Cytotherapy, 2015; 17: 359–368

International Society for Cellular Therapy
ISCT

PMID 25536863

ORIGINAL PAPERS

Effect of mild heat stress on the proliferative and differentiative ability of human mesenchymal stromal cells

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Abstract

Background aims. Mesenchymal stromal cells (MSCs) are an attractive candidate for autologous cell therapy, but regenerative potential can be compromised with extensive *in vitro* cell passaging. Development of viable cell therapies must address the effect of *in vitro* passaging to maintain overall functionality of expanded MSCs. *Methods.* We examined the effect of repeated mild heat shock on the proliferation and differentiation capability of human adipose-derived MSCs. Adipose tissue MSCs were characterized by means of fluorescence activated cell sorting analysis for expression of CD3, CD14, CD19, CD34, CD44, CD45, CD73, CD90 and CD105. Similarly, the expression of *SIRT-1*, p16^{INK4a} and p21 was determined by means of polymerase chain reaction. Measurements of population doubling, doubling time and superoxide dismutase activity were also determined. Differentiation of expanded MSCs into bone and adipose were analyzed qualitatively and quantitatively. *Results.* The strategy led to an increase in expression of *SIRT-1* concomitant with enhanced viability, proliferation and delayed senescence. The stressed MSCs showed better differentiation into osteoblasts and adipocytes. *Conclusions.* The results indicate that mild heat shock could be used to maintain MSC proliferative and differentiation potential.

Key Words: *heat shock, mesenchymal stromal cells, senescence*

Introduction

Mesenchymal stromal cells (MSCs) show therapeutic potential for tissue engineering and regenerative medicine applications. Adipose tissue represents a rich source of MSCs, with more abundant cells than other sources. MSCs from adipose tissue are easier to harvest, isolate, culture and expand than the bone marrow counterpart. However, several studies have suggested that age-related changes may make MSCs less effective in the treatment of certain diseases and disorders [1]. Similarly, *in vitro* passaging of cells has a negative impact on the regenerative potential of MSCs [2]. It has been shown that the adipogenic [3], osteogenic [4], chondrogenic [2] and myogenic [5] differentiation potential of MSCs declines with *in vitro* passaging. Because many promising tissue engineering applications require cell expansion after harvest, increased senescence would be a severe limitation for use.

The term "hormesis" is derived from a Greek word meaning "to excite" and refers to a cascade of beneficial biological effects in response to low doses of harmful stressors that are otherwise lethal in higher quantities. It has recently been shown that the hormetic effects of temperature can limit age-related dysfunction in cells and have recently been used with success to enhance stem cell functionality [6,7]. Culture temperatures below (32°C) [6] and above (41°C) [8] standard culture temperature have been shown to prevent stem cell aging and age-related impairments. Similarly, repeated heat stress has been shown to avert various characteristics associated with age [7,9]. However, the effects of heat stress on the growth characteristics and differentiation potential of MSCs largely remains unexplored.

The present study was designed to study the effect of repeated mild heat shock on the proliferation and

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(Received 26 February 2014; accepted 3 November 2014)

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http://dx.doi.org/10.1016/j.jcyt.2014.11.003

differentiation potential of human adipose-derived MSCs (AT-MSCs). AT-MSCs were analyzed for osteogenic and adipogenic potential along with various growth characteristics. The older "stressed" cells showed increased proliferative potential and better differentiation into osteoblasts and adipocytes as compared with older control cells. The results indicated that mild heat shock could be applied to AT-MSCs to maintain both proliferative and differentiation potential during *in vitro* expansion.

Methods

MSC isolation and expansion

Adipose tissue was harvested during a liposuction procedure ($n = 8$, 51.50 ± 5.17 years of age) with a 2.4-mm cannula or with a hand-held 10-mL syringe. All samples were obtained with written consent from the donors. MSCs from adipose tissue were isolated as described [10]. The tissue was washed with phosphate-buffered saline (PBS) and treated with 0.2% collagenase type IV in PBS for 20 min at 37°C. Collagenase activity was neutralized with 20 mL of fetal bovine serum (FBS) containing media and filtered through a sieve. The cell suspension was centrifuged for 10 min at 150*g* and the supernatant was discarded. The cells were seeded in 25-cm^2 culture flasks and incubated at 37°C with 5% CO_2 in humidity. The non-adherent cells were removed after 72 h to obtain a pure population of plastic adherent cells (MSCs).

MSCs were expanded in minimum essential medium (MEM, ThermoScientific) supplemented with 10% FBS and 1% each of non-essential amino acids, sodium pyruvate, L-glutamine and streptomycin/penicillin solution. The adipogenic AdvanceSTEM adipogenic differentiation medium (catalogue No. SH30886.02, ThermoScientific) supplemented with 10% AdvanceSTEM stem cell growth supplement (catalogue No. SH30b78.02) and osteogenic AdvanceSTEM osteogenic differentiation medium (catalogue No. SH30881.02, ThermoScientific) supplemented with 10% AdvanceSTEM stem cell growth supplement (catalogue No. SH30b78.02) media were used to induce MSCs into adipose and bone, respectively.

