Impact of early subcultures on stemness, migration and angiogenic potential of adipose tissue-derived stem cells and their resistance to in vitro ischemic condition

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Abstract Adipose tissue-derived stem cells (ADSCs) are capable of multipotential differentiation and express several angiogenic, anti-apoptotic and immunomodulatory markers. These features make adipose tissue as a promising source of stem cells for regenerative medicine. However, for efficient translational use, culture-induced changes in the gene expression profile and resistance of the ADSCs to ischemic environment should be taken into consideration. We compared the expression of some clinically important markers between the unpassaged and third-passaged ADSCs by RT-PCR, qPCR and flow cytometry. Our results demonstrated that the embryonic stem cell (ESC)-specific markers were expressed in the unpassaged ADSCs but were downregulated after three passages. The expression of stemness-related genes, *TGFB* and *FGF2*, was upregulated while *FGF4* and *LIF* were downregulated after three passages. The expression of angiogenic genes in the third-passaged ADSCs was higher than the unpassaged cells. Epithelial-mesenchymal transition (EMT) markers were either expressed in the third-passaged ADSCs or significantly upregulated

after three passages. In contrast, cell cycle inhibitors, *CDKN1A* and *TP53*, were downregulated with early subcultures. The unpassaged and third-passaged ADSCs showed nearly similar resistance to oxidative stress, hypoxia and serum deprivation. In conclusion, the primary cultures of human adipose tissue contain a subpopulation of cells expressing ESC-specific genes and proteins, but the expression of these pluripotency markers subsides rapidly in standard mesenchymal stem cell culture medium. The expression of angiogenic and EMT markers also varies with early subcultures. Altogether, early-passaged ADSCs may be better choices for transplantation therapy of injured tissues, especially after ischemic conditions.

Keywords Early subculture · Gene expression · ADSC · Hypoxia · Oxidative stress · Serum deprivation

Introduction

Adipose tissue has been identified as an abundant and easily accessible source of mesenchymal stem cells (MSCs) (Schaffler and Buchler 2007). It also contains a subpopulation of cells which express several embryonic stem cell (ESC)-related genes (Greco et al. 2007; Peroni et al. 2008). We previously showed that mouse stromal vascular fraction expresses Oct4, Sox2 and Nanog genes/proteins, but the expression of these pluripotency markers decreases with culture and subsequent passages (Taha and Hedayati 2010; Taha

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et al. 2014). These findings were in agreement with some reports regarding human ADSCs (Izadpanah et al. 2006; Peroni et al. 2008).

The pluripotent stem cells originated from adult tissues have useful characteristics which make them an alternative source of stem cells for regenerative medicine. These cells are non-tumorigenic and contribute to chimeric offspring when injected into a mouse model (Jiang et al. 2002; Kuroda et al. 2010; Simerman et al. 2014). However, isolation of a sufficient number of pluripotent stem cells, especially from older patients, and proper expansion of these cells need further investigation.

Adipose tissue-derived stem cells (ADSCs) have an inherent capacity for migration toward the injury sites (Baek et al. 2011) and produce several biologically active factors which mediate their immunomodulatory, proangiogenic, protrophic, and antiapoptotic properties (Mattar and Bieback 2015; Rasmussen et al. 2014; Rehman et al. 2004; Sawada et al. 2015). These characteristics have major contributions to the therapeutic potential of ADSCs.

During culture-expansion of the ADSCs some changes may happen in the expression of genes and proteins which play key roles in regenerative medicine. Moreover, resistance of the ADSCs to ischemic environment may vary with early subcultures. For efficient translational use, these culture-induced changes should be taken into consideration. In the current study, we determined the impact of early subcultures on the expression of some pluripotency, angiogenic and epithelial-mesenchymal transition (EMT)-related genes. The expression of cell cycle inhibitors, *CDKN1A* and *TP53*, and sensitivity of the ADSCs to oxidative stress, hypoxia and serum deprivation were also investigated.

Experimental procedures

Isolation and culture of human ADSCs

Adipose tissue samples were collected from 5 women who underwent elective abdominoplasty in Shariati Hospital of Tehran, Iran. This study was approved by the Ethics Committee of National Institute of Genetic Engineering and Biotechnology (7-8-93/NIGEB), and all patients gave their informed consent. The patients had not been on any type of dietary restriction but on a clear

liquid diet the day before surgery. Adipose tissues were washed in sterile phosphate buffer saline (PBS), minced into small pieces and digested at 37 °C in PBS containing 2% bovine serum albumin (BSA) and 2 mg/ml collagenase I (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) for 20–30 min on a stirrer. After digestion, stromal vascular fraction (SVF) was sedimented by centrifugation, resuspended in fresh medium, filtered through a 100 µm nylon filter mesh (BD Biosciences, Franklin Lakes, NJ, USA) and plated at 5×10^4 cells/ml in tissue culture flasks. Growth medium contained Dulbecco's Modified Eagles Medium (DMEM), 20% fetal bovine serum (FBS), 100 µ/ml penicillin and 100 µg/ml streptomycin (all from Gibco, Thermo Fisher Scientific, Grand Island, NY, USA). The cells were passaged after reaching 80–90% confluency. The culture medium was changed every 2 days.

