03% EDTA), and 5×10^5 cells in 3 ml complete culture media were seeded into 3.3 cm diameter culture plates for laser irradiation.

Differentiation of hADSCs into SMCs

Semi-confluent isolated hADSCs were differentiated with 10^{-7} M/L RA (Sigma Aldrich, R2625); 200 µg/ml heparin (Sigma Aldrich, H3149) was used as a positive control [11]. hADSCs were grown in Smooth Muscle Induction Medium (SMIM) which contained MCDB131 media (Sigma Aldrich, MCDB131) as the basal medium, supplemented with

1% FBS (PAA Laboratories GmbH, A15-101), 0.1% Penicillin-Streptomysin (PAA Laboratories GmbH, P11-010), 1 µg/ml Amphotericin B (PAA Laboratories GmbH, P11-001) and the respective inductive chemical. SMIM was added to the cells and they were allowed to grow and differentiate at 37°C in 5% CO_2 for either 2 weeks (RA) or 6 weeks (heparin). Media was changed every 3 to 4 days. Digital micrographs were taken every 2 days to assess the progress of differentiation. After differentiation, cells were kept in MCDB131 maintenance medium (without RA or heparin) until testing. SK-UT-1 cells (American Type Cell Culture, HTB-114) were used as a positive SMC control and were grown in DMEM-F12 supplemented with 10% FBS, 0.1% Penicillin-Streptomysin and 1 µg/ml Amphotericin B.

Laser Irradiation

Semi-confluent monolayers of hADSCs in complete medium were irradiated from above with the lid off in the dark at room temperature at 5 J/cm^2 with a 636 nm diode laser (Oriel, USA). An optical fibre, at a distance which produced a circular spot size of 9.1 cm^2, delivered laser light to the culture plates, uniformly covering the entire surface of the culture dish. On average a power output of 78 mW was measured using the Coherent Fieldmate detector and sensor, based on these readings it this was calculated to take 9 min and 46 s to deliver 5 J/cm^2 at a power density of 8.59 mW/cm^2. Non-irradiated cells (0 J/cm^2) were used as controls and were kept under the same conditions. Both irradiated and non-irradiated samples were re-incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24, 48 or 72 h.

Cellular Morphology

Cellular morphology of irradiated and non-irradiated hADSCs was assessed at 0, 24, 48 and 72 h post-irradiation. Morphological changes during SMC differentiation were also monitored. Digital micrographs were taken using an inverted light microscope (Olympus, CKX41) that was coupled to a digital camera (Olympus, C5060-ADUS). Differential interference contrast (DIC) photographs were taken using the Carl Zeiss Axio Observer Z1.

Cellular Viability

Post laser irradiation of hADSCs, cellular viability was determined 24, 48 or 72 h by differential staining with Trypan blue and determination of ATP luminescence.

Trypan blue

An equal mixture of cell suspension and Trypan blue reagent (Sigma Aldrich, T6146) was incubated at room temperature for 5 min. Using a Haemocytometer with Neubauer rulings and a light microscope (Olympus, CKX41) viable and non-viable cells were counted and the percentage viability was determined. The percentage viability was calculated by dividing the number of viable cells (translucent) by the total number of cells and multiplied by 100.

Adenosine Triphosphate (ATP) luminescence

Cellular ATP was measured using the Cell Titer-Glo® luminescent cell viability assay (Promega, G7573). Addition of reagent to the cells results in lysis and generation of a luminescent signal that is proportional to the amount of ATP present. Cells were re-

suspended at a concentration of 1 x 10^5 / 100 µl. According to the manufactures protocol, an equal volume of cell suspension (50 µl) and reagent was added to a 96 well micro plate, incubated at room temperature on a shaker (Labcon, 3081U) for 2 min to induce lysis, followed by a 10 min incubation period at room temperature in the dark. Luminescence, in Relative Light Units (RLU), was then determined (Perkin Elmer, Victor[3]).

Cellular Proliferation

Cell proliferation of hADSCs was measured using OD at 24, 48 or 72 h post-irradiation. One hundred microlitres of cell suspension in complete medium was read at A_{540} nm [22] (Perkin Elmer, Victor[3]).

