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Prolonged Exposure to Hypoxic Milieu Improves the Osteogenic Potential of Adipose Derived Stem Cells

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ABSTRACT

Mesenchymal stem cells (MSC) have been widely used in orthopedics for several applications. Conventionally, MSC are maintained under 21% O_2 which does not reflect the real O_2 tension in vivo. Recently, it was reported that different O_2 conditions can give different cellular responses. Here, we investigated whether prolonged exposure to hypoxia affects the osteogenic differentiation of adipose-derived stem cells (ASC). ASC from six individuals were cultured under "low" (2–3%) or "air" (21%) oxygen tensions, either without or with osteogenic stimuli. The effect of the O_2 tension was evaluated on cell proliferation, surface antigens, stemness and bone-related genes expression, alkaline phosphatase activity (ALP), mineralization activity, and release of osteogenic growth factors. Without differentiating stimuli, hypoxia favored ASC proliferation, reduced the number of CD184+ and CD34+ cells, and preserved the expression of *NANOG* and *SOX2*. The combination of hypoxia and osteogenic medium induced a high proliferation rate, a rapid and more pronounced mineralization activity, a higher expression of genes related to the MSC differentiation, a higher release of mitogenic growth factors (bFGF, PDGF-BB), and the decrease in TGF-β secretion, an inhibitor of the early stage of the osteoblast differentiation. We demonstrated that hypoxia acts dually, favoring ASC proliferation and the maintenance of the stemness in the absence of osteogenic stimuli, but inducing the differentiation in a bone-like microenvironment. In conclusion, prolonged cell culture in hypoxic microenvironment represents a proper method to modulate the stem cell function that may be used in several applications, for example, studies on bone pathophysiology or bone-tissue engineering. J. Cell. Biochem. 116: 1442–1453, 2015.

KEY WORDS: ADIPOSE-DERIVED STEM CELLS; PROLONGED HYPOXIA; STEMNESS; OSTEOGENIC DIFFERENTIATION

Multipotent mesenchymal stem cells (MSC) are described as cells owned of higher plasticity which give rise to different mesenchymal lineages, such as adipogenic, osteogenic, and chondrogenic lineage [Friedenstein et al., 1968; Ciapetti et al., 2012]. They represent the most effective "natural system" of bone repair, thus healing the majority of bony injuries with new tissue presenting the pre-existing properties [Phinney and Prockop, 2007]. However, there are cases in which bone regeneration is compromised, including delayed union, fracture non-union and avascular necrosis, or other orthopedic conditions in which the need of new formed bone exceeds the physiological capability of self-healing, such as for skeletal reconstruction of large bone defects following trauma. In such cases, MSC-based cell therapy may be a powerful strategy for repairing and/or replacing damaged tissue and restoring lost functionality. Bone progenitors are supplied to the injury site, either alone or in combination with artificial or biologic scaffolds [Rosset et al., 2014].

For a long time, the bone marrow has been considered the most appropriate source for MSC isolation, but other adult tissues have been shown to be good reservoirs of MSC [Moroni and Fornasari, 2013]. The ubiquity, the ease of retrieval, and the minimally invasive procedure required for harvesting the adipose tissue (AT), make it an ideal source for high yield MSC isolation. Adipose tissue is a highly complex tissue comprising a stromal vascular fraction (SVF) and a variety of other cells in addition to mature adipocytes, including MSC [Yoshimura et al., 2009]. Many in vitro and in vivo studies suggest that the use of expanded adipose-derived MSC (ASC) improve bone healing both directly, since they differentiate into mature osteoblasts, and indirectly, through paracrine effects that stimulate the migration and differentiation of the resident precursors [Barba et al., 2013;