Flow cytometry

Cultured cells were examined for surface markers by means of flow cytometry. The cells were stained with the following primary antibodies: AF-700–conjugated CD3 (BD BioSciences), phycoerythrin (PE)-conjugated CD14 (BD, Immunocytometry), Allophycocyanin (APC)-conjugated CD19 (BD BioSciences), PE-conjugated CD34 (BD, BioSciences), APC-conjugated CD44 (BD, Pharmingen), fluorescein isothyocyanate–conjugated CD45 (BD Pharmingen), PE-conjugated CD73 (BD Pharmingen), AF-700–conjugated CD90 (Biolegend) and APC-conjugated CD105 (Biolegend). Samples were analyzed on an LSR II flow cytometer (BD Biosciences), and at least 10,000 events were acquired for each population. Data acquisition and analysis were performed with the use of FACS DIVA software (BD Biosciences). Unstained cells were used to establish flow cytometer settings. Debris and auto-fluorescence were removed by means of forward scatter.

Mild heat shock

Confluent primary cultures of MSCs (at passage 1) after trypsinization were divided into 2 groups: group 1 (control) and group 2 (subjected to mild heat shock). MSCs in group 2 were subjected to heat shock for 60 min in a water bath set at 41°C once in a week. No heat shock was given for at least 24 h after sub-culturing, and no sub-culturing was done within 24 h of heat shock. These conditions were maintained throughout the study. MSCs in group 1 were kept in the 37°C incubator. MSCs were cultured through 8 cell passages (approximately 7 weeks) before differentiation was induced.

Senescence-associated β-galactosidase staining

Cellular senescence was detected by means of senescence-associated β-galactosidase (SA–β-gal) staining (Cell Signaling). Briefly, 5×10^3 cells were seeded in 12-well plates at passages 5 and 8. After 24 to 48 h, cells were incubated with freshly prepared β-gal staining solution for 60 min at 37°C in the absence of CO_2. MSCs were washed with water, and blue-colored senescent cells were observed under microscopy. Phase-contrast images were taken, and the percentages SA–β-gal–positive cells were calculated by dividing blue-stained cells by the total number of cells, multiplied by 100.

Superoxide dismutase assay

A calorimetric assay (Abcam) was used to evaluate the activity of superoxide dismutase (SOD) produced as a result of heat shock. Briefly, at passages 5 and 8, protein was extracted from both groups of MSCs and the total protein extract (10 µg) was used to determine SOD activity. Absorbance values were measured with the use of Spectra max PLUS 384 (Molecular Devices) at 450 nm.

Viability assay

MSCs of both types (at passages 5 and 8) were treated with H_2O_2 (100 µmol/L) for 90 min (for

oxidative stress), recovered in normal MEM for 1 h, and incubated in expansion medium for 1 h. Cells were mixed with trypan blue (1:1) to determine the number of viable cells. The percentage of viable cells was calculated by dividing the number of trypan blue−negative cells by the total number of cells examined, multiplied by 100.

Furthermore, viability of MSCs after heat shock was evaluated by means of flow cytometry with the use of the Annexin V apoptosis detection kit (BD Pharmagen). Briefly, MSCs were subjected to mild heat shock for either 5 or 8 passages ($n = 3$ each). The cells were detached with the use of trypsin−ehtylenediaminetetra-acetic acid (EDTA) solution and washed twice with PBS. The cells were treated with Annexin V binding buffer, followed by incubation with PE−Annexin V antibody at room temperature for 30 min. 7-AAD (7-amino-actinomycin D) viability staining solution was added in solution, vortexed and incubated at room temperature for 15 min. Samples were analyzed on an LSR II flow cytometer (BD), and data acquisition and analysis were performed as described above.

Cumulative growth index

Both groups of MSCs were serially passaged to determine the number and time of cell doublings. The first confluent cultures after first heat shock were designated as passage 1 (P1). The sub-confluent cultures were dissociated with the use of trypsin-EDTA, counted and re-plated at a 1:10 dilution in a new 25 cm^2-culture flask. The initial and final number of cells were recorded at each passage. The cell doublings and doubling time were calculated [11,12] by use of the following formulas:

$$cPDs = Log10\,(N/N_o) \times 3.33$$

$$DT = CT/cPDs,$$

where N is the final number of cells, N_o is the initial number of cells seeded CT is the time in culture (PD indicates population doubling; DT, doubling time).

Proliferation assay

Cell proliferation assay was assessed with the use of an XTT (sodium 3−[1-{phenylaminocarbonyl}-3,4-tetrazolium]-bis {4-methoxy-6-nitro} benzene sulfonic acid hydrate) assay according to the manufacturer's instructions (Roche). Briefly, at passages 5 and 8, 4×10^3 MSCs/well were plated in triplicate in a flat-bottom, 96-well plate containing MEM with 10% FBS and incubated overnight at 37°C. The absorbance values were measured in Spectra Max Plus 384 (Molecular Devices) at 450 nm, with 650 nm as reference wavelength.

In vitro *osteogenic differentiation*

For osteogenic differentiation, 25,000 MSCs per well in a six-well plate were seeded in expansion medium. When cells became 90% confluent, cultures were treated with osteogenic induction medium (ThermoScientific) for 3 weeks. During induction, cell cultures were subjected to heat shock at days 3, 10 and 17. MSCs maintained in expansion medium alone at 37°C for 3 weeks were used as controls. All experiments were performed in triplicate.

After 21 days, von Kossa staining [12] was performed to detect extracellular matrix calcification in both groups of MSCs. von Kossa staining was performed with the use of a commercially available kit (IHC World). Briefly, paraformaldehyde (PFA)-fixed cultures (4%) were treated with silver nitrate for 60 min at room temperature under ultraviolet light, followed by treatment with sodium thiosulphate for 5 min. The cells were counter-stained with nuclear fast red and then photographed by means of phase-contrast microscopy. Extracellular matrix calcification was carried out by detection of black extracellular deposits.