Protein Expression

Stem cells can be identified by their expression of certain proteins and genes. Two proteins that have previously been shown to be expressed by hADSCs are β1-integrin (CD29) and Thy-1 (CD90) [20,21]. Expression of two early SMC markers, smooth muscle alpha actin (SM-αa) and desmin, and two late SMC markers, smooth muscle myosin heavy chain (SM-MHC) and smoothelin were tested in differentiated SMCs and SK-UT-1 cells.

Immunofluorescence

Cells were seeded onto heat sterilised glass coverslips and grown to semi-confluence. hADSCs were irradiated and incubated at 37°C for 3, 24, 48 or 72 h to allow the cells to

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respond to the laser light; differentiated SMCs received no laser irradiations. Cells were rinsed twice with ice-cold phosphate buffered saline (PBS) bovine serum albumin (BSA)/azide buffer (PBS; 0.1% w/v BSA; 0.01% w/v azide). Isolated and commercial hADSCs were incubated with Integrin β1 CD29, very late antigen VLAb1 platelet (1:250, US Biological, 17661-390) and rabbit anti-human Thy-1 (1:200, Santa Cruz Biotechnology, SC-9163) in PBS BSA/azide for 30 min on ice. Differentiated SMCs and SK-UT-1 cells were incubated with 25 μg/ml mouse anti-human Desmin (1:200, US Biological, D3221-16), ready-to-use mouse anti-Actin, alpha, Smooth Muscle (US Biological, A0760-27B), 0.1 mg/ml mouse anti-human Smoothelin (1:200, US Biological, S1014-62B) or 1 mg/ml mouse anti-human Myosin, Smooth Muscle, heavy chain (3 μg/ml) (US Biological, M9850-14X) in PBS BSA/azide for 30 min on ice.

Cells were rinsed three times with PBS BSA/azide buffer and incubated with the secondary fluorescent antibody for β1-integrin (1:500; Texas Red Conjugated anti-Mouse IgG_1, $γ_1$ chain specific, Rockland, 610-4940), Thy-1 (1:500, goat anti-rabbit IgG-FITC, Santa Cruz Biotechnology, SC-2012), Desmin and Smoothelin (1:1,000, Fluorescein Conjugated anti-Mouse IgG_1, $γ_1$ chain specific, Rockland, 610-4240), SM-αa (1:1,000, Texas Red Conjugated anti-Mouse IgG_2, $γ_{2a}$ chain specific, Rockland, 610-4941) and SM-MHC (1:1,000, Texas Red Conjugated anti-Mouse κ, kappa chain specific, Rockland, 610-4910) in PBS BSA/azide for 30 min on ice, protected from light. Cells were rinsed three times as before and fixed in 3.7% formalin for 10 min. Following fixation, cells were rinsed briefly with PBS, and then with tap water before being stained for 1 min with 4′-6-Diamidino-2-Phenylinole (DAPI; Biomol, AP402-0010) and mounted

on glass slides (protected from light) using 0.1 M Propyl gallate (Sigma Aldrich, 02370) as the mounting medium. The slides were viewed through a fluorescent microscope (Carl Zeiss, Axio Observer Z1).

Real time RT-PCR

Real-time RT-PCR was used to assess the up- or down-regulation of stem cell markers β1-integrin (CD29) and Thy-1 (CD90) in response to laser irradiation 24, 48 or 72 h post-irradiation. Commercial hADSCs were run as a positive control. RT-PCR was also used to further quantify SMC differentiation by checking for expression of SMC-specific proteins desmin (early SMC marker) and smoothelin (late SMC marker). SK-UT-1 cells were used as a positive SMC control and heparin-induced cells as a positive growth factor control. The geNorm BioBank cDNA Kit (PrimerDesign) was also used as a positive control on all runs. Experiments were repeated twice, each biological sample was further split into two prior to reverse transcription, thus there were four repeats (n=4; 2 experimental and 2 biological repeats). PCR reactions were performed in duplicate. Primer sequences can be seen in Table 1. To check the specificity and alignment of the primers, each sequence was blasted using the Basic Local Alignment Search Tool (BLAST) on the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nhm.nih.gov). The primer sets were then developed by Whitehead Scientific S.A.

