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REGULAR ARTICLE

Hypoxia increases Sca-1/CD44 co-expression in murine mesenchymal stem cells and enhances their adipogenic differentiation potential

M. G. Valorani · A. Germani · W. R. Otto · L. Harper · A. Biddle · C. P. Khoo · W. R. Lin · M. I. Hawa · P. Tropel · M. P. Patrizi · P. Pozzilli · M. R. Alison

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Abstract Mesenchymal stem cells (MSCs) are usually cultured under normoxic conditions (21% oxygen). However, in vivo, the physiological "niches" for MSCs have a much lower oxygen tension. Because of their plasticity, stem cells are particularly sensitive to their environments, and oxygen tension is one developmentally important stimulus in stem cell biology and plays a role in the intricate balance between cellular proliferation and commitment towards differentiation. Therefore, we investigated here the effect of hypoxia (2% oxygen) on murine adipose tissue (AT) MSC proliferation and adipogenic differentiation. AT cells were obtained from the omental fat and AT-MSCs were selected for their ability to attach to the plastic dishes, and were grown under normoxic and hypoxic conditions. Prior exposure of MSCs to hypoxia led to a significant reduction of ex vivo expansion time, with significantly increased numbers of Sca-1^+ as well as Sca-1^+/CD44^+double-positive cells. Under low oxygen culture conditions, the AT-MSC number markedly increased and their adipogenic differentiation potential was reduced. Notably, the hypoxia-mediated inhibition of adipogenic differentiation was reversible: AT-MSCs pre-exposed to hypoxia when switched to normoxic conditions exhibited significantly higher adipogenic differentiation capacity

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M. G. Valorani (✉) · C. P. Khoo · W. R. Lin · M. I. Hawa · P. Pozzilli · M. R. Alison
Centre for Diabetes, Blizard Institute of Cell and Molecular Science, Barts & the London School of Medicine & Dentistry, Queen Mary University of London,
4 Newark Street,
London E1 2AT, UK
e-mail: m.g.valorani@qmul.ac.uk

M. G. Valorani · A. Germani · M. P. Patrizi
Fondazione Livio Patrizi,
Rome, Italy

M. G. Valorani
BIOS S.p.A.,
Rome, Italy

W. R. Otto · W. R. Lin · M. R. Alison
Histopathology Laboratory, Cancer Research UK,
London, UK

C. P. Khoo · P. Pozzilli
Area di Endocrinologia, Universita' Campus Bio-Medico,
Rome, Italy

L. Harper · A. Biddle · P. Tropel
Centre for Cutaneous Research, Blizard Institute of Cell and Molecular Science, Barts & The London School of Medicine & Dentistry, Queen Mary University of London,
London, UK

W. R. Lin
Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital, Chang Gung University College of Medicine,
Taipei, Taiwan

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compared to their pre-exposed normoxic-cultured counterparts. Accordingly, the expression of adipocyte-specific genes, peroxisome proliferator activated receptor γ (*Pparγ*), lipoprotein lipase (*Lpl*) and fatty acid binding protein 4 (*Fabp4*) were significantly enhanced in hypoxia pre-exposed AT-MSCs. In conclusion, pre-culturing MSCs under hypoxic culture conditions may represent a strategy to enhance MSC production, enrichment and adipogenic differentiation.

Keywords Hypoxia · mMSCs · Adipose Tissue · Sca-1 · CD44 · Differentiation · Cell culture (Mouse)

Introduction

The therapeutic potential of mesenchymal stem cells (MSCs) is limited by the lack of specific surface antigens, as well as by an incomplete understanding of in vitro culture conditions that maintain their stem-cell phenotype and retain the cell native characteristics. MSCs can be isolated from a variety of adult tissues including bone marrow (BM) and adipose tissue (AT); these cells can be characterized by their ability to adhere to plastic and acquire osteogenic, adipogenic and chondrogenic phenotypes in specific culture conditions. However, there is a general agreement in defining the murine mesenchymal compartment in the bone marrow as cells expressing CD44, CD49, CD29 and Sca-1 (Baddoo et al. 2003; Eslaminejad et al. 2006; Kanemaru et al. 2005; Meirelles Lda and Nardi 2003; Nadri and Soleimani 2007; Nadri et al. 2007; Sun et al. 2003; Sung et al. 2008), but negative for the haematopoietic and endothelial markers CD45, CD11b, TER119 and CD31 (Anjos-Afonso et al. 2004). Similarly to BM-MSC, CD44 and Sca-1 together with CD49, CD29 also identifies MSCs in adipose tissue (Sung et al. 2008; Zheng et al. 2006; Gomillion and Burg 2006).

Hypoxia is an important microenvironmental factor in major aspects of stem cell biology including survival, proliferation, viability, differentiation and migration, playing a role in the balance between cellular proliferation and commitment towards differentiation (Simon and Keith 2008). Conventionally, cells are grown in ambient or normoxic conditions (21% oxygen) which does not reflect physiological conditions, exposing cells to an unphysiologically high concentration of O_2. Hypoxic conditions are required to maintain the pluripotency of mammalian ES cells (Ezashi et al. 2005). Although, in adults, low oxygen levels are commonly associated with pathologic conditions, including ischaemia, inflammation and tumours, hypoxic conditions are also present in physiologic conditions, i.e., in the bone marrow where the architecture of medullary sinuses and arterial blood flow generates an O_2 gradient (with oxygen tension ranging from 1 to 7%) (Hung et al. 2007). It has been proposed that hematopoietic stem cells (HSCs) and their proliferating progenitors are naturally distributed along this gradient, with the HSCs occupying the most hypoxic niches (Parmar et al. 2007). The hypoxic environment seems required for maintaining HSC proliferation and self-renewal capability (Ivanovic et al. 2000a, b).