Flow cytometry

The ADSCs were detached using Trypsin–EDTA and fixed in 70% cold ethanol. After washing, the cells were incubated with the primary antibodies against CD73, CD90, CD105 and CD45 proteins (all from Abcam, Cambridge, UK), SSEA4 (Invitrogen) and OCT4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C for 30 min. For OCT4 staining, the cells were also permeabilized with 0.2% Triton X-100 solution. Then, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO, USA) or phycoerythrin (PE)-conjugated donkey anti-rabbit IgG (Abcam) for 30 min at room temperature. The cells only stained with the secondary antibodies were considered as negative controls. Flow cytometry was performed by a BD FACSCalibur™ (BD Biosciences). Analysis of the results was performed by FlowJo 7.6.1 (Tree Star Inc., Ashland, OR, USA).

Multipotential differentiation capability

For adipogenic differentiation, third-passaged ADSCs were cultured in a medium containing DMEM, 10% FBS, 1 µM dexamethasone, 100 µM indomethacin, 5 µg/ml insulin and 500 µM isobutylmethylxanthine (IBMX) for three weeks. Then, the differentiated cells were fixed using 4% paraformaldehyde and stained using Oil red O for lipid accumulation.

For osteogenic differentiation, the third-passaged cells were cultured in a medium containing DMEM, 10% FBS, 10^{-8} M dexamethasone, 10 mM glycerol phosphate, 3.7 g/l sodium bicarbonate and 0.05 g/l ascorbic acid (all from Sigma) for two weeks. Calcium deposits were assessed using Alizarin Red S staining.

For cardiomyogenic differentiation, third-passaged ADSCs were cultured in a medium containing DMEM, 10% FBS, 10 ng/ml basic fibroblast growth factor (bFGF; Sigma) and 20 ng/ml BMP4 (Invitrogen) for four days. After this initial phase of induction, differentiation of the cells was continued in 10% FBS-containing medium up to three weeks. Then, the expression of cardiac troponin I was evaluated by immunocytochemistry.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) and treated with DNase I to avoid genomic DNA contamination. Then, 1 µg of total RNA was subjected to cDNA synthesis using cDNA Synthesize Kit (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was used as template for PCR using Maxima Hot Start PCR Master Mix (Thermo) and specific primers described in Table 1. PCR products were visualized by 2% agarose gel electrophoresis.