Approximately 5×10^5 hADSCs were irradiated with 5 J/cm^2 and left to incubate for 24, 48 or 72 h (n=2). In the case of differentiated SMCs, 5×10^5 cells were seeded and not

exposed to laser irradiation (n=2). The RNeasy Mini kit (Qiagen, 74134) was used in conjunction with the Qiagen QIAcube to isolate total RNA. RNA concentration was measured using the Quant-iT™ RNA Assay Kit (Invitrogen, Q32852) in conjunction with the Qubit™ fluorometer (Invitrogen, Q32857). RNA (680 ng for hADSCs and 800 ng for SMCs) was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, 205311). cDNA was stored at -20°C. A 2-fold dilution series (1:25; 1:50; 1:100; 1:200; 1:400) was made from pooled calibrator cDNA (hADSCs, 0 J/cm^2, 0 h or RA differentiated SMCs). PCR reactions were carried out on the Corbett RotorGene 6000 using SensiMix™ dT PCR kit (Celtic Molecular Diagnostics, QT6T3-05), with SYBR green as a fluorescent marker. Unknown samples were diluted 1:100 (hADSC) or 1:50 (differentiated SMC). PCR cycles are shown in Table 1. A melt was performed at the end of each PCR run on PCR products (72 – 95 °C, 5 s in 1 °C increments), and results were quantified using the Relative Expression Software Tool (REST).

Prior to real-time RT-PCR of β1-integrin and Thy-1, reference genes were validated to determine which gene, GAPDH or UBC (PrimerDesign, geNorm Housekeeping Gene Selection Kit), was expressed at a constant level and not affected by experimental conditions (results not shown). Results were quantified using REST. It was found that that expression of UBC in irradiated isolated hADSCs at 24, 48 or 72 h was no different to the calibrator (P=0.766, P=0.589 and P=0.305 respectively). In contrast, the expression of GAPDH was found to be down-regulated at 24 and 48 h post-irradiation by a mean factor of 0.710 (P=0.003) and 0.853 (P=0.023) respectively. At 72 h, there was no difference (P=0.187). Thus expression of β1-integrin and Thy-1 was normalised

against UBC. No reference genes were used in the case of differentiated SMCs as this experiment was purely to see whether these cells displayed gene expression of desmin and smoothelin at a transcriptional level.

Statistical Analysis

Experiments were repeated six times (n = 6). All assays were performed in duplicate and the mean was used. Results were graphically presented and statistically analysed using SigmaPlot Version 8.0. A student t test and one-way ANOVA was performed to detect differences between the control and experiments and between experimental groups respectively. REST was used to analyse RT-PCR results. Results were considered to be statistically significant when P < 0.05. Statistical significance, compared to their respective control (0 J/cm^2) is shown in tables and figures as P < 0.05 (*), P < 0.01 (**) or P < 0.001 (***).

RESULTS

Irradiated ADSCs

Cellular Morphology

Morphological characteristics of hADSCs were determined by inverted light microscopy and DIC (Figure 1). Morphologically, isolated and commercial hADSCs grew as a monolayer of flat, elongated cells resembling fibroblasts. No noticeable difference in morphology was observed between the irradiated and the non-irradiated control cells at 3, 24, 48 or 72 h. However, compared to both isolated and commercial non-irradiated

hADSCs, (Figure 1 a and d respectively), irradiated cells (Figure 1 b and e respectively) showed a greater density of cells. Using DIC microscopy, isolated and commercial hADSCs displayed an elongated, fibroblast-like appearance (Figure 1 c and f respectively).

Cellular Viability

Cellular viability of cells was determined in isolated and commercial hADSCs 24, 48 and 72 h post-irradiation by the Trypan blue exclusion test and ATP luminescence. The Trypan blue exclusion test indicated a significant increase in percentage viability in irradiated isolated hADSCs as compared to non-irradiated controls at 24 and 48 h ($P \leq 0.001$) (Table 2). At 72 h the increase was not significant (P=0.090). In the commercial cell line, irradiated hADSCs showed a significant increase in percentage viability as compared to their controls at 24, 48 and 72 h (P ≤ 0.01, P ≤ 0.05 and P ≤ 0.01 respectively) (Table 2).