The effect of hypoxia on MSC behavior has been widely investigated. The consensus in the field is that either short- or long-term hypoxic culture promotes MSC proliferation and migration (Grayson et al. 2006, 2007; Hung et al. 2007; Potier et al. 2007a; Rosova et al. 2008), even though hypoxia, concomitant with serum deprivation, has been demonstrated by two different groups to induce apoptosis in MSCs (Potier et al. 2007b; Zhu et al. 2006). Moreover, low oxygen levels (2%) reduces adipogenic, chondrogenic and osteogenic differentiation in murine and human AT-derived-MSC (Lee and Kemp 2006; Malladi et al. 2006), while moderate hypoxia (8%) stimulates adipogenesis in murine BM-MSCs (Ren et al. 2006).

In this study, we analyzed the effect of hypoxia on the expression of AT-MSC antigens Sca1 and CD44; moreover, we evaluated whether low oxygen levels affected AT-MSC proliferation and adipogenic differentiation. It was found that the expression of Sca-1^+ as well as Sca1^+/CD44^+ double-positive cells increased in hypoxic-cultured AT-MSC. Although hypoxia inhibits adipogenic differentiation, pre-hypoxic-cultured AT-MSCs demonstrated a higher differentiation potential in normoxia compared to their prior normoxic-cultured counterparts.

Materials and methods

Cell culture

Mesenchymal stem cells (MSCs) were isolated from adipose tissue (AT), bone marrow (BM), pancreas (P) and testis (T) tissues of 8- to 12-week-old non-obese diabetic (NOD) male mice, a suitable model for Type 1 diabetes disease studies (n=82). At this time, mice had not developed diabetes as assessed by the evaluation of their glycaemic levels using a glucometer (Accu-Chek Tests; Roche Diagnostics, Mannheim, Germany); in normal NOD mice, glucose levels were <11.5 mmol/l. NOD mice were housed in individually ventilated cages with an exhaust system (Sealsafe IVC) and under the relevant safety standards. Mice were kept in specific pathogen-free conditions at 21°C, and a relative humidity of 50% and were given autoclaved food and water ad libitum. The NOD mice were sacrificed by cervical dislocation according to UK Home Office regulations. Briefly, AT cells were obtained from the pooled omental fat (epiploon) of five 8- to 12-

week-old NOD mice, fat was cut into small pieces and digested with 1 mg/ml Accutase (Chemicon, Millipore) for 2 h at 37°C with shaking every 15 min. This cell detachment solution of proteolytic and collagenolytic enzymes was used for a more gentle tissue digestion and cells were centrifuged and filtered through a 40-μm nylon filter (Becton Dickinson Labware, Franklin Lakes, NJ, USA). P- and T-MSCs were isolated as AT-MSCs. BM-MSCs were collected by flushing femurs, tibias and iliac crests with 5 ml PBS supplemented with 2% foetal bovine serum (FBS; Gibco, Paisley, UK). Cells were plated at a density of 1×10^4 cells/cm^2 and cultured in complete medium: MesenCult MSC Basal Medium with 20% Mesenchymal Stem Cell Stimulatory Supplements (Stem Cell Technologies, Vancouver, Canada) further supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere in 21% oxygen (normoxia). Hypoxic conditions were created using an Invivo2 1000 hypoxia workstation (Ruskinn Technology, Pencoed, Wales) according to the manufacturers' instructions. Briefly, the workstation atmosphere is continually monitored for CO_2 and O_2 concentrations and adjusted by adding a mixture of 3 gases (compressed medical air, medical N_2 and medical CO_2). A final and maintained concentration of 2% O_2,5% CO_2 was achieved before placing the cultures in the workstation. The workstation was kept at 37.5°C with humidity set above 90%.

Non-adherent cells were eliminated, in normoxic as well as in hypoxic-cultured cells, by a complete medium change at day 1 and a wash with PBS of the adherent cells remaining in the cultures. Then, cells were cultured with fresh Complete Medium and a half volume of medium was replaced twice a week. The whole adherent fraction was detached by trypsinization at 80% confluence (after 4–5 days) using Accutase (Chemicon Europe, Hampshire, UK) and re-plated. The FACS analysis was performed at day 5 (after 10 days of exposure to normoxia and hypoxia).

Proliferation studies

Normoxic and hypoxic AT-MSCs were isolated from a pool of 8-week-old Balb/c male mice ($n=5$), cultured in normoxic and hypoxic conditions for 5 days, then detached and plated in 12.5 cm^2 flasks for the indicated time points (3,000 cells/cm^2). Cells were used at passage P1.

Flow cytometry analysis

The phenotypes of cultured AT-MSCs, BM-MSCs, P-MSCs and T-MSCs were analyzed by fluorescence activated cell sorter (FACS) analysis using a BD FACSAria analyzer fitted with BD FACSDiva software.

The following rat anti-mouse IgG monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin-cyanin7 (PECy7)-conjugated Sca-1; and FITC-conjugated CD44. FACS analysis was performed on haematopoietic and endothelial lineage-negative cells (Anjos-Afonso et al. 2004) which were identified following incubation with phycoerythrin (PE)-conjugated CD45, CD11b, TER119 and CD31 rat anti-mouse IgG (BD Biosciences Pharmingen, Palo Alto, Ca, USA). As controls, cells were stained with FITC, PECy7, PE-labeled isotype rat anti-mouse IgG. The compensation was performed using single color controls. Samples were analyzed to compare the negative selection antibodies against Sca-1-PE-Cy7 or CD44-FITC. CD44$^+$/Negative Selection were then gated to show percent double-positive for CD44 and Sca-1.