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Kim and Heo, 2014]. The first requirement for an effective regenerative strategy is the good yield of stem cells. MSC resident in the bone marrow stroma constitute about 0.001–0.01% of the total marrow nucleated cells, whereas the amount of ASC isolated from equivalent amounts of adipose tissue is approximately 500-fold higher [Strioga et al., 2012]. In addition, ASC possess a higher proliferation capacity and are more genetically stable in long-term culture [Zuk et al., 2002; Lindroos et al., 2011]. A desirable feature is that the cells retain the ability to proliferate preserving their multipotentiality, but the essential condition is that they exhibit a physiologic osteogenic potential in order to obtain a fully functional bone tissue. Over the years, a variety of different culture strategies have been investigated to optimize the expansion and the differentiation of MSC, such as the use of different types of serum and growth factors [Bieback et al., 2009; Bieback, 2013] and the engineering of the microenvironment by using bioreactors [Liu et al., 2013]. However, in vitro simulation of the microenvironment in which cells will be transplanted offer a more realistic scenario of how they may preserve their features and achieve their function.

Recent studies have shown that hypoxia is an important feature of cellular microenvironment able to modify characteristics in various types of stem cells, including embryonic stem cells [Simon and Keith, 2008], induced pluripotent stem cells [Yoshida et al., 2009], and some adult somatic stem cells [Grayson et al., 2007]. For most of these cells, the low oxygen tension has to be considered as a physiological condition, also called "physiological normoxia," rather than the 21% air oxygen tension which is commonly used in standard culture conditions [Nakayama, 2009]. Hypoxia facilitates proper embryonic development, maintain stem cell pluripotency, induce differentiation, and regulate the signaling of multiple cascades, including angiogenesis [Haque et al., 2013].

In the last years, several studies have evaluated the effect of low oxygen tension on MSC, but only few researches have been performed on human ASC and the results on cell expansion and differentiation are contrasting and no consensus has been reached. The majority of the literature indicates that low oxygen concentration (<5%) favors ASC proliferation but inhibits osteogenic differentiation [Lee and Kemp, 2006; Merceron et al., 2010; Sahai et al., 2013; Choi et al., 2014; Russo et al., 2014], while other authors have shown the opposite effect [Valorani et al., 2012]. However, in these studies the experimental conditions are quite different, in particular with respect to the time and the sequentiality of the exposure to the low oxygen concentrations (Table I). Recently, we observed that the low concentrations of oxygen promote cell proliferation and stemness of human ASC without inhibiting their multipotency, thus enriching the pool of cells potentially able to differentiate [Fotia et al., 2014].

Since the data from the literature are controversial, in this study we aimed to investigate the influence of a prolonged hypoxia exposure on the osteogenic potential of ASC obtained from intact adipose tissue. We chose an in vitro method to mimic better what happens when ASC containing osteoprogenitors are transplanted in a hypoxic microenvironment. We examined changes in immunophenotype, gene expression, biochemical markers, bone matrix production and release of pro-osteogenic factors by ASC continuously maintained under "low oxygen tension" (2–3% O_2, hereinafter named hypoxia) or "air oxygen tension" (21% O_2, hereinafter named normoxia), either without or with osteogenic stimuli.

MATERIALS AND METHODS

CELL CULTURE

ASC were isolated as already described [Fotia et al., 2014] from intact adipose tissues of six subjects (five women; mean age 42.6 years; one men; age: 37 years) undergoing abdominal plastic surgery following a remarkable weight loss (BMI < 30). Informed consent was obtained from enrolled patients, all of whom agreed that discarded biological samples could be used for research purposes. A code number was assigned to each sample in order to assure subject anonymity. All samples were processed within 24 h.