ATP luminescence showed that there was a statistically significant increase in ATP concentration in isolated irradiated cells when compared to non-irradiated cells at 48 h ($P \leq 0.05$) but at 24 and 72 h the increase was not significant (P=0.061 and P=0.185 respectively) (Table 2). ATP luminescence showed no significant increase in irradiated commercial hADSCs as compared to their non-irradiated controls at 24, 48 or 72 h (P=0.226, P=0.352 and P=0.306 respectively) (Table 2).

Cellular Proliferation

OD (A_{540} nm) was used to determine proliferation in isolated and commercial hADSCs. Statistical analysis of irradiated isolated hADSCs showed a statistically significant increase at 24 and 72 h compared to their respective non-irradiated controls (P≤0.05), however at 48 h the increase was not significant (P=0.213) (Table 2). In the commercial cell line, OD results showed no statistically significant increase in irradiated cells at 24, 48 or 72 h compared to their respective controls (P=0.373, P=0.482 and P=0.368, respectively) (Table 2).

Expression of β1-integrin (CD29) and Thy-1 (CD90)

Expression of the stem cell markers, β1-integrin and Thy-1, was determined by immunofluorescence and real-time RT-PCR post-irradiation. Indirect immunofluorescent staining showed expression of β1-integrin and Thy-1 in irradiated cells from 3 to 72 h (Figure 2). These results were supported by Western blot and dot blot analysis (results not shown).

Expression of β1-integrin and Thy-1 24, 48 or 72 h post-irradiation was determined by real-time RT-PCR. UBC was chosen to normalise results. A 2-fold standard curve was run with each PCR run, and an efficiency of 99% and 98%, and R^2 value of 0.999 and 0.999, was achieved for β1-integrin and Thy-1 respectively. A single product was obtained for the melt for both genes (Figure 3). PCR product for Thy-1 melted at around 87.3°C, and at around 83.5°C for β1-integrin. Results from REST showed that β1-integrin in isolated hADSCs is not up or down regulated at either 24, 48 or 72 h post irradiation (P=0.530, P=0.172, P=0.911 respectively). REST showed that Thy-1

expression in isolated hADSCs is not regulated 24 or 72 h post-irradiation (P=0.905 and P=0.295, respectively). In contrast Thy-1 expression is down-regulated 48 h post-irradiation as compared to the non-irradiated control group by a mean factor of 0.793 (P=0.002).

Differentiated SMCs

Cellular Morphology

Morphological characteristics of differentiated SMCs were determined by inverted light microscopy and DIC (Figure 4). Isolated hADSCs were differentiated into SMCs using RA, with heparin used as the positive growth factor control and SK-UT-1 cells as the positive SMC control. Pre-differentiation cells (hADSCs) displayed the usual fibroblast-like morphology, smooth and elongated (Figure 4 a and d). As time progressed there were extending cytoplasmic extensions, forming new branches as the cells started to become more angular looking, and assumed a more compact and spindle-like shape and a "hill and valley" morphology (Figure 4 c and f) resembling SMCs (Figure 4 b and e).

Expression of SM-αa, desmin, SM-MHC and smoothelin

Expression of two early (SM-αa and desmin) and two late (SM-MHC and smoothelin) SMC markers was determined by indirect immunofluorescence, while real-time RT-PCR looked at the transcritptional expression of desmin and smoothelin.

Indirect immunofluorescent staining showed expression of all four SMC markers in SK-UT-1, and heparin and RA differentiated isolated ADSCs (Figure 5), in particular RA differentiated SMCs. These results were supported by Western blot and dot blot analysis (results not shown).

To confirm differentiation of hADSCs into SMCs, real-time RT-PCR was performed on desmin and smoothelin (Figure 6). A 2-fold dilution series (1:25; 1:50; 1:100; 1:200) was used to form standard curves while samples were diluted 1:50 and run in triplicate. The BioBank cDNA Kit was run as a positive control. Results showed that RA differentiated cells expressed desmin with an average C_t value of 27.4. The positive controls, SK-UT-1 and heparin-differentiated cells, also expressed desmin with an average C_t value of 35.7 and 29.8 respectively. The melt curve analysis showed that a single product was formed in all tested sample cells for desmin during the PCR run and was melted at approximately 87.7°C (Figure 6 c). A number of optimising steps was performed with smoothelin. The best results were obtained with a primer concentration of 5 µM with 4.0 mM $MgCl_2$. Results showed that RA differentiated cells expressed smoothelin with an average C_t value of 36.6. The positive controls, SK-UT-1 and heparin-differentiated cells, also expressed smoothelin with an average C_t value of 37.2 and 39.7 respectively. The melt curve analysis showed that the samples displayed a single product during the PCR run and was melted at 89°C ± 2°C (Figure 6 d).