In vitro adipogenic, osteogenic and chondrogenic differentiation

For adipogenic differentiation, AT-MSCs were cultured in complete medium with 0.5 μM hydrocortisone, 0.2 μM isobutyl methyl xanthine, 100 μM indomethacin and 5 μg/ml insulin (Nagai et al. 2007). The differentiation culture medium was changed three times per week for up 3 weeks. Then, cells were fixed with 4% PFA in PBS for 20 min at room temperature, incubated in 60% iso-propyl-alcohol (IPA) and stained with 1% Oil Red O (Raymond Lamb, Eastbourne, UK) in IPA for 15 min and further incubated in IPA to remove background staining. Nuclei were stained with half-strength Harris' haematoxylin for 30 s, and then mounted in Glycergel. The positive fat vacuoles appeared as red-stained droplets.

Chondrogenesis was assessed by culturing cells for up to 3 weeks in complete medium containing 1 ng/ml basic fibroblast growth factor (bFGF) and 5 ng/ml transforming growth factor β1 (TGFβ1). Chondrocytes were stained with 1% alcian blue (BDH, Poole UK) in 3% acetic acid, pH 2.5 for 5 min, with a 1-min neutral red nuclear counterstain, which revealed sulphated proteoglycan production by MSCs (Mouiseddine et al. 2007).

For osteogenic differentiation, cells were grown for up to 3 weeks in complete medium supplemented with 10 nM dexamethasone, 0.2 mM vitamin C phosphate and 10 mM Na-β-glycerophosphate. Von Kossa staining for calcium salts was used to detect osteocytes as described (Bancroft and Gamble 2002).

RNA isolation, array analysis and qPCR

Total RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany). Taqman RNA to Ct 2 step kit (Applied Biosystems, Warrington, UK) was used for reverse transcription of total RNA (1 μg) into complementary DNA and

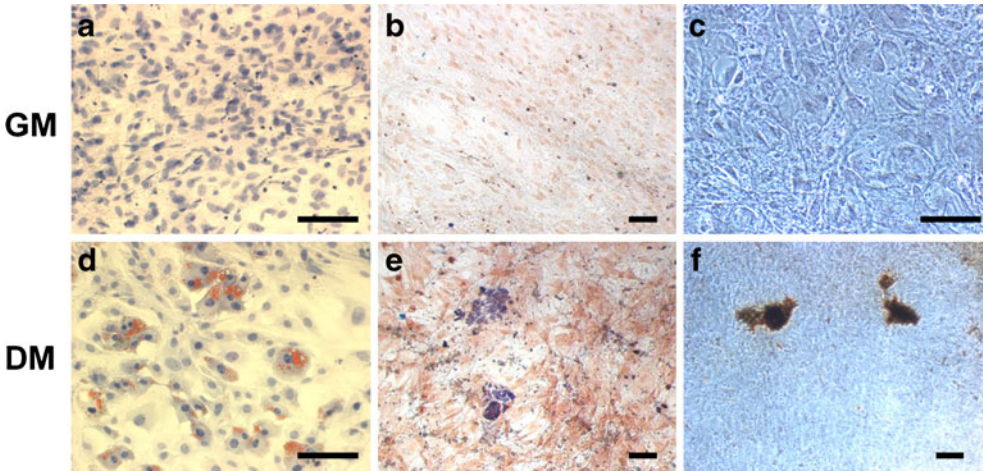


Fig. 1 Differentiation potential of normoxic cultured AT-MSC. AT-MSC differentiation toward adipogenic, chondrogenic, osteogenic lineages in culture. Representative images of AT-MSC cultured under normoxic conditions for 10 days in growth control medium (*GM*, *upper panels*) (**a**–**c**) and later cultured for 3 weeks in the specific differentiation cocktail media (*DM*, *lower panels*) (**d**–**f**). Adipogenic cells were identified by Oil Red O staining of intracellular lipid droplets (**d**). Alcian blue staining revealed sulphated proteoglycan production by MSCs showing chondrogenic differentiation (**e**). Von Kossa staining for calcium salts was used to detect osteocytes (**f**). *Bars* 100 μm

quantitative PCR according to the manufacturer's instructions. The following gene specific assays (Applied Biosystems) were used: *Nanog* (Mm02019550_s1); *Sox2* (Mm00488369_s1); *Oct4* (Mm00658129_gh); *Pparγ* (Mm00440945_m1); *Lpl* (Mm00434764_m1); *Fabp4* (Mm00445880_m1). Expression levels were normalized against *Gapdh* using Mouse *Gapdh* TaqMan as an endogenous control (Applied Biosystems) and as a reference control for quantitative PCR gene-expression analysis. To assess the linearity and sensitivity of the assay, a standard curve was generated using serial dilutions of Stratagene QPCR Mouse Reference Total RNA (Stratagene, CA, USA). qPCR measurements were performed in triplicate. All quantitative PCR were carried out using a 7500 Real-Time instrument (Applied Biosystems).