Table 1 Primers used for RT-PCR and quantitative real-time PCR

Target	Forward	Reverse	Size	Accession
B2M	5'-TCCAGCGTACTCCAAAGATTCA-3'	GTCAACTTCAATGTCGGATGGAT-3'	113	NM-004048
GAPDH	5'-CAAATGAGCCCCAGCCTTCT-3'	5'-TCACCATCTTCCAGGAGCGA-3'	116	NM_002046
OCT4A	5'-GATGTGGTCCGAGTGTGGTT-3'	5'-AAGGGACCGAGGAGTACAGT-3'	202	NM_002701
OCT4B	5'-AGACTATTCCTTGGGGCCACAC-3'	5'-AAATAGAACCCCCAGGGTGAGC-3'	228	NM_203289
OCT4B1	5'-AGACTATTCCTTGGGGCCACAC-3'	5'-CTTAGAGGGGAGATGCGGTCA-3'	272	NM_001285986
NANOG	5'-GAAGCATCCGACTGTAAAGAATC-3'	5'-TGGTGGAAGAATCAGGGCTGT-3'	169	NM-024865
SOX2	5'-AGAACCCCAAGATGCACAAC-3'	5'-TCCTTCTTCATGAGCGTCTTG-3'	184	NM-003106
KLF4	5'-ATTACCAAGAGCTCATGCCACC-3'	5'-GTGTGCCTTGAGATGGGAAC-3'	158	NM-004235
MYC	5'-CTACCCTCTCAACGACAGCAG-3'	5'-CTCATCTTCTGTTCCTCCTCAG-3'	190	NM-002467
LIN28	5'-TTCCATGTGCAGCTTACTCT-3'	5'-CAGCAGTTTGAGGTGGC-3'	228	NM_024674
NODAL	5'-TGGCTGGTGGAAAATCTCAATG-3'	5'-GCAGAAGATGTGGCAGTGGA-3'	165	NM_018055
TGFB1	5'-CTATTGCTTCAGCTCCACGGA-3'	5'-AGGACCTTGCTGTACTGCGT-3'	171	NM_000660
FGF2	5'-CCCTCACATCAAGCTACAAC-3'	5'-GTGTATTTCCCTTGACCGGTAA-3'	192	NM_002006
FGF4	5'-TATGGCTCGCCCTTCTTCAC-3'	5'-TCGGTTCCCTTCTTGGTCT-3'	141	NM_002007
LIF	5'-TCTTGGCGGCAGGAGTTGT-3'	5'-CCTGGGCTGTGTAATAGAGAA-3'	204	NM_002309
VEGFA	5'-ATGTCCACCAGGTCTCGATT-3'	5'-AACTTTCTGCTGTCTTGGGTG-3'	179	NM_003376
HIF1A	5'-TCCTGTGGTGACTTGTCTTTA-3'	5'-TTTCTCAGTCGACACAGCCT-3'	187	NM_001530
HIF1B	5'-TGATTTTCCCTGGCAAGTCTCT-3'	5'-AGGGCTGGATTTTGATGATGATG-3'	147	NM_001668
ENG	5'-ATCCTTGAAGTCCATGTCCTCT-3'	5'-TGGAGATGCAGGAAGACACT-3'	149	NM-001114753
SNAI1	5'-CACTGGTACTTCTTGACATCTG-3'	5'-ATGCACATCCGAAGCCACAC-3'	203	NM_005985
SNAI2	5'-ACCTGTCTGCAAATGCTCTGTT-3'	5'-GCGATGCCAGTCTAGAAAAT-3'	225	NM_003068
TIMP1	5'-CCTTTATACATCTTGGTCATCTTG-3'	5'-TCCTGTTGTTGCTGTGGCTGA-3'	181	NM_003254
MMP2	5'-GAGTCCGTCCTTACCGTCAAA-3'	5'-TTGGCTACACACCTGATCTGG-3'	185	NM_004530
CXCR4	5'-AATAGTCAGCAGGAGGGCAG-3'	5'-CAGTCAACCTCTACAGCAGTG-3'	133	NM_001008540
FOXC2	5'-AGTATTTTCGTGCAGTCGTAGGA-3'	5'-ATGTTCAACTCCCACCGGCT-3'	148	NM_005251
CDKN1A	5'-CTGTCTTGTACCCTTGTGCCTC-3'	5'-TGGAGTGGTAGAAATCTGTCATG-3'	118	NM_000389
TP53	5'-TAATCTACTGGGACGGAACA-3'	5'-CAGTGCTCGCTTAGTGCTC-3'	139	NM_000546

Quantitative real-time PCR (qPCR) analysis

After cDNA synthesis, qPCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) on a Rotor-GeneTM 6000 (Corbett Research, Mortlake, NSW, Australia) real-time PCR machine. Comparative quantification was performed between different groups using REST 2009 software (Relative Expression Software Tool, Qiagen GmbH, Hilden, Germany) (Pfaffl et al. 2002). *B2M* and *GAPDH* were used as reference genes. Four biological replicates of each group were included in the qPCR experiments.

Immunocytochemistry

For immunostaining, the cells were fixed by 4% paraformaldehyde and permeabilized using 0.2% Triton X-100 (Sigma). After blocking with 10% goat serum (Gibco), the cells were incubated with monoclonal antibodies against OCT4A (Santa Cruz Biotechnology), SOX2 (Santa Cruz Biotechnology) and cardiac troponin I (EMD Millipore, Billerica, MA, USA), for 45 min at 37 °C. Anti-mouse FITC-conjugated IgG antibody (Sigma) was used as the secondary antibody. Preparations were examined and photographed by an inverted-phase fluorescent microscope (Nikon, Elipse TE 2000U, Tokyo, Japan).

Oxidative stress, hypoxia and serum deprivation assessments

Primary cultured and third-passaged ADSCs were isolated by trypsinization and 5×10^3 cells were seeded into each well of 96-well tissue culture plates. For induction of oxidative stress, the ADSCs were cultured in a medium containing 20% FBS and 2 mM H₂O₂ for 60 min. Then the cells were washed with PBS and cultured in growth medium for 23 h. For hypoxia culture, the cells were cultured in a growth medium containing 20% FBS and 150 μ M CoCl₂ for 24 h. For serum deprivation, the cells were cultured in DMEM without FBS for 24 h. Control groups consisted of cells cultured in a complete growth medium without CoCl₂ or H₂O₂. All experiments were performed in quadruplicates.