For quantification of mineralized matrix deposition, ImageJ software (http://rsbweb.nih.gov/ij/) was used, which measured the amount of cellular staining (black) in a given field of view. Percentage-positive area was calculated by dividing the positively stained area divided by the total area, multiplied by 100.

Adipogenic differentiation

When the heat-shocked MSCs at passage 7 became confluent, the cells were seeded in triplicate in 12-well plates at a final cell density of 5000 cells per cm^2 in complete expansion medium. Differentiation of MSCs was initiated with the use of adipogenic induction medium (ThermoScientific), according to the manufacturer's instructions. During induction, cells were subjected to heat shock at days 3, 10 and 17. The induction medium was changed every 3 to 4 days, and experiments were terminated after 3 weeks. MSCs at the same cell density were maintained in expansion medium alone to serve as controls.

Adipogenic differentiation of MSCs was assessed by means of oil red O staining of cytoplasmic lipid-rich vacuoles [10,13] according to the manufacturer's instructions (IHC World). Briefly, paraformaldehyde PFA-fixed MSCs were washed with pre-stain solution and incubated with oil red O solution for 30 min at 60°C. Oil red O staining was followed by washing with 60% isopropanol and then several

changes of distilled water. Cells were counterstained with hematoxylin and eosin solution for 1 mine and visualized under phase-contrast microscopy.

Oil red O uptake was quantified by use of a previously published method [14]. Briefly, oil red O was extracted with isopropanol containing 4% nonidet P-40 detergent overnight at room temperature, and optical density was then measured at 490 nm [10,14]. All analyses were carried out in triplicate.

Total RNA extraction

Total RNA was extracted with the use of TRIzol reagent (Invitrogen) and an Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was determined with the use of an ND-1000 spectrophotometer (NanoDrop Technologies). Reverse transcription was performed with the use of a SuperScript III First-Strand synthesis system (Invitrogen), with the use of 1 μg of total RNA in a 20-μL reaction mixture containing 1 μL of 10 μmol/L oligo dt primer and 1 μL of reverse transcriptase enzyme (RT-enzyme).

Quantitative RT–polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed with the use of iTaq SYBR green supermix with ROX (Bio-Rad) in an ABI PRISM 7300 sequence detection system. The final reaction contained template complementary DNA, iTaq SYBR green and gene-specific primers (Table I). β-Actin was used as an internal control. The CT (cycle threshold) values of β-actin and other specific genes were acquired after PCR. The normalized fold expression was obtained by use of the $2^{-\Delta\Delta CT}$ method. The results were expressed as the normalized fold expression for each gene. To minimize PCR reaction variations, all samples were transcribed simultaneously.

Statistical analysis

Experimental data were analyzed with the use of Graphpad Prism 5 Software. One-way analysis of variance was used when three or more groups within one variable were compared. To analyze two groups, the unpaired *t*-test was used. The data are expressed as mean ± standard error of the mean. Values of $P < 0.05$ were considered significant.

Results

Expression of surface markers in isolated MSCs

Flow cytometry was performed to confirm MSC identity. Analysis showed that cells isolated from adipose tissue exhibited phenotypic characteristics of MSCs: being strongly positive for CD44, CD73, CD90 and CD105 while lacking expression of hematopoietic markers CD3, CD14, CD19, CD34 and CD45 (data shown in Supplementary Figure 1). These results are in agreement with previously published reports from our laboratory and other laboratories [10,12,15,16].

Table I. Gene-specific primers.

Gene	Primer sequences (5′-3′)
Collagen type 2	GGCAATAGCAGGTTCACGTACA (F)
	CGATAACAGTCTTGCCCCACTT (R)
Osteocalcin	GGCAGCGAGGTAGTGAAGAG (F)
	CTGGAGAGGAGCAGAACTGG (R)
Lipoprotein lipase	GTCCGTGGCTACCTGTCATT (F)
	TGTCCCACCAGTTTGGTGTA (R)
NSE	CTGATGCTGGAGTTGGATGG (F)
	CCATTGATCACGTTGAAGGC (R)
Aggrecan	TCAACAACAATGCCCAAGAC (F)
	AGCGACAAGAAGAGGACACC (R)
Alkaline phosphatase	GACCCTTGACCCCCACAAT (F)
	GCTCGTACTGCATGTCCCCT (R)
PPAR-γ	AAGACCACTCCCACTCCTTTG (F)
	GTCAGCGGACTCTGGATTCA (R)
NFM	TGGGAAATGGCTCGTCATTT (F)
	CTTACTGGAAGCGGCCAATT (R)
p16	CCCAACGCACCGAATAGTTA (F)
	ACCAGCGTGTCCAGGAAG (R)
p21	GGCAGACCAGCATGACAGATT (F)
	GCGGATTAGGGCTTCCTCT (R)
Sirt-1	GCCAGAGTCCAAGTTTAGAAGA (F)
	CCATCAGTCCCAAATCCAG (R)
β-Actin	AGAGCTACGAGCTGCCTGAC (F)
	AGTACTTGCGCTCAGGAGGA (R)

MSC morphology

Cells freshly isolated (~10 days) from adipose tissue showed plastic adherent growth and a fibroblastic morphology (Figure 1A). Culture-related alterations in cell morphology were reduced in group 2 MSCs (~8 weeks) as compared with group 1 MSCs (~8 weeks). The "stressed" cells were thin and spindle-shaped as compared with control. Figure 1B shows that control cultures had cells with increased cell size and a flattened appearance, a typical age-related alteration in morphology as compared with stressed cells (Figure 1C).