Briefly, adipose tissue was washed with PBS, minced into small pieces, and incubated with 0.075% type II collagenase (Gibco, Invitrogen, Monza, Italy) under continuous shaking for 60 min at 37°C. Mature adipocytes and connective tissues were separated by centrifugation and filtration with a 100-mm mesh (Sigma-Aldrich, Milan, Italy). Freshly isolated stromal vascular fraction (SVF) was resuspended in standard medium (SM), consisting on Alpha-MEM (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml Fungizone (Gibco). The cells were seeded and maintained at 37°C in 5% CO_2 and 21% O_2 in humidified atmosphere. As previously demonstrated [Fotia et al., 2014] after 96–120 h cells reached the 80% confluence. At this time point, ASC were seeded for the different experiments under the two oxygen tensions. To create the hypoxic conditions the cells were incubated in the Hypoxia Incubator Chamber (Stem Cell Technology, Vancouver,

TABLE I. Relationship Between Osteogenic Potential of Human ASC and Timing of Exposure to Low Oxygen Tension (Summary of Recent Studies)

References	Source of human ADMSC	Expansion	Timing of expansion (passage)	Osteogenic differentiation	Timing of osteogenic differentiation (days)	Mineralization (hypoxia vs. normoxia)
Lee and Kemp [2006]	Intact tissue	Normoxia	Unknown	Hypoxia vs. normoxia	21	↓
Merceron et al. [2010]	Lipoaspirate	Normoxia	Passage 2	Hypoxia vs. normoxia	28	↓
Valorani et al. [2012]	Lipoaspirate	Hypoxia vs. normoxia	Passage 1	Normoxia	22	↑
Sahai et al. [2013]	Lipoaspirate	Normoxia	Passage 2–7	Hypoxia vs. normoxia	28	↓
Choi et al. [2014]	Intact tissue	Hypoxia vs. normoxia	Passage 3	Hypoxia vs. normoxia	21	↓
Russo et al. [2014]	Intact tissue	Normoxia	Passage 2	Hypoxia vs. normoxia	28	↓

Canada) and flushed with a mixture of gasses (95% N_2 and 5% CO_2). The final oxygen tension was 2–3%, measured by oximeter Oxybaby M+ (Witt Technology, Solza, Italy). The culture medium was discarded and replaced with fresh medium every 3–4 days. For osteogenic differentiation, the culture medium was replaced with the osteogenic medium (OM) consisting of α-MEM, 10% FBS, supplemented with 50 μg/ml L-ascorbic acid 2-phosphate (Sigma), and 10^{-8} M dexamethasone (Sigma). For mineralization medium (MM), OM was supplemented with 10 mM β-glycerophosphate (Sigma). The effect of hypoxia was evaluated by analyzing different parameters (immunophenotype, gene expression, cell proliferation, growth factor release in the supernatant, ALP activity, and mineralization activity) at different time points, that is, T0: ASC selected by adhesion after 96–120 h from the seeding of the SVF, before the hypoxia conditioning; T1: after 6 days of culture in SM or OM; T2: only in OM conditions on cells reaching the confluence; T3: on cells cultured for 9–12 days in MM. The protocol for cell culture characterization is summarized in Figure 1.

FLOW CYTOMETRY

Immunophenotyping of ASC was performed using monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), or R-Phycoerythrin (RD1), or RD1-Cyanin 5.1 (PC5) (Biolegend, Milan, Italy). The description of the tested antigens is shown in Table II. Monoclonal antibodies and cells (10^5/test) were incubated for 20 min at 4°C, and the percentage of positive cells was evaluated on 10,000 events by using a flow cytometer EPICS XL-MCL (Beckman Coulter, Fullerton, CA). Moreover, for highly expressed antigens (>95%), we evaluated the mean fluorescence intensity (MFI), which indicates the mean expression of the antigen on the cell surface.

PROLIFERATION ASSAY

ASC were seeded at 100,000/well on six-well plates in duplicate. According to the proliferation kinetics of our cell model [Fotia et al., 2014], cell proliferation was evaluated after 6 days of culture in hypoxia or normoxia. The cells were detached with 0.05% Trypsin-EDTA (Gibco) and the cell counting was performed on harvested cells using eritrosin B (Sigma) dye exclusion assay.