MTT assay

For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, medium of each well was changed to 100 μ l RPMI 1640 (Gibco), and 10 μ l of 12 mM MTT stock solution was added. The cells were incubated for 4 h at 37 °C. The MTT tetrazolium crystals were then solubilized in 100 μ l DMSO. The spectrophotometrical absorbance was read at 490 nm using a microplate reader (Labsystem Multiskan MS, Artisan Technology Group, Champaign, IL, USA). The percentage of viability (%) was calculated by dividing the 490 nm absorbance of every treatment group to that of the control group. Data analysis and production of charts were performed by unpaired *t* test and Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Isolation and characterization of human ADSCs

Within a few hours after plating, ADSCs adhered to the plastic surfaces of tissue culture plates. The medium was renewed after 5–6 h. ADSCs rapidly proliferated and were passaged 2–3 times a week, after reaching 80–90% confluency. Flow cytometric analysis indicated that 87.3, 98.4 and 99.7% of the third-passaged ADSCs were positively stained with antibodies against CD73, CD90 and CD105 proteins, respectively (Fig. 1a–c). Only 0.99% of the ADSCs were positive for the expression of hematopoietic marker, CD45 (Fig. 1d).

Third-passaged ADSCs showed a fibroblast-like morphology (Fig. 1e). To evaluate the multipotential differentiation capacity, ADSCs at passage three were treated with different induction media. Within the first week of adipogenic differentiation, some small fat droplets appeared in the cytoplasm of ADSCs, and the size of lipid droplets increased during the next weeks. At the end of experiment (day 21), lipid accumulation was confirmed using Oil Red O staining (Fig. 1f). When the cells were differentiated in osteogenic medium for two weeks, calcium deposits were detected using Alizarin Red S staining (Fig. 1g). In cardiogenic medium, three-week differentiated ADSCs showed positive immunostaining for cardiac troponin I protein (Fig. 1h).

Fig. 1 **a–d** The third-passaged ADSCs were analyzed by flow cytometry for the expression of mesenchymal (CD73, CD90 and CD105) and hematopoietic (CD45) markers. Third-passaged ADSCs showed a fibroblast-like morphology (**e**). To evaluate multipotential differentiation capability, the ADSCs were cultured in different induction media, as described in the methods. **f** After three weeks of differentiation in adipogenic

medium, lipid accumulation was confirmed using Oil Red O staining. **g** After two weeks of differentiation in osteogenic medium, calcium deposits were detected using Alizarin Red S staining. **h** After three weeks of differentiation in cardiogenic medium, cardiomyocyte-like cells were detected by immunostaining with anti-cardiac troponin I monoclonal antibody

Gene/protein expression analysis

Pluripotency markers

ESC-specific genes, *OCT4A*, *SOX2*, *NANOG*, *c-MYC*, *KLF4*, *LIN28* and *NODAL*, were expressed in both the unpassaged and third-passaged ADSCs. The Other *OCT4* variant, *OCT4B*, was also expressed in the cultured ADSCs both before passage one and at passage three. *OCT4B1* was only expressed in the unpassaged ADSCs (Fig. 2a).

Quantitative real-time PCR analysis showed that after three passages, the expression of *OCT4A*, *SOX2* and *NANOG* was significantly downregulated by mean factors of 0.008, 0.008 and 0.011, respectively. The expression of *c-MYC*, *KLF4*, *LIN28* and *NODAL* mRNAs was also significantly downregulated in the third-passaged ADSCs compared to the unpassaged cells by mean factors of 0.476, 0.038, 0.183 and 0.006, respectively (Fig. 2b).

Immunocytochemistry and flow cytometric analyses were used to confirm the expression of some pluripotency markers at protein level. As shown in

Fig. 3, a significant proportion of the unpassaged ADSCs were positively immunostained for OCT4A and SOX2 proteins, but the population of OCT4A- and SOX2-expressing cells decreased after three passages. Flow cytometry analysis showed that 9.28 and 9.74% of the unpassaged ADSCs were positive for the expression of OCT4A and SSEA3 proteins, respectively (Fig. 4a, c). The population of OCT4A and SSEA3-expressing cells decreased, respectively, to 0.96 and 5.35% after three passages (Fig. 4b, d).

Stemness-related genes

Both the unpassaged and third-passaged ADSCs expressed *TGFBI*, *FGF2* and *LIF*. *FGF4* was rarely detected in the third-passaged ADSCs (Fig. 5a). The expression levels of *TGFBI* and *FGF2* in the third-passaged ADSCs were 4.29 and 2.028 folds higher than in the unpassaged ADSCs, respectively. In contrast, *FGF4* and *LIF* were significantly downregulated in the third-passaged ADSCs by mean factors of 0.001 and 0.485, respectively (Fig. 5b)

Fig. 2 **a** RT-PCR analyses of some pluripotency markers in unpassaged and third-passaged ADSCs. **b** Quantitative analysis of some pluripotency markers by comparative method showed significant downregulation after three passages; the expression level of each gene in the unpassaged ADSCs has been assumed 1

(indicated by the red line) and its expression in the third-passaged ADSCs was compared to that. *Significant differences are indicated by their respective P values (Pair Wise Fixed Reallocation Randomization Test® performed by REST 2009 software). (Color figure online)

Angiogenic factors

Vascular endothelial growth factor A (*VEGFA*), Endoglin (*ENG/CD105*) and *HIF1B* were expressed in the unpassaged ADSCs (Fig. 6a), and their expression levels were significantly upregulated in the third-passaged ADSCs by 6.957, 3.827 and 3.032 folds, respectively (Fig. 6b). *HIF1A* was only expressed in the third-passaged ADSCs (Fig. 6a).