DISCUSSION

The science of TE and regenerative medicine has evolved in parallel with recent biotechnological advances. These future cell-based therapies will benefit from a source

of autologous stem cells that are pluripotential and readily accessible [8]. Subcutaneous adipose tissue is a particularly attractive reservoir of stem cells as it is easily accessible, abundant and self replenishing, and donor morbidity is minimal [6,23]. ADSCs are capable of proliferating to attain larger cell numbers, and of differentiating to obtain a tissue of interest [24]. ADSCs have the ability to differentiate into several lineages such as bone, cartilage, fat, endothelium, haematopoietic cells, hepatocytes, nerves, and skeletal, smooth and cardiac muscle cells [7]. TE of mesenchymal organs and tissues such as bone, cartilage, fat and muscle is of major interest in human diseases, such as traumatic, inherited, or degenerative bone, joint and soft tissue defects [5]. hADSCs can be isolated from a patient's own adipose tissue, cultured on specific matrices or scaffolds with lineage-specific growth factors that allow differentiation into a required tissue type, and ultimately the differentiated tissue could be grafted back into the same patient [3].

LILI involves the application of low power, monochromatic and coherent light to damaged tissue. LILI is a treatment that utilises light of a single wavelength; it emits no heat, sound, or vibration. Instead of producing a thermal effect, LILI acts via non-thermal or photochemical reactions in the cells [25]. In this instance, light is absorbed by a photoacceptor, known as a chromophore, which is excited and leads to primary molecular processes that can be measured [26]. Basically, photonic energy is absorbed by photon acceptor sites on the cell membrane which increases ATP production and membrane perturbation to lead to permeability changes, which will trigger a secondary messenger to initiate a cascade of intracellular signals that initiate, inhibit or accelerate

biological processes [27]. Karu suggested that the mechanism of LILI at the cellular level is based on the electronic excitation of chromophores in cytochrome c oxidase which modulates a redox status of the molecule and enhances its functional activity [28,29].

LILI causes a variety of biostimulatory effects, such as stimulation of cell proliferation, anti-inflammatory action, pain control, increase of local microvasculation, increased ATP synthesis, acceleration of collagen synthesis, facilitation of growth factor release, increased fibroblast activity and keratinocyte motility [30,31]. Studies on LILI and stem cells have shown that laser irradiation has been found to promote proliferation [20,32-34], increase stem cell migration [19], and enhance differentiation of stem cells [34-36]. LILI has been shown to increase cellular viability and protein expression in ADSCs up to 48 h post-irradiation [20,21]. An increase in ATP was seen 10 min post-irradiation in neural progenitor cells irradiated at 808 nm with 0.05 J/cm^2 [37]. Hou and colleagues found that irradiation of BM-MSCs at 635 nm with different fluencies up to 5 J/cm^2 did not induce damage cellular membrane damage as there was no increase in release of lactate dehydrogenase (LDH) [36]. A fluence of 0.05 J/cm^2 was found to be an optimal fluence to stimulate proliferation, while 5 J/cm^2 significantly increased secretion of vascular endothelial growth factor (VEGF) and nerve growth factor (NGF). A fluence of 5 J/cm^2 was also found to dramatically facilitate myogenic differentiation with 5-azacytidine (5-aza) induction [36]. Oron *et al.* concluded that following acute hepatectomy LILI stimulates the regeneration of the liver and is conducive to both the formation of new hepatocytes and MSCs and angiogenesis [38].