Mild heat shock delays cellular senescence in MSCs

Cell senescence after mild heat shock was determined by use of SA–β-gal staining and by measuring messenger RNA expression of the p16 and p21 genes. The messenger RNA levels of the p16 and p21 genes, which are associated with senescence, were

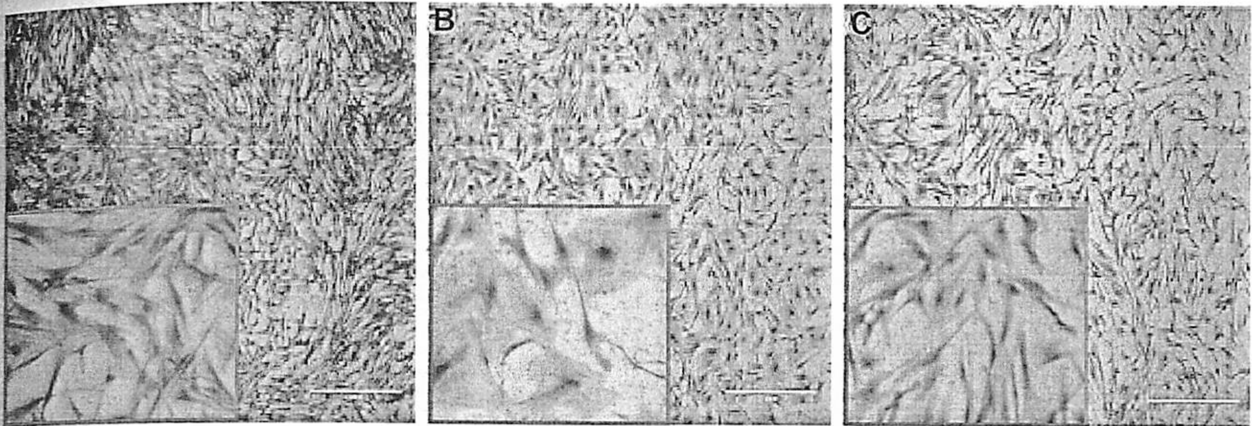

Figure 1. Heat-stressed MSCs show fewer alterations in morphology. Freshly isolated (~10 days) MSCs showed a spindle-shaped morphology (A). However, during expansion (~8 weeks), culture-related alterations in cell morphology were observed (B). MSCs subjected to heat shock (~10 days) during expansion (C) exhibited reduced alterations and were thin and spindle-shaped as compared with control expanded MSCs. Insets show morphology of each group at higher magnification.

analyzed by means of PCR [17]. The expression of both genes was significantly higher in the controls (group 1) than in the heat shock group, as shown in Figure 2A. Similarly, SA–β-gal activity was analyzed. This assay has been a commonly used biomarker for the identification of senescent cells [18]. A significant increase in the percentage of positive SA–β-gal cells was observed with passaging. However, in group 2, the rate of SA–β-gal–positive cells decreased at passage 8 (Figure 2B). In addition, expression of the *Sirt-1* gene was higher in MSCs after treatment with repeated mild heat shock as shown by PCR analysis. This upregulation of *Sirt-1* expression might have contributed to lowering senescence [19]. These results showed that mild heat shock is associated with a decrease in the expression

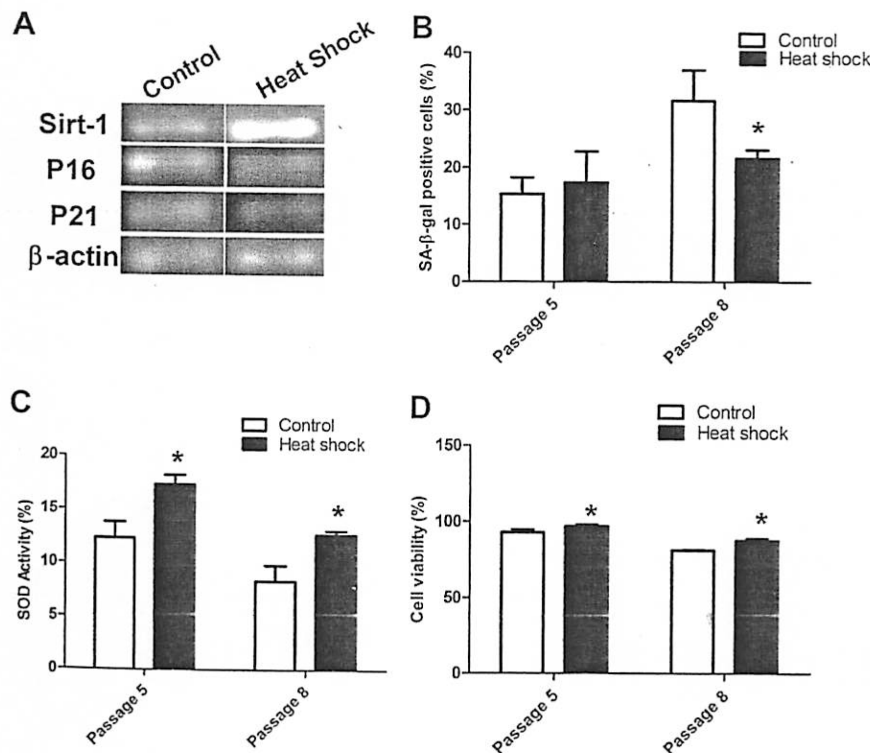

Figure 2. Assessment of age-related parameters in *in vitro*–expanded AT-MSCs subjected to heat shock. (A) Gene profiling of MSCs indicated that gene expression of *Sirt-1* was higher after heat shock. In contrast, gene expression of p16 and p21 was downregulated after heat shock. (B) Similarly, cultures subjected to heat shock showed reduced senescent features as determined by SA–β-gal staining compared with control cultures. Concomitantly stressed AT-MSCs had higher levels of SOD activity (C) and overall cell viability (D) after heat stress at both passages 5 and 8. Results are expressed as mean ± standard deviation. *$P < 0.05$.

of SA−β-gal activity and thus delayed the onset of senescence at later cell passages.