Epithelial to mesenchymal transition (EMT) markers

CXCR4, *MMP2*, *FOXC2* and *SNAIL* were only expressed in the third-passaged ADSCs (Fig. 7a). *SNAI2* and *TIMP1* were weakly expressed in the unpassaged ADSCs, but their expression was upregulated in the third-passaged ADSCs by 39.975 and 11.792 folds, respectively (Fig. 7a, b).

Cell cycle inhibitors

CDKN1A and *TP53* were significantly downregulated in the third-passaged ADSCs compared to the unpassaged cells by mean factors of 0.632 and 0.125, respectively (Fig. 8a).

Resistance of ADSCs to oxidative stress, hypoxia and serum deprivation

The effect of oxidative stress, hypoxia and serum deprivation on cell viability/proliferation of the unpassaged and third-passaged ADSCs was determined by MTT assay (Fig. 8b). Following 60 min exposure to 2 mM H_2O_2 , a significant inhibition of metabolic activity was observed in both the unpassaged (6.9% decrease, $P = 0.003$) and third-passaged ADSCs (11.4% decrease, $P = 0.0023$). Culture in serum-free media for 24 h also significantly inhibited the metabolic activity of the unpassaged (45.5% decrease, $P < 0.001$) and third-passaged ADSCs (41.9% decrease, $P < 0.001$). 24 h exposure to 150 μ M hypoxia-mimetic agent $CoCl_2$ inhibited the viability/proliferation of the unpassaged ADSCs (7.7% decrease, $P = 0.0094$) but had no statistically significant effect on third-passaged ADSCs (2.7% decrease, $P = 0.0987$). Moreover, a combination of 150 μ M $CoCl_2$ and serum deprivation for 24 h significantly inhibited the metabolic activity of the unpassaged (24.3% decrease, $P < 0.001$) and third-passaged ADSCs (16.9% decrease, $P < 0.001$). It's also interesting to note that the metabolic activity of the ADSCs which were exposed to a combination of

Fig. 3 OCT4A and SOX2 proteins (*green color*) were expressed in a significant proportion of unpassaged ADSCs, but the population of OCT4A- and SOX2-expressing cells decreased after three passages. *Red color* shows the staining of all nuclei with propidium iodide (PI). (Color figure online)

CoCl₂ and serum deprivation was significantly lower than for the cells treated with CoCl₂ alone but significantly higher than for the cells only exposed to serum deprivation (Fig. 8b).

Statistical analysis showed no significant difference between the unpassaged and third-passaged ADSCs in all experimental conditions, i.e. oxidative stress, hypoxia and serum deprivation (Fig. 8b).

Discussion

ADSCs are characterized by the expression of MSC-associated surface antigens, the lack of hematopoietic and endothelial markers and their multipotential differentiation capability (Dominici et al. 2006). The differentiation of ADSCs is not limited to mesodermal lineages. In specific induction media, ADSCs

Fig. 4 Flow cytometric analysis showed the expression of OCT4A and SSEA3 proteins in a significant proportion of the unpassaged (respectively, shown by **a** and **c**) and third-passaged (respectively, shown by **b** and **d**) ADSCs

transdifferentiate into multiple cell types of neuroectodermal and endodermal origins (Kwon et al. 2015; Nam et al. 2014; Zuk et al. 2002).

ADSCs hold a great potential for regenerative medicine. For transplantation therapy purposes, in vitro expansion is needed to produce a sufficient number of ADSCs. However, during culture-expansion, some changes may happen in the gene expression profile

of the ADSCs which should be taken into consideration. Moreover, viability of the ADSCs after transplantation is a major determining factor in treatment of ischemic, degenerative and inflammatory disorders. It is therefore essential to investigate resistance of the ADSCs to oxidative stress, hypoxia and serum deprivation which are three important factors occurring in pathological conditions. Hence, we examined the culture-induced

Fig. 5 RT-PCR (a) and quantitative real-time PCR (b) analyses for the expression of some stemness-related genes in the unpassaged and third-passaged ADSCs; the expression level of each gene in the unpassaged ADSCs has been assumed 1 (indicated by the red line) and its expression in third-passaged ADSCs was compared to that. The expression levels of *TGFB1*

and *FGF2* were upregulated while the expression levels of *FGF4* and *LIF* were downregulated significantly after three passages. *Significant differences are indicated by their respective *P* values (Pair Wise Fixed Reallocation Randomization Test[®] performed by REST 2009 software). (Color figure online)