Smooth muscle is an active component of the cardiovascular, reproductive, urinary and intestinal systems, and has been the subject of intense research in the field of regenerative medicine. A restriction to date has been a reliable source of healthy SMCs, as biopsies normally lead to low cell harvest that needs to be expanded at length before therapeutic use. In addition, previous research has shown that SMCs that are obtained from diseased tissue can differ phenotypically and functionally from normal healthy SMCs, which consequently restricts their use [8]. ADSCs have the potential to differentiate into functional SMCs and consequently may prove a useful source of autologous cells for reconstruction of diseased human organs and tissues containing smooth muscle [8]. RA induces numerous genes during development and influences vascular SMC differentiation in vivo [15]. RA induced an embryonic carcinoma cell line to display a number of features of SMCs, such as expression of smooth muscle specific markers SM-αa and SM-MHC [39].

In this study, the effects of laser irradiation on isolated hADSCs after 24, 48 and 72 h were investigated *in vitro* and their differentiation potential into SMCs using RA. This study aimed to add to the current knowledge on the effects of laser irradiation on hADSCs by investigating an extended post-irradiation time-lapse of up to 72 h, which ultimately could aid in the elucidation of whether laser irradiation can maintain hADSCs in culture over extended periods of time.

Morphologically isolated and commercial hADSCs did not change post-irradiation at 636 nm with 5 J/cm^2 between 3 and 72 h, cells remained spindle shaped. This indicates that

irradiation at this fluence and wavelength does not appear to encourage differentiation into another cell type with varied morphological characteristics. Morphologically, irradiation at the current parameters does not appear to damage hADSCs, no dead cells were seen floating in the media, and irradiated cultures appeared denser than non-irradiated cultures.

The cellular viability results (Trypan blue staining and ATP luminescence) do not correlate with one another. It should be remembered that these two assays are based on different principles. Trypan blue staining is dependent on cellular membrane integrity, while ATP luminescence is dependent on the amount of ATP generated by viable active cells. There was an increase in cellular viability, as determined by Trypan blue staining, in both isolated and commercial hADSCs. This shows that under the specified laser parameters, irradiation does not affect the cellular membrane, and increases cellular viability. This effect is still evident 72 h post-irradiation. LILI modulates various biological processes, and has been found to increase mitochondrial respiration and ATP synthesis [34]. ATP luminescence only showed a significant difference in irradiated isolated hADSCs at 48 h. This increase was not seen in any of the other groups. An increase in ATP generation is an immediate, primary effect of LILI, leading to other secondary effects later on. It is possible that had ATP been measured sooner post-irradiation, an increase in irradiated cells may have been detected. Oron *et al.* saw an increase in ATP in neural progenitor cells 10 min post-irradiation [37].

Significant increases in cellular proliferation were seen in the isolated hADSCs 24 and 72 h post-irradiation as compared to their non-irradiated controls. This corresponds to the results found by Hawkins and Abrahamse [40] who observed an increase in cellular proliferation of human skin fibroblast cells (WS1) when treated with a Helium-Neon laser (632.8 nm) with 5 J/cm^2. Irradiating mitochondria with red light causes them to produce cytochromes, which increases their efficiency, and research has shown that muscle cells and fibroblasts grow five times faster when treated with red light [41]. Mvula *et al.* found an increase in proliferation in hADSCs at both 24 and 48 h post-irradiation [20]. Hou *et al.* found an increase in proliferation in BM-MSCs 2, 4, and 6 days post-irradiation [36].

Stem cell markers β1-integrin (CD29) and Thy-1 (CD90) have been observed in hADSCs in various laboratories [5,9,10,42,43]. Isolated and commercial hADSCs labelled with β1-integrin and Thy-1 was observed in both irradiated and non-irradiated groups up to 72 h post-irradiation. These results were further supported with Western blot and dot blot (results not shown). Mvula *et al.* [20,21] found expression of β1-integrin 24 h post-irradiation via immunofluorescence, and expression of Thy-1 24 and 48 h post-irradiation via Western blot. These results show that the cells isolated from the donated tissue are in fact stem cells, and that LILI maintained hADSC character throughout this time period and did not induce differentiation into another cell type where these proteins are not expressed. Expression of these stem cell markers were further confirmed by real-time RT-PCR. Expression of β1-integrin was consistent and

unchanged post-irradiation, while Thy-1 was down regulated by a mean factor of 0.793 48 h post-irradiation. The reason for this decrease is unknown.