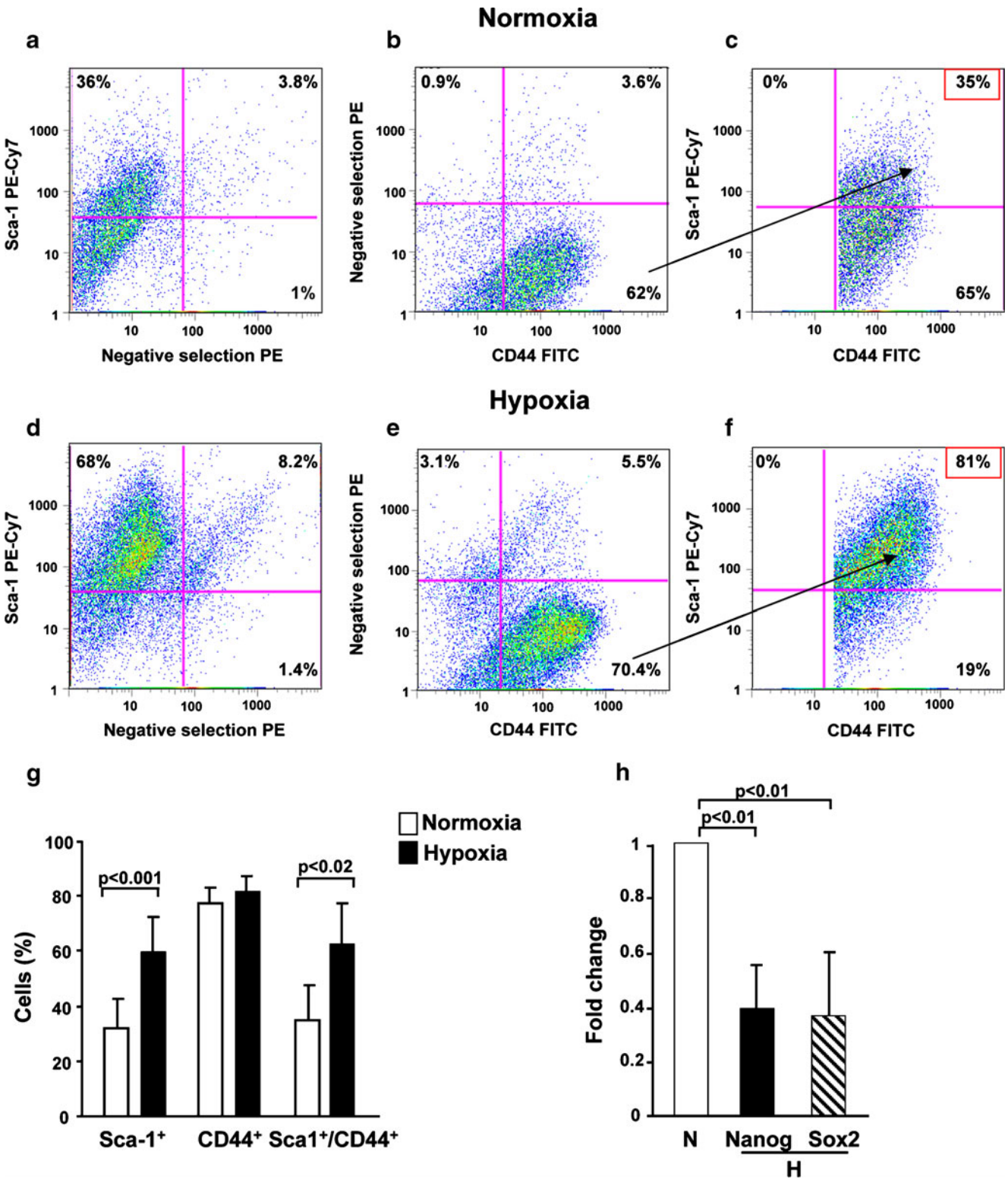
The amplified transcripts were quantified using the comparative CT method with the formula for relative fold change $= 2^{-\Delta\Delta CT}$.

Apoptosis studies

Normoxic- and hypoxic-cultured AT-MSCs were trypsinized, resuspended in 200 μl of calcium rich annexin V buffer (BD Biosciences, Oxford, UK) and incubated for 15 min at RT with 15 μl of annexin V-AlexaFluor-647 (Invitrogen, Paisley , UK). Propidium iodide (PI) (5 μg/ml) was added and samples were analyed on a Becton Dickinson LSRII cytometer, using the 660/20 nm channel from the red laser for annexin V-AlexaFluor-647 detection and the 576/26 nm channel from the argon laser was used to detect PI (10,000 events were collected). No compensation controls were required as PI and AlexaFluor-647 did not spectrally overlap. Quadrant gating was used to detect live cells (annexinVneg/PIneg), apoptotic cells (annexinVpos/PIneg), and dead cells (annexinVneg/PIpos) and (annexinVpos/PIpos).

For cell cycle distribution analysis, annexin V-labeled cells were fixed in 70% ice-cold ethanol, spin-washed in PBS and incubated with 100 μg/ml RNAse (Sigma) at 37° C for 15 min and resuspended in 50 μg/ml PI in PBS. Then, samples were analysed (10,000 events collected) on a Becton Dickinson LSRII cytometer using the 610/10 nm channel from the argon laser to detect PI in a linear manner with the width parameter used to exclude doublets of cells. Histogram analysis of the PI signal allowed the determination of the percentage of cells that have lost DNA due to DNA fragmentation. The result was a population of cells with a reduced DNA content and the cells were stained with a DNA intercalating dye, PI. A DNA profile representing

Fig. 2 Effect of hypoxia on the expression of stem cell markers in AT-MSC. FACS analysis of normoxic (**a**–**c**) and hypoxic (**d**–**f**) cultured AT-MSCs at day 10 (P1). Representative dot plots of $Sca1^+$ (**a**,**d**), $CD44^+$ (**b**,**e**) and $Sca1^+/CD44^+$ (**c**,**f**) cells. Negative selection was performed incubating cells with phycoerythrin (PE)-conjugated CD45, CD11b, TER119 and CD31 rat anti-mouse IgG and measuring PE fluorescence at 576 nm. $CD44^+$ cells in the middle panels were then gated to show percent double-positive for CD44 and Sca-1. **g** *Bar graphs* showing results from FACS analysis (n=3). There was a significant increase in the frequency of Sca1 positive cells when grown in hypoxia compared to normoxia ($p < 0.001$) and likewise a significant increase in $Sca1^+/CD44^+$ ($p < 0.02$). **h** Real-time RT-PCR showing the fold change of *Nanog* and *Sox2* expression in AT-MSC cultured for 10 days either in hypoxic (*H*) and in normoxic (*N*) conditions (n=3). Under normoxia, the expression levels of both Nanog and Sox2 were similar: deltaCT=15.6±0.4 and 15.3±1.2, respectively. Nanog and Sox2 decreased under hypoxic conditions (p<0.01)