GENE EXPRESSION ANALYSIS

The gene expression was analyzed in duplicate at T0 and T1, by quantifying the transcripts of genes useful to monitor the stemness [Riekstina et al., 2009] and the osteogenic potential [Granchi et al., 2010] of ASC (Table III). RNA was extracted with NucleoSpin RNA II (Macherey-nagel, Düren, Germany) and the retrotranscription was performed with MuLV Reverse Transcriptase (Applied Biosystems,

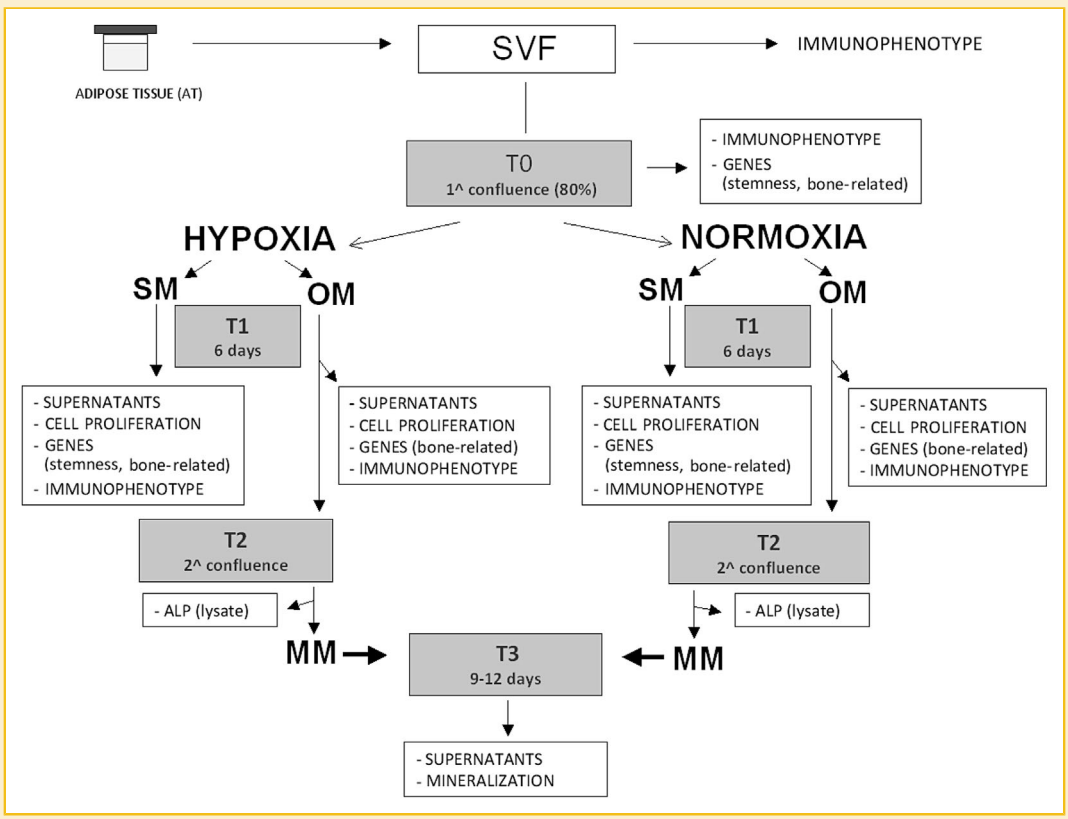

Fig. 1. Flow diagram for the evaluation of hypoxia effect on stemness properties and osteogenic differentiation of human ASC. ASC were isolated from SVF by adhesion, after 96–120 h from the seeding. T#: time point (for details see Materials and Methods Section); SM, standard medium; OM, osteogenic medium; ALP, alkaline phosphatase; MM, mineralization medium. Stemness genes analyzed were *NANOG*, *SOX2*, and *OCT4*. Bone-related genes analyzed were *COL1A1*, *COL12A1*, *IBSP*, *SPARC*, *POSTN*, *CLEC3B*, *BGLAP*, and *ALPL*. Supernatants were used for ELISA assays to quantify the release of growth factors, such as bFGF, PDGF-BB and TGFβ.