Fig. 6 RT-PCR (a) and quantitative real-time PCR (b) analyses for the expression of some angiogenic genes in the unpassaged and third-passaged ADSCs; the expression level of each gene in the unpassaged ADSCs has been assumed 1 (indicated by the red line) and its expression in third-passaged ADSCs was compared to that. *HIF1A* was only expressed in the third-

passaged ADSCs. *VEGFA*, *ENG* and *HIF1B* were expressed in the unpassaged ADSCs and their expression was significantly upregulated after three passages. *Significant differences are indicated by their respective *P* values (Pair Wise Fixed Reallocation Randomization Test[®] performed by REST 2009 software). (Color figure online)

Fig. 7 RT-PCR (a) and quantitative real-time PCR (b) analyses of the expression of some epithelial to mesenchymal transition (EMT) genes in the unpassaged and third-passaged ADSCs; the expression level of each gene in the unpassaged ADSCs has been assumed 1 (indicated by the red line) and its expression in the third-passaged ADSCs was compared to that. *CXCR4*, *MMP*, *FOXC2* and *SNAI1* mRNAs were only expressed in the

third-passaged ADSCs. *SNAI2* and *TIMP1* mRNAs were weakly expressed in the unpassaged ADSCs, but their expression was upregulated after three passages. *Significant differences are indicated by their respective *P* values (Pair Wise Fixed Reallocation Randomization Test[®] performed by REST 2009 software). (Color figure online)

alterations in the expression of some genes which are of great significance in regenerative medicine. Moreover, the impact of early subcultures on resistance of the ADSCs to pathological condition was investigated.

The expression of pluripotency markers

Unpassaged and third-passaged ADSCs expressed some pluripotency markers including *OCT4A*, *SOX2*, *NANOG*, *c-MYC*, *KLF4*, *LIN28* and *NODAL*. These cells also showed expression of OCT4A, SOX2 and SSEA3 proteins. As reported previously, OCT4, SOX2 and NANOG are essential transcription factors for self-renewal of ESCs (Mitsui et al. 2003; Nichols et al. 1998). These factors together with KLF4, c-MYC and LIN28 are required for maintenance of the pluripotency feature (Voutsadakis 2015). Synergistic action of *OCT4* and *SOX2* regulate their own expression (Chew et al. 2005) and also the expression

of *NANOG* (Rodda et al. 2005), *UTF1* (Nishimoto et al. 1999), *FGF4* (Ambrosetti et al. 1997; Yuan et al. 1995) and *ZFP206* (Wang et al. 2007), which are all important transcription factors for maintaining pluripotency.

The expression of pluripotency genes in the adult tissues is one of the important fields of research today. Jiang et al. (2002) found a population of progenitor cells expressing some pluripotency markers in bone marrow. More recently, some investigators have demonstrated the existence of very small embryonic-like stem cells (VSEL-SC) in bone marrow and cord blood (Kucia et al. 2006). Also, multilineage differentiating stress enduring (Muse) cells have been reported in bone marrow aspirates and human skin fibroblasts (Kuroda et al. 2010). All of these reports indicate the existence of ESC-like populations in different adult tissues, although the nomenclatures are different.

Fig. 8 **a** Quantitative real-time PCR analysis to compare the expression of cell cycle inhibitors, *CDKN1A* and *TP53*, between the unpassaged (assumed 1 and indicated by the red line) and third-passaged ADSCs. *Significant differences are indicated by their respective *P* values (Pair Wise Fixed Reallocation Randomization Test[®] performed by REST 2009 software). **b** The effect of oxidative stress (H₂O₂), hypoxia (CoCl₂) and

serum deprivation on the viability/proliferation of the unpassaged and third-passaged ADSCs was determined by MTT assay. *a* The difference with the control ADSC P0 is significant ($P < 0.05$); *b* the difference with the control ADSC P3 is significant ($P < 0.05$); *** and **: $P < 0.001$ and $P < 0.01$, respectively; P0: unpassaged ADSCs; P3: third-passaged ADSCs; SD: serum deprivation. (Color figure online)

Previously, the expression of pluripotency markers in mouse and human ADSCs has been reported by our team (Taha and Hedayati 2010; Taha et al. 2014) and some other investigators (Heneidi et al. 2013; Izadpanah et al. 2006; Peroni et al. 2008; Simerman et al. 2014). Using flow cytometric analysis, here we showed that a significant proportion of unpassaged human ADSCs are positive for the expression of OCT4A and SSEA3 proteins, but the positively stained populations decrease after three passages. These changes together with the downregulation of *c-MYC*, *LIN28* and *KLF4* show a significant decrease in the ESC-like population after several passages. Furthermore, downregulation of *c-MYC*, *LIN28* and *KLF4* expression may show a considerable compromise on the tumorigenic potential of the passaged ADSCs compared to the freshly isolated cells.