SOD activity

SOD activity was determined in MSC cultures after heat shock treatment. SOD is an antioxidant enzyme that catalyzes the conversion of superoxide radical anions (O^{2-}) to hydrogen peroxide, which is then catalyzed to O_2 and H_2O by glutathione peroxidase and catalase. The accumulation of intracellular oxidative damage caused by reactive oxygen species is thought to be involved in decreasing regenerative potential of cells. The activity of antioxidant enzymes such as SOD decreases progressively with *in vitro* aging [20]. SOD activity was significantly higher in group 2 (heat shock) cultures as compared with group 1 cultures at all cell passages studied (Figure 2C).

Effect of mild heat stress on cell viability

Trypan blue staining showed a significant change in the viability of heat shock and expanded MSCs compared with control expanded MSC. The results showed a significantly higher percentage of viable MSCs in group 2 as compared with group 1 at both passage 5 (92.3 ± 1.2 versus 97.7 ± 0.8) and passage 8 (81.3 ± 0.3 versus 87.7 ± 1.5) (Figure 2D). The percentage of viable cells after mild heat shock was also determined by means of flow cytometric analysis with the use of the 7-AAD/Annexin V apoptosis detection kit. These results were comparable with those obtained with trypan blue (Supplementary Figure 2).

Mild heat shock alters MSC proliferative potential

MSCs underwent cellular senescence (as evidenced by upregulation of p16 and p21 gene expression and increased number of SA−β-gal−positive cells) with decreasing proliferation capacity with increasing cell passaging. The effect of mild heat stress on MSC growth characteristics was thus analyzed. MSC numbers recorded at each cell passage were used to calculate the maximum number of population doublings and the doubling time. There was a significant difference in the number of maximum population doublings between group 1 and group 2 (Figure 3A), with shorter doubling times in group 2 (Figure 3B).

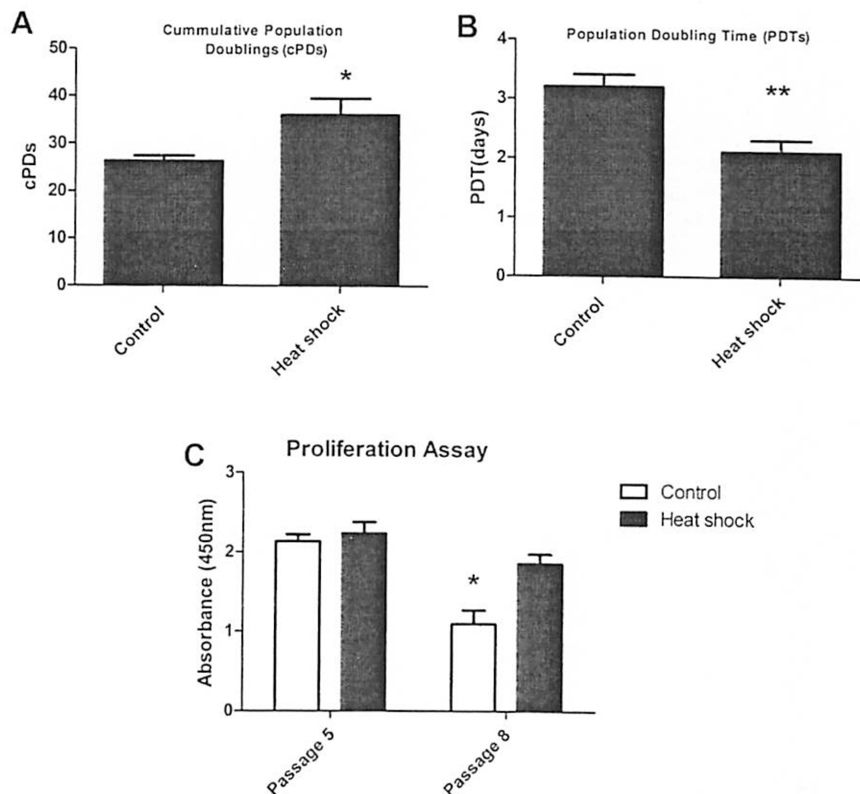

Figure 3. Growth characteristics of expanded and heat-shocked AT-MSCs. MSC proliferation was significantly higher in cultures that were subjected to repeated mild heat shock (A) during expansion. The number of population doublings increased, whereas the time per population doubling decreased (B) in stressed cells as compared with control cells. (C) The proliferative potential of cells was also assessed at passages 5 and 8 and was observed to be greater in MSCs subjected to heat shock during expansion. Results are expressed as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$ for heat-shocked AT-MSCs versus control AT-MSCs.