Normoxia
a
b
c
36%
3.8%
1%
0.9%
3.6%
62%
0%
35%
65%
Sca-1 PE-Cy7
Negative selection PE
CD44 FITC
Hypoxia
d
e
f
68%
8.2%
1.4%
3.1%
5.5%
70.4%
0%
81%
19%
g
h
Normoxia
Hypoxia
Cells (%)
p<0.001
p<0.02
Sca-1+
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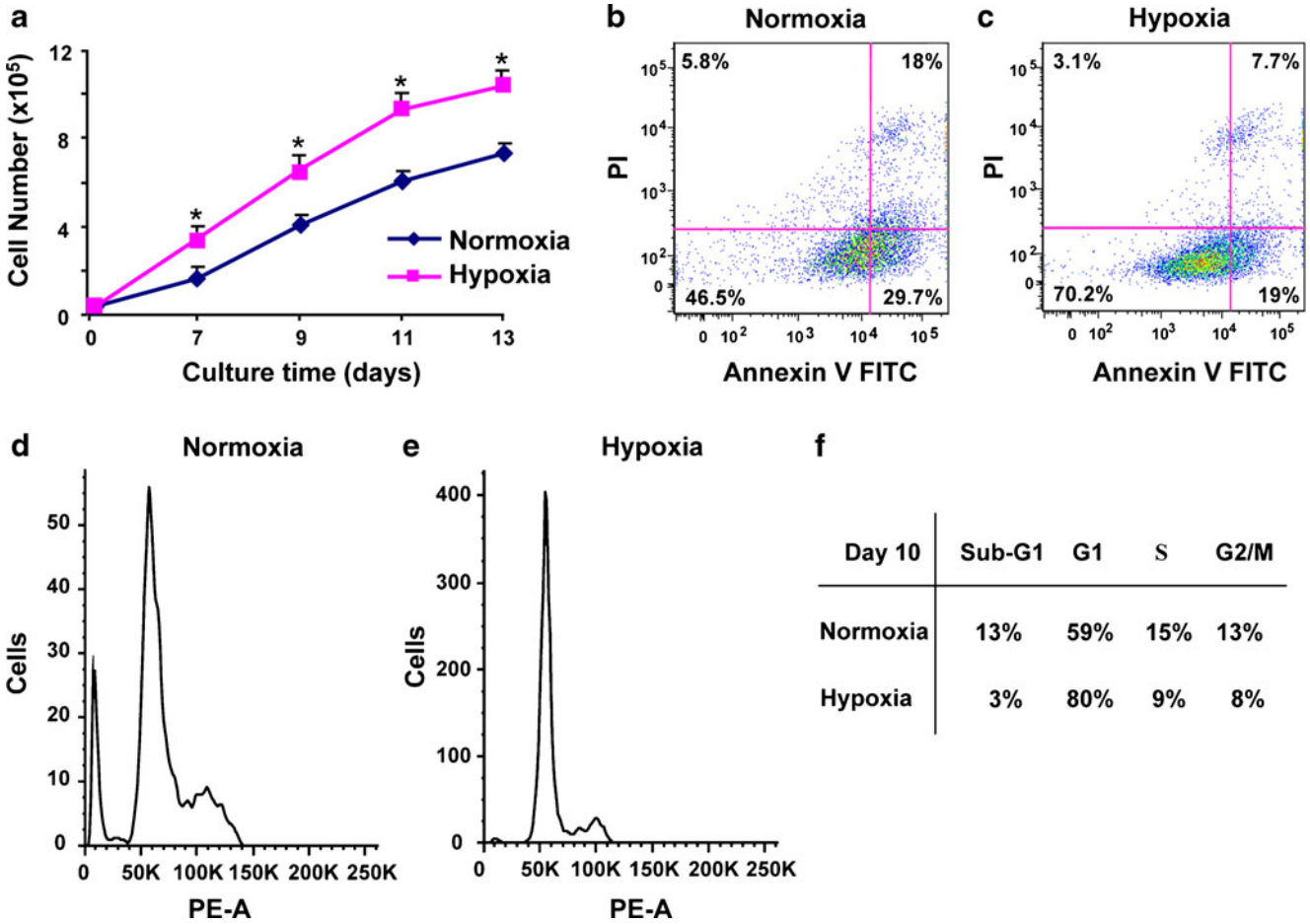

Fig. 3 Effect of hypoxia on cell growth, survival and cell cycle distribution. **a** Growth curve of normoxic and hypoxic AT-MSC (n=4, p<0.001). Cells were detached and counted at the indicated time points. Note that at day 13 hypoxic cells were at 80–85% confluency. **b**,**c** Representative dot plots of annexin V- and PI-labeled AT-MSCs after 10 days culture either in normoxia or in hypoxia. Data representative of two independent experiments. **d**,**e** Cell cycle distribution and percent (**f**) of PI-labeled AT-MSCs after 10 days culture either in normoxia or in hypoxia

cells in G1, S-phase and G2M was observed with apoptotic cells being represented by a sub G1 population seen to the left of the G1 peak.

Statistical analysis

Results are presented as mean ± standard error (SE). Statistical significance between two measurements was evaluated by Student's t test. A probability value of p<0.05 was considered significant.

Results and discussion

Characterization of normoxic and hypoxic cultured AT-MSC

AT-MSCs express a variety of cell surface antigens that are shared with different stem cell populations. Among them, Sca-1 and CD44 define the MSC compartment in murine bone marrow (Baddoo et al. 2003; Eslaminejad et al. 2006; Kanemaru et al. 2005; Meirelles Lda and Nardi 2003; Nadri and Soleimani 2007; Nadri et al. 2007; Sun et al. 2003; Sung et al. 2008), and adipose tissue (Sung et al. 2008; Zheng et al. 2006).