The expression of OCT4 variants in the ADSCs

The human *OCT4* gene has 5 exons and potentially encodes at least three different spliced variants known as *OCT4A*, *OCT4B* and *OCT4B1* (Atlasi et al. 2008). OCT4A protein is localized within the nucleus of

zygote, inner cell mass (ICM), ESCs, embryonic carcinoma cells, germ cell lines and cancer stem cells (Prusa et al. 2003) and seems to be the main variant responsible for stemness properties. OCT4B is localized within the cytoplasm of somatic cells, cell lines and primary tumors. OCT4B cannot sustain the self-renewal property but may respond to cell stress (Farashahi Yazd et al. 2011). *OCT4B1* is expressed in human pluripotent and nonpluripotent cells and is downregulated during differentiation (Atlasi et al. 2008). In the current study, we showed the expression of all three *OCT4* variants in the unpassaged ADSCs, but the expression of *OCT4A* and *OCT4B1* were downregulated with serial passages which further supports a decrease in the ESC-like population.

The expression of critical regulators of pluripotency

Maintaining self-renewal and pluripotency in human ESCs is a complicated process which requires the activity of FGF2 and TGFβ/Activin/Nodal pathways (James et al. 2005; Vallier et al. 2005). In contrast to mouse ESCs (Ying et al. 2003), SMAD1/5 activation

by BMP4 induces differentiation of human ESCs (Xu et al. 2002), and LIF/STAT3 signalling is not sufficient for undifferentiated propagation of human ESCs (Daheron et al. 2004).

In the present study, ADSCs expressed *TGFBI*, *FGF2*, *NODAL*, *FGF4* and *LIF* genes which is in agreement with some previous reports (Hwang et al. 2010). Hwang et al. (2010) showed that the expression of Activin A, FGF4, TGF β , FGF2, PDGF and IL-6 in the ADSCs is comparable to those of bone marrow-derived MSCs (BM-MSCs), placental cells and human dermal fibroblasts which are commonly used as human feeder layers for undifferentiated propagation of ESCs. Altogether, these findings demonstrate three important possibilities: (1) Adipose tissue contains a subpopulation of ESC-like cells which may be the remnants of embryonic pluripotent stem cells (migrating epiblast), and the expression of pluripotency regulators, probably from the adjacent cells, may be necessary for preservation of these ESC-like cells. (2) Theoretically, some cancer stem cells may be derived from adult ESC-like cells. Dysregulation of pluripotency maintenance factors in the adult tissues may be one important cause of tumor formation and progression. This idea is supported by previous studies which show the active role of TGF β (Friedman et al. 1995; Gupta et al. 2014) and FGF (Halaban et al. 1988; Rykala et al. 2011) signalling pathways in tumor progression. (3) As shown previously, the ADSCs can be used as an alternative source of feeder cells to support the expansion of both human and mouse ESCs and maintenance of pluripotency (Hwang et al. 2010; Kim et al. 2013). However, we showed that the expression levels of *TGFBI* and *FGF2* in the third-passaged ADSCs were almost 4.3 and 2 folds higher than the unpassaged cells, while *FGF4* expression was downregulated severely and *LIF* expression decreased to half after three passages. According to these findings, it may be concluded that the ADSCs at passage three may act as better feeder cells for the expansion of human ESCs, while the unpassaged ADSCs may be a preferred feeder layer for mouse ESCs.

The expression of angiogenic factors

Low oxygen level is a critical problem in ischemic diseases. The primary responses to hypoxia is mediated through the HIF pathway. The HIF pathway mediates

long-term adaptation to hypoxia by stimulation of angiogenesis through regulation of pro-angiogenic genes, including VEGF, angiopoietin-1, angiopoietin-2, Tie2, PDGF and basic bFGF and monocyte chemoattractant protein-1 (MCP-1) (Krock et al. 2011).

As described previously, ADSCs secrete some angiogenic and anti-apoptotic factors, including VEGF and HGF (Rehman et al. 2004). Here, we demonstrated that the cultured ADSCs express *VEGFA*, *HIF1A*, *HIF1B* and *ENG*, and that the expression of these angiogenic factors in the third-passaged ADSCs were significantly higher than the unpassaged cells. This property makes the early-passaged ADSCs a valuable cell source for vascular repair and regeneration in ischemic tissues.

EMT markers

MSCs have an inherent capacity for migration toward the injury sites, ischemia inflammation, and tumor microenvironment. This behavior is mediated by numerous EMT-inducing transcription factors, including forkhead box protein C2 (*FOXC2*), *SNAIL*, *SNAIL2*, *TWIST*, *ZEB1*, *ZEB2* and *MMP2* (Chiotaki et al. 2016; De Becker et al. 2007; Momin et al. 2010; Son et al. 2006; Thiery et al. 2009).