Heat-shocked MSCs achieved 36.0 ± 3.4 doublings, whereas MSCs in group 1 (control) achieved 26.2 ± 1.1 doublings. The doubling time was 3.2 ± 0.2 days in group 1 as compared with 2.1 ± 0.2 days in group 2.

Similarly, an XTT assay was performed to compare the proliferative potential of control and heat-shocked cultures at passages 5 and 8. Significant differences in proliferation between the two groups were observed in later passages (Figure 3C). Higher proliferation was observed in cells treated with mild heat shock. These results demonstrated that heat shock can alter the proliferative potential of MSCs when it is applied to expand cells before final use.

MSC osteogenic potential increases with repeated mild heat shock

After mild heat shock, MSCs were induced to differentiate into osteoblasts at passage 8. In the osteogenic induction medium, cells underwent dramatic morphology changes; undifferentiated MSCs remained fibroblastic, whereas induced MSCs formed aggregates containing calcium deposits. At 3 weeks, von Kossa staining [21] revealed a positive extracellular matrix formation in the induced AT-MSCs (Figure 4A–C). Cell aggregates were found along with dark regions of minerals. Heat-shocked MSCs (Figure 4C) produced more matrix than did non–heat-shocked MSCs (Figure 4B). Comparative quantification of von Kossa staining by means of ImageJ software is shown for all groups (Figure 4D). Significant differences between group 2 (19.8% ± 1.3%) and group 1 (11.0% ± 1.3%) were observed in this regard.

Lineage-specific expression of two osteogenic genes, alkaline phosphatase and osteocalcin [22], was assessed by means of real-time RT-PCR 21 days after induction. The expression of both of these genes was

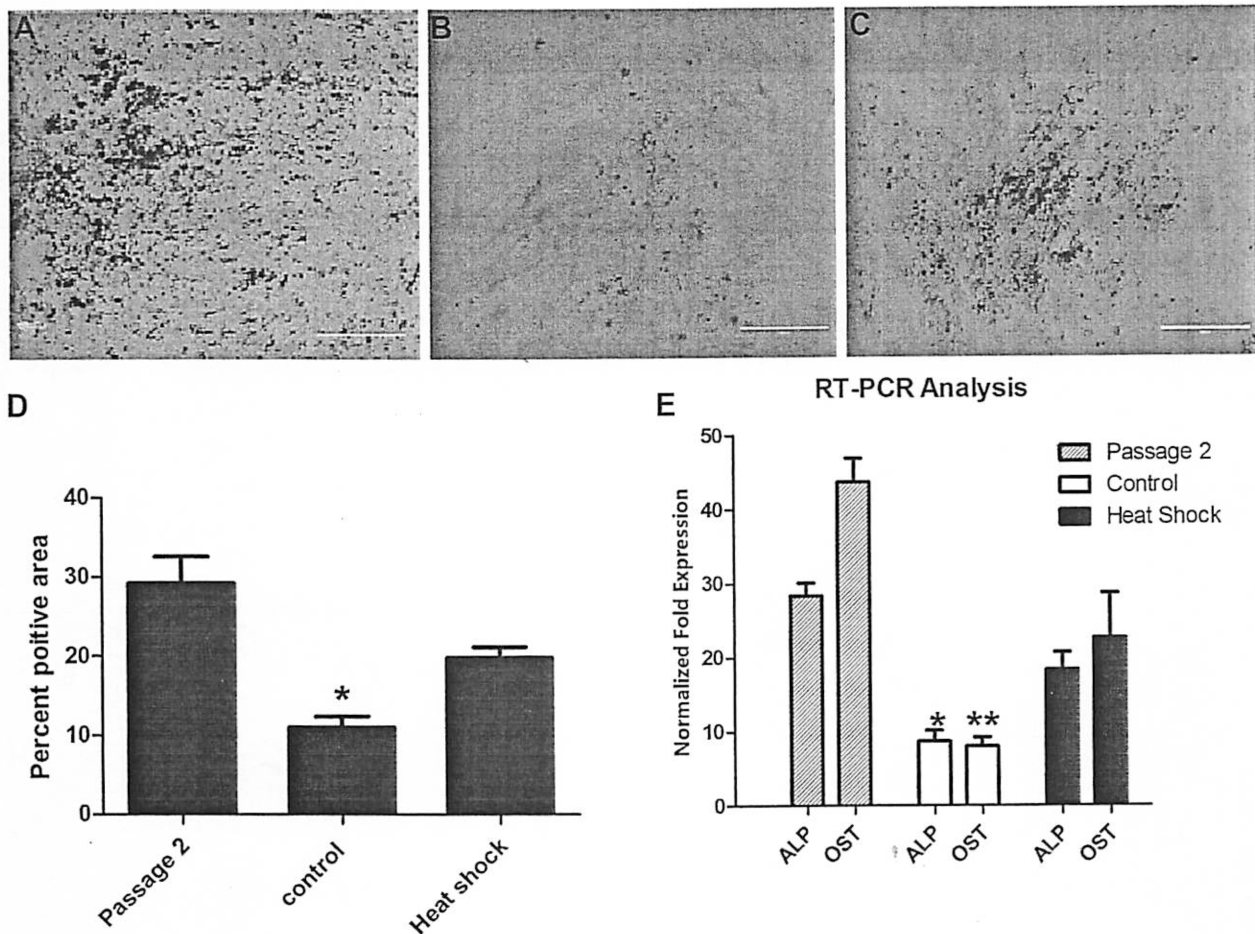

Figure 4. Expanded AT-MSCs subjected to heat shock have higher osteogenic potential. Osteogenesis was assessed by use of von Kossa staining after MSC expansion. (A–C) Representative figures show matrix mineralization in cultures of induced MSCs at passage 2, passage 8 (non–heat shock) and passage 8 (heat shock), respectively. Differences in osteogenic potential between heat-stressed and control expanded MSCs were measured with the use of ImageJ software. AT-MSCs subjected to heat shock showed more matrix mineralization than did control cultures (D). Similar results were obtained when gene expression of the osteocalcin (*OST*) and alkaline phosphatase (*ALP*) genes were analyzed by means of quantitative RT-PCR (E). Results are expressed as mean ± standard deviation. $*P < 0.05$, $**P < 0.01$, for heat-shocked AT-MSCs versus control AT-MSCs.