To confirm that in vitro-cultured cells maintained MSC potential, we investigated their ability to differentiate along the osteogenic, adipogenic and chondrogenic lineages. AT-MSCs were grown in normoxia for 10 days and, after that, cultured for 3 weeks in either growth media (Fig. 1a–c) or specific differentiation media (Fig. 1d–f). These studies showed that AT-MSCs were capable of giving rise to adipocytes, visualized by intracellular lipid droplets using Oil Red O staining (Fig. 1d), and chondrocytes, had sulphated proteoglycan production confirmed by alcian blue staining (Fig. 1e), and osteogenic cells with calcium salt deposition were identified by von Kossa staining (Fig. 1f).

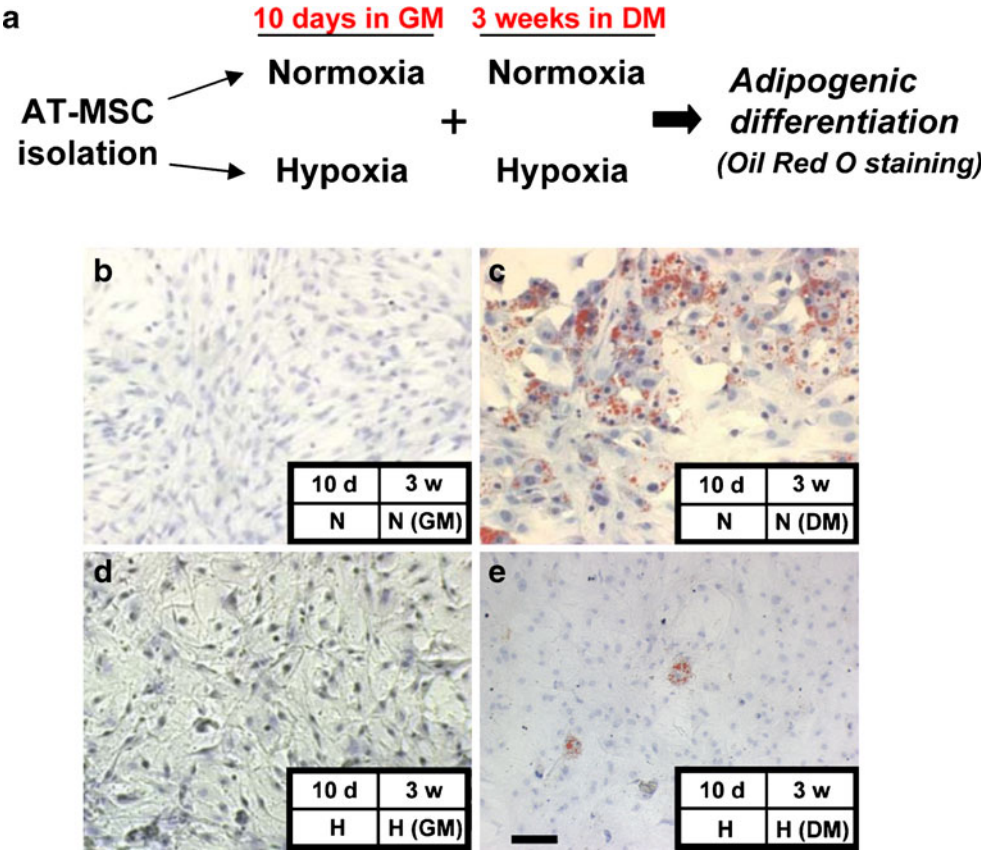

Fig. 4 Hypoxia promotes proliferation and inhibits AT-MSC adipogenic differentiation. **a** Experimental plan. AT-MSCs were pre-cultured in growth control medium (*GM*) for 10 days in normoxia (**b**,**c**) and hypoxia (**d**,**e**). Then, cells were cultured for a period of 3 weeks either in differentiation cocktail medium (*DM*) (**c**,**e**, *right panels*) or *GM* (**b**,**d**, *left panels*). Oil red O staining was performed to detect adipogenic differentiation and showed lipid vacuole accumulation in normoxic-DM cultured cells (**c**,**e**). Adipogenic differentiation was strongly reduced when AT-MSC were cultured in GM and seldom in adipogenic medium (DM) under hypoxia. *Bar* 100 μm

To evaluate the effects of low oxygen levels, AT-MSCs were cultured in parallel both in normoxia (21% O_2) and in hypoxia (2% O_2). Flow cytometric analysis performed on 10 days AT-MSCs culture showed that hypoxia significantly enhanced the frequency of Sca-1$^+$ as well as Sca-1$^+$/CD44$^+$ cells in AT-MSC in comparison to normoxic conditions (59.5±13 vs.32±11% and 62±15 vs 34.5±13%) (Fig. 2a–g). Notably, low oxygen levels enhanced the number of Sca-1$^+$/CD44$^+$ cells in the MSC fraction obtained from both pancreas and testis (Electronic supplementary material, ESM, Fig. S1a–d). In contrast, FACS analysis of normoxic cultured bone marrow (BM)-MSCs revealed that Sca-1$^+$ and CD44$^+$ cells were less abundant compared to AT-MSCs (7.6±2.7 vs 32±11%, $p<0.05$; and 3.9±3 vs 67±12%, $p<0.001$); these percentages did not significantly change between 20 days normoxic and hypoxic BM-MSC culture; however, after 20 days culture, 70±4% of CD44 cells also expressed Sca-1 (ESM, Fig. S1e).