EMT plays a central role in embryonic development (Thiery et al. 2009). The process also occurs during tissue regeneration, organ fibrosis, cancer progression and metastasis (Kalluri 2009; Thiery et al. 2009). In our study, EMT-related transcription factors, *FOXC2*, *SNAIL*, matrix metalloproteinase 2 (*MMP2*), *CXCR4* and *TIMP1* were only expressed in the third-passaged ADSCs. Moreover, the expression of *SNAIL2* in the third-passaged ADSCs was almost 40 fold higher than in the unpassaged cells. These changes may be in favor of migration of the third-passaged ADSCs toward the injury sites. However, our results are in contrast to some previous studies which report the downregulation of a key homing receptor, *CXCR4*, in the culture-expanded MSCs (Honczarenko et al. 2006; Wynn et al. 2004).

Cell cycle inhibitors

P21 and P53 are two well-known cell cycle inhibitors which have significant roles in regulation of stem cell proliferation and differentiation (Armesilla-Diaz et al.

2009). In addition, P53 induces apoptosis via direct interaction with pro- and antiapoptotic proteins (Moll et al. 2005). We showed that the expression of *CDKN1A* (P21) and *TP53* in the unpassaged ADSCs was 1.6 and 8 fold higher than in the third-passaged ADSCs, respectively. This observation suggests that the mincing, mechanical agitation and collagenase digestion of adipose tissue may result in stress-induced cell cycle arrest and apoptosis. Moreover, downregulation of *CDKN1A* and *TP53* after three passages may be implying that the early subcultures are necessary to eliminate the apoptotic cells and to restore the proliferative capacity of the ADSCs.

Resistance to oxidative stress, hypoxia and serum deprivation

Oxidative stress, hypoxia and serum deprivation are three important factors which may induce apoptosis in mesenchymal stem cells after transplantation into the pathological locations. In previous studies, H_2O_2 exposure has been used as a common inducer of oxidative stress in vitro, because it has a relatively long half-life and is soluble in both lipids and aqueous media (Liu et al. 2015; Wei et al. 2010). In the current study, ADSCs were exposed to a relatively high dose of H_2O_2 (2 mM) for 1 h. As shown by MTT assay, the ADSCs were somewhat resistant to H_2O_2 -induced cell death; the viability/proliferation in the unpassaged and third-passaged ADSCs decreased by 6.8 and 11.4%, respectively.

Serum deprivation and hypoxia are two components of ischemia in vivo (Huang et al. 2009). To analyse the effect of hypoxia on the viability/proliferation of the ADSCs, we used $CoCl_2$ which is a chemical inducer of HIF-1 α and is widely used to mimic the effects of reduced atmospheric oxygen levels (Triantafyllou et al. 2006). Based on MTT analysis, 24 h exposure to 150 μ M $CoCl_2$ decreased the viability/proliferation of the unpassaged ADSCs by 7.7%, but the third-passaged ADSCs were resistant to this condition. 24 h culture in the serum-free medium decreased the viability/proliferation of the unpassaged and third-passage ADSCs by 45.5 and 41.9%, respectively. This finding shows that both the unpassaged and third-passaged ADSCs are highly sensitive to serum deprivation. However, a combination of $CoCl_2$ and serum deprivation was unexpectedly better tolerated by the ADSCs. Under this condition, the viability/proliferation of the unpassaged and third-passage ADSCs decreased by 24.3 and 16.9%,

respectively. This finding is in agreement with some previous reports which show the protective role of hypoxia/ $CoCl_2$ against different pro-apoptotic conditions, such as serum and glucose deprivation and oxidative stress (Piret et al. 2002, 2004; Wu et al. 2012). It has been suggested that several possible mechanisms are responsible for this protective role of $CoCl_2$, including its antioxidant effect which reduces generation of reactive oxygen species (ROS) and its mitochondrial protective function which inhibits dissipation of mitochondrial membrane potential following serum and glucose deprivation (Wu et al. 2012). $CoCl_2$ also induces several hypoxia-related protective factors, such as HIF-1 (Piret et al. 2002, 2004), EPO (Fisher and Langston 1968), VEGF (Endoh et al. 2000), glucose transporters (Ybarra et al. 1997), HSP90 (Yang et al. 2011) and heme oxygenase-1 (Eyssen-Hernandez et al. 1996).

In conclusion, the results of current study have revealed that the expression of some genes with key importance in regenerative medicine is altered during culture-expansion of human ADSCs. The expression of pluripotency markers and cell cycle inhibitors decreases, while the expression of some angiogenic and EMT-related genes is upregulated during early passages of the ADSCs. Both the unpassaged and third-passage ADSCs are highly sensitive to serum deprivation and moderately sensitive to oxidative stress and hypoxia. A combination of serum deprivation and hypoxia is better tolerated by the ADSCs than serum deprivation alone. Altogether, considering the different gene expression profile but nearly similar sensitivity of the unpassaged and early-passaged ADSCs to oxidative stress, hypoxia and serum deprivation, it seems that the early-passaged ADSCs may be better choices for vascular repair and regeneration in ischemic tissues.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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