upregulated in differentiation conditions compared with undifferentiated samples. Furthermore, heat shock treatment enhanced expression of these osteo-specific genes (Figure 4E). However, as compared with induced MSCs at passage 2, the expression of both genes was lower in the heat shock group.

Adipogenic differentiation potential of AT-MSCs

In adipogenic-induced MSC cultures, significant morphological changes were observed. The induced cells had a larger, flatter morphology, full of lipid vacuoles. Adipogenic experiments were terminated after 3 weeks, and oil red O was used to stain for lipid-rich vacuoles [10,12,23] (Figure 5A–C). Quantification of oil red O uptake indicated a statistically significant difference between initial (passage 2) and expanded MSC cultures. However, oil red O uptake was higher in expanded MSC cultures that were exposed to repeated heat shock as compared with expanded but non–heat-shocked cultures (Figure 5D). Real-time RT-PCR was used to confirm histochemistry findings through the use of two different genes that are known markers of adipogenesis: peroxisome proliferator-activated-receptor-γ (PPAR-γ) and lipoprotein lipase (LPL) [14,23]. As shown in Figure 5E, the expression of both adipogenic specific genes was significantly higher in expanded cultures treated with heat shock as compared with control cultures at passage 8.

Discussion

Human MSCs isolated from various sources may be used for therapeutic applications, as indicated by the increasing number of ongoing clinical trials (www.clinicaltrial.gov). This promising therapeutic potential of MSCs for regenerative medicine applications still has challenges to clinical applications. One such challenge is the replicative senescence during *in vitro* expansion that reduces cell functions such as proliferation and differentiation. Therefore, development of viable cell therapies for treatment of various diseases requires optimization of the *in vitro* cell