TABLE II. Immunophenotypic Characterization of ASC

Cluster differentiation	Description	SVF	T0
CD49e	α5 integrin; fibronectin receptor	4% ± 4	93% ± 5
CD45	Receptor-type tyrosine-protein phosphatase C	23% ± 9	6% ± 6
CD90	Thymus cell antigen 1 (Thy-1)	63% ± 12	99% ± 1
CD105	Endoglin	3% ± 0	88% ± 15
CD73	Ecto-5′-nucleotidase	43% ± 13	97% ± 1
CD184	CXC chemokine receptor type 4 (CXCR-4)	4% ± 1	5% ± 1
CD117	Mast/stem cell growth factor receptor kit	3% ± 3	7% ± 6
CD140b	Platelet-derived growth factor receptor-β (PDGFRβ)	15% ± 13	44% ± 35
CD146	Melanoma cell adhesion molecule (MCAM)	18% ± 9	43% ± 11
CD34	Hematopoietic progenitor cell antigen 1 (HPCA1)	45% ± 5	47% ± 11
CD36	Thrombospondin receptor, collagen receptor (type I, IV, V)	23% ± 10	31% ± 24

Percentage (mean value ± SEM) of cells expressing markers before seeding of the SVF and after the first confluence (T0) in normoxic condition.

Foster City, CA). Real-Time Polymerization Chain Reaction (Real-time PCR) was performed by amplifying 1 μg of cDNA using the Light Cycler instrument and the Universal Probe Library system (Roche Applied Science, Monza, Italy). Probes and primers were selected using the web-based assay design software (ProbeFinder: http://www.roche-applied-science.com). β-Actin was used as housekeeping gene to normalize the expression of the genes of interest. The results were expressed as a ratio between gene of interest and β-actin reference gene.

ALP ACTIVITY

Cell lysates of ASC were obtained with 0.01% SDS. The ALP activity was measured in duplicate by a chromogenic assay based on conversion of p-nitrophenyl phosphate substrate to p-nitrophenol. ALP reaction buffer (Sigma) was added 1:1 to cell lysates and the mixture incubated at 37°C for 15 min. The absorption was measured at 405 nm wavelength with a spectrophotometer for microplates (Infinite F200pro, Tecan, Männedorf, Switzerland), and ALP activity calculated using a standard curve by serial dilution of p-nitrophenol solution in a range of concentrations between 0 and 40 mmol. The results were expressed as millimoles of p-nitrophenol formed in 1 min and normalized to the cell protein content evaluated by the BCA protein assay kit following the manufacturer's protocol (Pierce, Thermo Fisher Scientific, Milan, Italy).

MINERALIZATION ASSAY

The deposition of mineralized matrix was evaluated in duplicate on cells cultured with OM until reaching the confluence, then the