Since hypoxia enhanced the proportion of Sca-1$^+$- as well as Sca-1$^+$/CD44$^+$-expressing cells but left CD44$^+$ cells unchanged (Fig. 2g), we investigated whether low oxygen levels affected the expression of the pluripotency stem cell markers *Nanog*, *Sox2* and *Oct4*. By real-time PCR, we showed that 10 days hypoxic culture conditions markedly inhibited *Nanog* and *Sox2* mRNA levels in AT-MSCs (Fig. 2h). In contrast, *Oct-4* levels were not detectable either in normoxia or in hypoxia (data not shown). Previous studies have reported the opposite effect of hypoxia on *Oct-4* levels in human and murine bone marrow MSCs (Grayson et al. 2006, 2007; Ren et al. 2006); however, different times of exposure to low oxygen levels may account for such a discrepancy.

Effect of hypoxia on AT-MSC on cell growth and adipogenic differentiation

Reduced oxygen levels have been shown to enhance the proliferation of multiple types of stem and progenitor cells including MSCs (Grayson et al. 2006, 2007; Fehrer et al. 2007; Ren et al. 2006). In agreement with these reports, the growth curve showed that from day 7 AT-MSCs number significantly increased in hypoxic-cultured cells at all time points analyzed (Fig. 3a). Both hypoxic and normoxic cells exhibited a small, spindle-shaped morphology (ESM, Fig. S2). Annexin V and propidium iodide (PI) staining revealed that hypoxia protected AT-MSCs from death. Specifically, a combination of both parameters, PI and annexin V, allowed for the discrimination between necrotic PI-positive and apoptotic PI-negative/annexinV-positive cells. At day 10, the number of apoptotic cells was higher in normoxic-cultured AT-MSCs compared to their hypoxic