The differentiation potential of isolated hADSCs into SMCs using RA was successfully shown. Results were comparable with heparin induction. There were definite morphological changes over the 14 day period, cells changed from thin elongated shape, to a more angular, star shape, with "hills and valleys". These changes were also observed by Wu and colleagues who differentiated ADSCs into myoblasts using 5-aza [44].

To establish that a particular cell is a mature SMC, certain unique markers must be present [11]. Some of the best characterised markers for contractile SMCs include SM-αa, caldesmon, SM22, calponin, SM-MHC and smoothelin. SM-αa is an early marker of developing SMCs, whereas the other markers are highly restricted to differentiated smooth muscle, particularly SM-MHC and smoothelin which are not expressed in any other cell type other than contractile (therefore functional) SMCs [11]. Differentiation into SMCs by RA and heparin as a control was confirmed by the expression of SM-αa, desmin, SM-MHC and smoothelin by immunofluorescence. Western blot and dot blot further supported their expression (results not shown). Expression of desmin and smoothelin was further confirmed by RT-PCR. Both immunofluorescence and RT-PCR showed that there was higher expression of desmin in both RA and heparin differentiated cells. During development, desmin expression precedes all the other muscle-specific structural genes and it may play some modulating role in myogenic

commitment and differentiation [45]. The low copy number of smoothelin and high copy number of desmin suggests that the majority of cells are in the early stages of differentiation. Growing cells for longer in SMC maintenance medium may increase the expression of late SMC proteins. The effect of LILI on differentiation of hADSCs should be investigated further.

In conclusion, the present study indicates that LILI at a wavelength of 636 nm and a fluence of 5 J/cm^2 (8.59 mW/cm^2) positively affects isolated hADSCs *in vitro* by increasing cellular proliferation and viability, and by maintaining stem cell morphology and protein expression for up to 72 h. Laser irradiation at this fluence and wavelength does not appear to induce differentiation into another cell type, even after an extended period of time. This study also showed that stem cells isolated from human adipose tissue can be differentiated into SMCs using RA. Since low yields of stem cells are isolated, and their proliferation rate is low, LILI could be a useful tool in relation to TE and stem cell therapies as the initial number of isolated stem cells could be significantly expanded before commencing differentiation, which would lead to a higher yield of differentiated cells. This information could be used to aid researchers in future TE and other regenerative medicine applications. Cell therapies that involve SMC differentiation may offer an alternative treatment for diseases that involve SMC pathology such as gastrointestinal diseases, bladder dysfunction, urinary incontinence and cardiovascular diseases.

ACKNOWLEDGEMENTS

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Table 1 PCR primers and three step PCR cycling program.

Genes	Primer Sequence	Product Size (bp)	Cycles	Temperature (°C)	Duration
[a]RG (UBC & GAPDH)	PrimerDesign, geNorm Housekeeping Gene Selection Kit	-	1	95	10 min
				95	5 s
			40	60	15 s
				72	15 s
β1-integrin	F:5'- CCTTACATTAGCACAACACC-3'	284	1	95	10 min
	R:5'-CATCTCCAGCAAAGTGAAAC-3'			94	10 s
			40	55	10 s
				72	20 s
Thy-1	F:5'-ATGAACCTGGCCATCAGCATCGC-3'	486	1	95	10 min
	R:5'-TCACAGGGACATGAAATCCGTGG-3'			95	5 s
			40	63	10 s
				72	15 s
Desmin	F: 5'-CCAACAAGAACAACGACG-3'	408	1	95	10 min

	R: 5'-TGGTATGGACCTCAGAACC-3'			95	5 min
			45	60	15 s
				72	20 s
Smoothelin	F: 5'- ATGGCGGACGAGGCCTTAG -3'	358	1	95	10 min
	R: 5'- CCTCAATCTCCTGAGCCC-3'			95	5 min
			45	60	15 s
				72	20 s

[a]RG Reference Gene

Table 2. The Trypan blue exclusion test (percentage viability, %) and ATP luminescence (Relative Light Units, RLU) was used to determine cellular viability, while optical density (A_{540} nm) was used to assess cellular proliferation. Statistical differences between irradiated (5 J/cm^2) and non-irradiated (0 J/cm^2) cells are shown as P≤0.05 (*), P≤0.01 (**), and P≤0.001 (***). ± Standard error.