TABLE III. Gene Expression in ASC

Gene	ID number	Primer sequence (5′–3′)		Probe[a]
ALPL: alkaline phosphatase, liver/bone/kidney	NM_000478.3	Sense	gggtcagctccaccacaa	#52
		Antisense	gcattggtgttgtacgtcttg	
BGLAP: bone gamma-carboxyglutamate (gla) protein (osteocalcin)	NM_199173.2	Sense	ggcgctacctgtactaatgg	#1
		Antisense	tcagccaactcgtcacagtc	
CLEC3B: C-type lectin domain family 3, member B (tetranectin)	NM_003278.1	Sense	ctcaaggagccgtctggaca	#10
		Antisense	gtccccttcaggcagacc	
COL1A1: collagen, type I, alpha 1	NM_000088.3	Sense	cccctggaaagaatggagat	#60
		Antisense	aatcctcgagcaccctga	
COL12A1: collagen, type XII, alpha 1	NM_004370.5	Sense	actggaaaagacggtgcaat	#81
	NM_080645.2	Antisense	ccacttggtccttgtgactcc	
IBSP: integrin-binding sialoprotein (bone sialoprotein)	NM_004967.2	Sense	gcaagaaaatggagatgacagtt	#35
		Antisense	cttcattgttttctccttcatttg	
NANOG: Nanog homeobox	NM_024865.2	Sense	atgcctcacacggagactgt	#69
		Antisense	agggctgtcctgaataagca	
OCT-4: POU class 5 homeobox 1, transcript variant 1	NM_002701.4	Sense	cttcgcaagccctcatttc	#60
		Antisense	gagaagggaaatccgaag	
POSTN: periostin, osteoblast specific factor	NM_006475.1	Sense	atgggagacaaagtggcttc	#47
		Antisense	ctgctcctcccataatagactca	
SOX-2: SRY (sex determining region Y)-box 2	NM_003106.3	Sense	gggaatggaccttgtatag	#65
		Antisense	gcaaagctcctaccgtacca	
SPARC: secreted protein, acidic, cysteine-rich (osteonectin)	NM_003118.2	Sense	acccgcttttcgagacc	#85
		Antisense	caagatccttgtcgatatccttct	
β-Actin (housekeeping gene)	NM_001101.2	Sense	ccaaccgcgagaagatga	#64
		Antisense	agggctgtcctgaataagca	

List of primers and probes selected to analyze the expression of genes related to the cell stemness and the osteogenic phenotype.
[a]Number of probe specific for each gene expression analysis (ProbeFinder: http://www.roche-applied-science.com).

medium was supplemented with 10 mM β-glycerophosphate (Sigma). At the T3 time point, cells were fixed in 3.7% paraformaldehyde for 20 min and stained with 1% Alizarin Red (pH 4.2) (Sigma) for 1 h at room temperature.

IMMUNOENZYMATIC ASSAY

The concentration of human basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF-BB) and transforming growth factor beta 1 (TGFβ1) released in the supernatants by ASC was measured using commercially available reagents, based on a sandwich enzyme immunoassay technique (human FGF basic DuoSet; human PDGF-BB DuoSet; human TGFβ1, R&D Systems, Minneapolis, MN), and following the manufacturer's protocol. For each assay a standard reference curve was prepared, using the appropriate recombinant human protein, between 0 and 1,000 pg/ml (bFGF) and 0–2,000 pg/ml (PDGF-BB, TGFβ1). Briefly, 100 μl of standards and samples were pipetted into a 96-well microplate pre-coated with a mouse anti-human antibody, and a biotinylated mouse anti-human antibody was used to detect the growth factor bound by the immobilized antibody. To analyze the content of bFGF and PDGF-BB, the supernatants were used intact, whereas for the TGFβ1 the supernatant was activated with 1 N HCl for 10 min at room temperature, then the reaction was stopped with NaOH/0.5 M HEPES. Following washing to remove any unbound antibody-enzyme reagents, a tetramethylbenzidine substrate solution was added to the wells. The reaction was stopped with 2 N H_2SO_4, and the optical density was read using a microplate reader set to 450 nm (Infinite F200pro, Tecan). The concentrations of growth factors were extrapolated by means of a dedicated software, Magellan V 7.1 SP-1 (Tecan).

STATISTICAL ANALYSIS

The statistical analysis was performed by StatView5.01 software (SAS Institute, Inc., Cary, NC). Quantitative results were expressed as arithmetic mean plus or minus the standard error of the mean (SEM). Mann–Whitney test was performed as unpaired comparison for two independent variables. All P values <0.05 were considered as statistically significant.