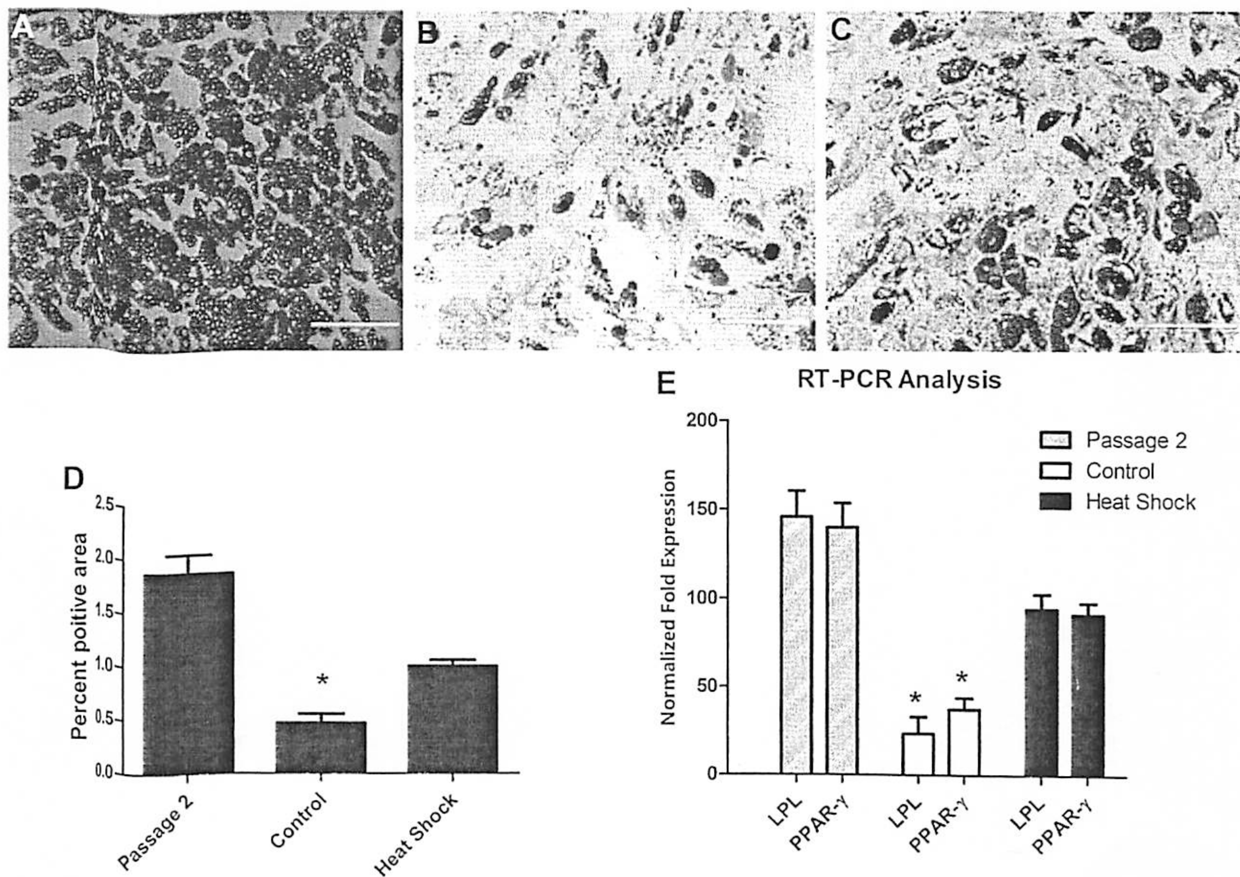

Figure 5. Mild heat shock enhances adipogenic potential of expanded AT-MSCs. Adipogenic differentiation of expanded AT-MSCs was performed after heat shock. Adipogenesis was confirmed through oil red O staining (A–C) followed by quantification of oil red O uptake. Heat shock enhanced adipogenic differentiation levels as indicated by oil red O uptake (D). Adipogenic differentiation was further confirmed by means of real-time RT-PCR (E). Results are expressed as mean ± standard deviation. $^*P < 0.05$ for heat-shocked AT-MSCs versus control AT-MSCs.

expansion methods to maximize the therapeutic benefits.

In the current study, we investigated the effects of mild heat shock on cell proliferation and differentiation potential. The results presented in this study provided evidence for the beneficial effects of repeated mild heat shock in maintaining youthful characteristics while human adipose-derived MSCs undergo *in vitro* expansion. We found that MSC function is severely affected with increasing cell passage, whereas these characteristics were well-maintained with mild heat shock treatment. These results may serve as a starting point for the development of clinically compliant procedures for *in vitro* MSC expansion needed for cell-based therapies.

Sirt-1 is a mammalian homologue of *Sirt-2* that has a protective role in endothelial regeneration [24], but its expression decreases progressively in culture during senescence [17]. Several studies indicate that *Sirt-1* is involved in inhibiting apoptosis and senescence [25] and enhances proliferation [26]. PCR analysis indicates that expression of *Sirt-1* was upregulated after treatment with mild heat shock. This finding agreed with observations of decreased expression of p16 and p21 in treated cells, along with fewer SA–β-gal–positive cells after heat shock. Similarly, SOD activity was also improved, and mild heat stress also restored MSC function. Another important parameter that describes the ability of cells to cope with stressful conditions is the survival rate of cells. We stressed the cells by means of hypoxic insult, and viability was measured with the use of two parameters, namely, the trypan blue exclusion assay and an Annexin V (coupled with 7-AAD) apoptosis detection system. Mild heat stress enabled the cells to better endure hypoxic conditioning. At different time points (passages 5 and 8), the viability was significantly higher in the treated groups as compared with control. The results of both assays were comparable in this regard. In addition, passaged and heat-shocked MSCs maintained a typical MSC phenotype as CD44+, CD73+, CD90+ and CD105+ while being CD3−, CD14−, CD19−, CD34− and CD45− [27].

A self-renewal capability and an ability to differentiate along both mesenchymal and non-mesenchymal lineages makes AT-MSCs an ideal source for regenerative medicine applications. The mild heat shock treatment used in our experiments showed that this strategy maintains the above-mentioned features of MSCs that are otherwise lost with *in vitro* expansion. There was a significant increase in the number of maximum population doublings with heat shock, in agreement with shorter doubling times. In addition, mild heat shock treatment enhanced differentiation of MSCs into adipocytes and osteoblasts, as shown by histology and confirmed by RT-PCR, during expansion; as recommended by recent reports from the International Federation for Adipose Therapeutics and Science and International Society for Cellular Therapy societies [27]. Adipogenesis was measured through oil red O staining followed by quantification of oil red O uptake. Periodic heat shock enhanced adipogenic differentiation levels, as indicated by oil red O uptake. These results of adipogenic differentiation were further confirmed by real-time RT-PCR of lineage-specific genes *LPL* and *PPAR-γ*, which are lipid exchange enzyme and fat transcription factor, respectively. Similarly, differentiation into osteogenic lineage was confirmed by means of matrix mineralization detection and by comparing osteogenic markers (alkaline phosphatase and osteocalcin) quantitatively. Greater matrix mineralization and higher expression of these genes was observed in MSCs treated with mild heat shock. Overall, our results demonstrated that periodic mild heat shock during *in vitro* expansion can be a potential approach to enhance MSC differentiation and in turn their potential use for cell-based therapies and tissue engineering.

MSC therapy as seen with tissue engineering and regenerative medicine applications is expanding rapidly. However, because of limited availability in adult tissues, there is a need for *in vitro* cell expansion before treatment, which raises concerns for their use. Safe strategies that can maintain cell potential during expansion are required. In the current study, we used one such strategy that might be useful in this regard. The present study demonstrated the beneficial effect of mild and repeated heat shock on the expansion and differentiation potential of AT-MSCs. Our results demonstrated that the growth kinetics and the osteogenic and adipogenic potentials of AT-MSCs were enhanced when treated with mild heat shock, particularly at later cell passages.

Acknowledgments

This work was supported in part by Adicyte, Inc. The authors wish to acknowledge the assistance of John Pierce, MD, for the adipose tissue collections. The authors also wish to acknowledge our undergraduate students, especially Faryal Sharif, Briana Choi and Hee Ju, for their help in this study.

Disclosure of interests: Harris is the Chief Science Officer for Adicyte, Inc. Badowski is a consultant to Adicyte. The other authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcyt.2014.11.003.