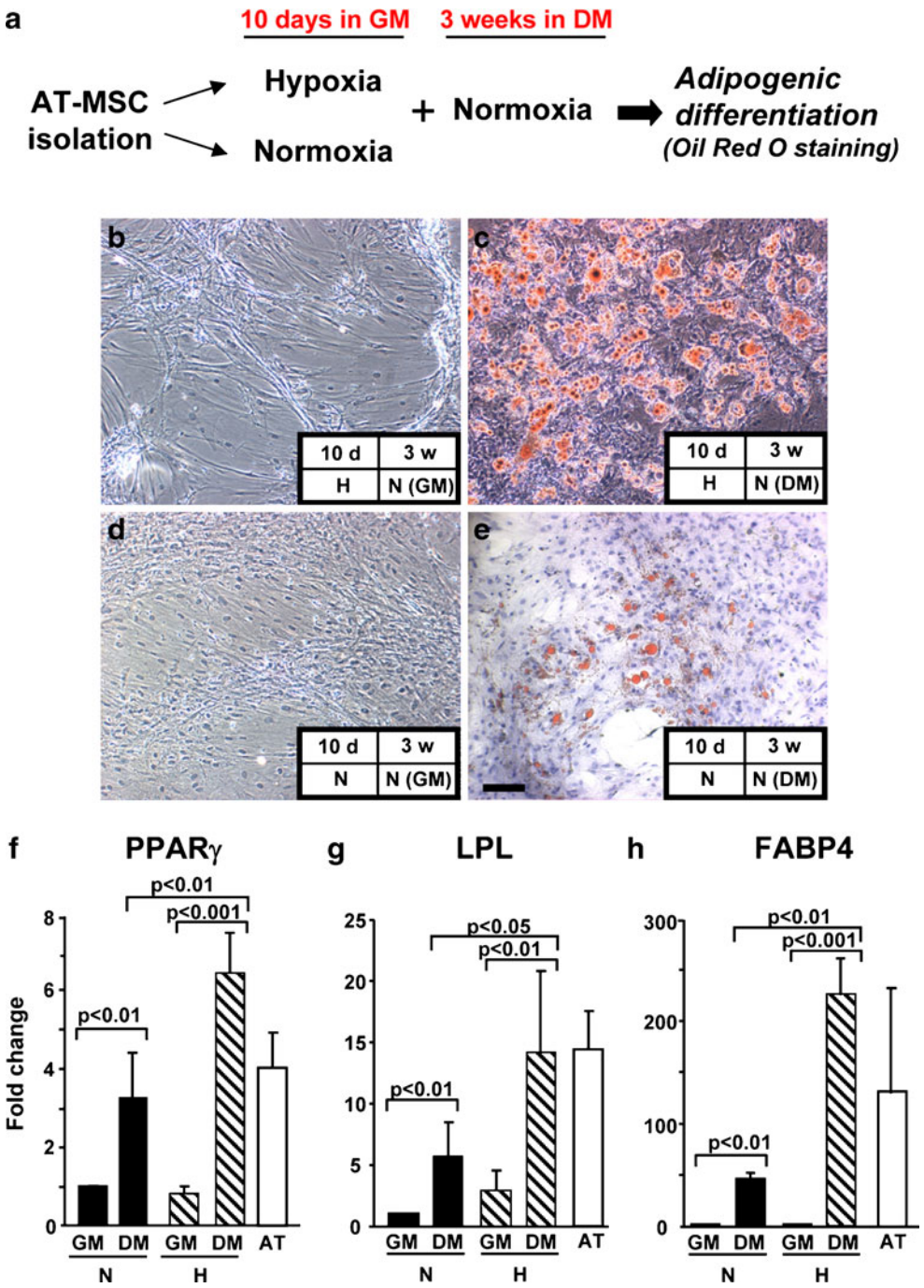

Fig. 5 Pre-hypoxic-cultured AT-MSCs display enhanced adipogenic differentiation potential when exposed to normoxia. **a** Experimental plan. AT-MSCs were cultured under hypoxia in growth control medium (GM) for 10 days and transferred to normoxia in the presence of GM (**b**) or the adipogenic differentiation cocktail medium (DM) (**c**) for a period of 3 weeks. As controls, AT-MSCs were cultured in normoxia and then exposed for the same time either to GM (**d**) or DM (**e**). Oil red O staining showed that lipid vacuoles accumulated to a greater extent in pre-hypoxic AT-MSCs exposed to normoxic conditions under DM (**c**) compared to pre-normoxic AT-MSCs cultured in DM (**e**). *Bar* 100 μm. **f**–**h** Real-time RT-PCR showing the expression of genes involved in adipogenesis *Pparγ, Lpl* and *Fabp4*, in the culture conditions described in (**b**–**e**). Briefly, cells pre-exposed to either normoxia (*N*) or hypoxia (*H*) for 10 days were induced to differentiate after 3 weeks of culture in adipogenic medium (*DM*) under normoxic conditions ($n=3$). As negative controls, cells were left in *GM*. As positive controls fresh murine adipose tissue (*AT*) was used for the expression of the indicated genes. Data are expressed as fold change from normoxic GM-cultured cells

counterpart (Fig. 3b, c). Flow cytometric analysis of cell cycle distribution was performed to further confirm the presence of apoptotic cells. Apoptosis can result in the progressive generation of particles corresponding to hypodiploid DNA content, which reflects DNA fragmentation. By flow cytometry, apoptotic cells appear as a peak in 'sub-G1'. We found that at day 10 of culture, the percentage of sub-G1 cells was lower in hypoxia compared to the normoxic counterpart (Fig. 3d–f). Taken together, these data suggest that low oxygen levels enhanced the proliferative activity of AT-MSCs and protected them from death.

To evaluate the effect of hypoxia on adipogenic differentiation, AT-MSCs were cultured for 10 days in growth medium under either normoxic or hypoxic conditions. We then analyzed their ability to undergo adipogenic differentiation in presence of adipogenic differentiation medium (Fig. 4a). As expected, Oil-Red O-positive colonies were detected in AT-MSCs pre-exposed for 10 days to normoxia and cultured for 3 weeks in adipogenic differentiation medium under normal oxygen levels (Fig. 4c). This phenomenon was strongly attenuated in AT-MSCs pre-exposed for 10 days to hypoxia and then cultured for

3 weeks in adipogenic differentiation medium under hypoxic conditions (Fig. 4e), confirming previous studies that hypoxia affects the adipogenic differentiation capacity of MSCs (Fehrer et al. 2007; Fink et al. 2004; Lee and Kemp 2006).

To analyze whether the adipogenic differentiation program was temporarily or permanently inhibited by hypoxia, AT-MSCs were cultured in growth medium (GM) under normoxia or hypoxic conditions for 10 days, the cells were then transferred to normoxia and the GM was replaced with the adipogenic differentiation medium (DM) (Fig. 5a–e). Oil Red-O staining performed after 3 weeks of differentiation showed that the cells pre-exposed to hypoxia differentiated into adipocytes to a greater extent compared to the cells pre-exposed to normoxia (Fig. 5c, e). The expression of the adipogenic genes peroxisome proliferator activated receptor γ (*Pparγ*), lipoprotein lipase (*Lpl*) and fatty acid binding protein 4 (*Fabp4*) was evaluated by real-time RT-PCR to quantify the adipogenic differentiation of normoxic and hypoxic pre-grown cells that were all transferred to normoxia. As expected, the addition of differentiation medium to pre-normoxic-cultured cells resulted in increased expression of *Pparγ*, *Lpl* and *Fabp4*. However, when pre-hypoxic-cultured AT-MSCs were exposed to differentiation medium under normoxic conditions, the expression of adipogenic genes was significantly higher compared to their normoxic counterparts (Fig. 5f-h).

In conclusion, this study provides the basis for understanding the key impact that in vitro manipulation of oxygenation has on MSCs. Pre-hypoxia exposure enhances proliferation, protects from death, and inhibits adipogenic differentiation of AT-MSCs. Importantly, under this condition, re-oxygenation potentiates the differentiation ability of these cells into adipocytes. Previous studies have demonstrated that the expression of several growth factors including VEGF, HGF and IGF-1, are upregulated both in hypoxic-cultured BM- and AT-MSCs (Gnecchi et al. 2006; Rosova et al. 2008; Sadat et al. 2007), suggesting the existence of a paracrine effect which may positively affect cell proliferation and survival. Emerging evidence indicates that some of the effects of hypoxia on stem cell function are directly regulated by hypoxia-inducible factor (HIF) proteins (Simon and Keith 2008). However, it is not clear how hypoxia affects the differentiation potential of MSC. Low oxygen levels in MSC enhanced the expression of HIF2α, as well as the stem cell gene Oct-4, which is required to maintain embryonic stem cells in their undifferentiated state (Grayson et al. 2006, 2007). Although, a possible hypoxia-mediated reprogramming of MSC has been suggested, it is noteworthy that in our hypoxic culture conditions, Oct-4 was undetectable while the expression of other stem cell genes, Nanog and Sox2, was reduced. Since Oct-4 expression has been shown to increase from days 3 through 7 in hypoxic-cultured human BM-MSCs (Grayson et al. 2007), while an opposite effect in Oct-4 expression was found in murine BM-MSCs cultured in presence of low oxygen levels (8%) up to 6 days (Ren et al. 2006), different cell types and culture time may account for this discrepancy.

Our results are in agreement with previous reports showing that MSC-hypoxic preconditioning is an important factor in enhancing cell survival, proliferation and differentiation in vitro and in vivo (Hu et al. 2008; Hung et al. 2007; Rosova et al. 2008). The exploitation of whether pre-hypoxic exposure affects the differentiation potential of AT-MSCs in other cell lineages including chondrogenic and osteogenic may impact upon the therapeutic potential of these cells.

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