RESULTS

EXPRESSION OF TYPICAL ASC ANTIGENS

The immunophenotype of the isolated cells was characterized at three time points. Before the seeding of the SVF; at T0, namely the first confluence; at T1 time point, namely after 6 days of culture with SM or OM under hypoxic and normoxic condition. The SVF was characterized by the presence of a heterogeneous population of cells, demonstrated by the existence in the same pool of cells of both hematopoietic and mesenchymal antigens (Table II). At the first confluence, the cells selected by adhesion showed an increase in the expression of mesenchymal markers and the loss of expression of hematopoietic markers (Table II). The percentage of cells CD36+, a typical marker of MSC derived from adipose tissue, was enriched at the later stages of culture (Table II). When the cells were cultured under the two oxygen tensions, hypoxia reduced significantly the number of cells expressing CD184 and CD34 detectable in the presence of SM (Fig. 2), and the intensity of CD105 expression with OM (Table IV).

CELL PROLIFERATION

The effect of hypoxia on cell proliferation was evaluated on ASC cultured in SM and OM for 6 days. As reported in Figure 3, we found that the low oxygen tension favored the proliferation of cells cultured under both standard and differentiating conditions ($P = 0.04$, hypoxia vs. normoxia for both culture conditions).

EXPRESSION OF STEMNESS GENES

In order to evaluate if hypoxia affected the stemness, the expression of *OCT4*, *NANOG*, and *SOX2* was analyzed by RT-PCR at T0 and after 6 days of culture in SM (T1). While *OCT4* transcripts were not detectable in all the ASC cultures (data not shown), under low oxygen tension the transcript levels of *NANOG* and *SOX2* increased from T0 to T1 (Fig. 4).

EXPRESSION OF BONE RELATED GENES

To determine if hypoxia could affect the osteogenic differentiation of ASC, we evaluated the expression of extracellular matrix proteins, such as collagenous (*COL1A1*, *COL12A1*) and non-collagenous proteins (*CLEC3B*, *IBSP*, *POSTN*, *SPARC*, and *BGLAP*), and *ALPL*. After 6 days in OM (T1) hypoxia favored the expression of most of the analyzed genes, even if with a large variability and at different extent. In particular, in air oxygen tension the expression of *ALPL* and *IBSP* decreased from T0 to T1, while in hypoxic condition the expression increased. The increase in *BGLAP* and *CLEC3B* expression was significant in hypoxic conditions, while it was not significant in normoxia. Finally, the changes in *COL1A1*, *SPARC*, and *POSTN* were more pronounced in hypoxic environment, and were higher than that observed in normoxia (Fig. 5).

MINERALIZATION

The biochemical activity of alkaline phosphatase was evaluated on cells cultured with OM until reaching the second confluence (T2). Figure 6A shows that the oxygen tension did not influence ALP activity. After the addition of β-glycerophosphate we performed a daily observation of the cultures, and at 9–12 days we found that ASC maintained in hypoxia showed a high number of mineral nodules that were microscopically visible even without staining. Since the cellular multilayer tended to detach showing signs of suffering, the end point T3 was fixed at 9 days instead of 21 days as required by our standard operating procedure for testing in vitro mineralization [Fotia et al., 2014]. We found that the deposition of mineral nodules was strongly promoted by hypoxia. Indeed, the number of mineral nodules formed under low oxygen tension was significantly higher, and their size varied in a wide range of dimension (Fig. 6B,C).

QUANTIFICATION OF GROWTH FACTORS RELEASED BY ASC

We also analyzed if hypoxia affected the release of growth factors involved in proliferation and osteogenic differentiation, thus performing the immunoenzymatic assays of PDGF-BB, bFGF, and TGF-β at T1, T2, and T3 (Fig. 7A–C).