Irradiation Time (h)	Percentage Viability (%)		ATP Luminescence (RLU)		Optical Density (A_{540} nm)	
	0 J/cm^2	5 J/cm^2	0 J/cm^2	5 J/cm^2	0 J/cm^2	5 J/cm^2
Isolated ADSC						
24	91 (±1)	93 (±0.5) ***	12820 (±4449)	20568 (±7799)	0.108 (±0.04)	0.159 (±0.03) *
48	92 (±1.4)	95 (±0.7) ***	17131 (±7185)	27854 (±4927) *	0.160 (±0.02)	0.180 (±0.03)
72	94 (±1.1)	95 (±0.9)	23598 (±14695)	33473 (±8532)	0.151 (±0.02)	0.196 (±0.04) *
Commercial ADSC						
24	91 (±1.6)	95 (±1.1) **	30069 (±6068)	34993 (±7120)	0.045 (±0.004)	0.047 (±0.004)
48	93 (±1.1)	95 (±1.3) *	36711 (±29959)	52030 (±24076)	0.053 (±0.01)	0.057 (±0.01)
72	95 (±1.1)	97 (±0.7) **	44774 (±22267)	56420 (±14253)	0.057 (±0.01)	0.063 (±0.01)

Fig. 1 Morphology (400x magnification) of non-irradiated isolated (a) and commercial (d) hADSCs at 24 h. Twenty four hours post-irradiation, isolated (b) and commercial (e) hADSCs showed no changes in cellular morphology. Differential interference contrast (DIC) microscopy also showed the same morphology between isolated (c) and commercial hADSCs (f).

Fig. 2 Expression of β1-integrin (red) and Thy-1 (green) in isolated (IADSCs) and commercial (CADSCs) hADSCs 72 h post incubation. Nuclei were counter stained with DAPI (blue). Non-irradiated (0 J/cm^2) isolated and commercial hADSCs showed expression of β1-integrin, and Thy-1, as did irradiated (5 J/cm^2) hADSCs.

Fig 3 Real-time RT-PCR was performed on β1-integrin and Thy-1 in isolated hADSCs 24, 48 and 72 h post laser irradiation, at 636 nm with a fluence of 5 J/cm^2. Results were normalised against UBC. Melt curve analysis shows a single product for both β1-integrin (a) and Thy-1 (b), which melted at 87.3 and 83.5°C respectively. The product on the far left is geNorm BioBank cDNA which was used as a positive control. REST was used to quantify and analyse results. β1-integrin expression was not altered (c), while Thy-1 was down regulated by a mean factor of 0.793 (d). Statistical differences are shown as P≤0.01 (**).

Fig. 4 Morphology was determined by inverted light microscopy (a-c) and differential interference contrast (DIC) microscopy (d-f), 200x magnification. Isolated hADSCs (a and d) were differentiated into SMCs with 10^{-7} M/L retinoic acid (c and f) over a 21 day period. SK-UT-1 cells were used as a positive SMC control (b and e). hADSCs were more long and slender in morphology, while the SK-UT-1 and differentiated SMCs had a more angular morphology.

Fig. 5 Isolated hADSCs (IADSCs) were differentiated into SMCs using retinoic acid (RA), heparin was used as a positive control, while SK-UT-1 cells was used as a SMC control. Expression of SMC markers smooth muscle alpha actin (SM-αa, red), desmin (green), smooth muscle myosin heavy chain (SM-MHC, red), and smoothelin (green) in differentiated SMCs was determined by indirect immunofluorescence. Nuclei were counter stained with DAPI (blue). Expression of all four markers can be seen in all the cells, particularly in RA differentiated SMCs.

Fig. 6 To confirm SMC differentiation, Real-Time RT-PCR was performed on SMC markers desmin (early marker) and smoothelin (late marker). A standard curve was performed on both desmin (a) and smoothelin (b) primer sets. Melt curve analysis shows a single product for desmin (c) and smoothelin (d), which melted at 87.7 and 89°C respectively. The product on the far left is geNorm BioBank cDNA which was used as a positive control between PCR runs.