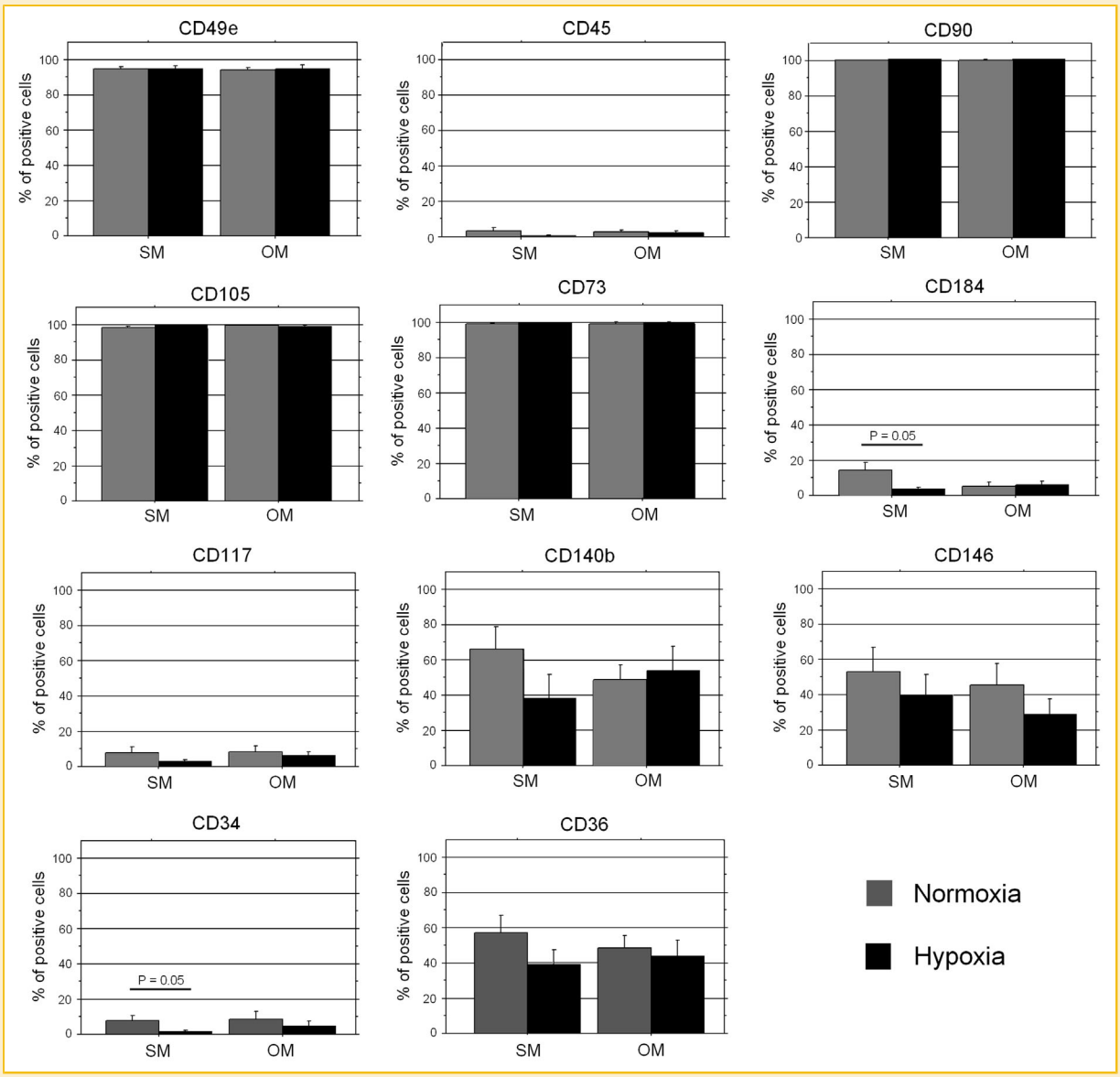

Fig. 2. Expression of surface markers in ASC cultivated in hypoxia and normoxia (n = 6). Expression of surface antigens was evaluated at the T1 time point by flow cytometer. The analysis reveals that hypoxia induced a significant reduction in the percentage of CD34+ and CD184+ cells in SM. Mean ± SEM.

PDGF-BB and bFGF were detectable only in three of six samples, and their secretion was increased in hypoxia, in all culture conditions. In particular, the release of PDGF-BB was steadily under the detection limit when cells were maintained in normoxic condition, while discrete amounts were assessable under hypoxia (Fig. 7A). When ASC were cultured with SM, bFGF was released under both oxygen tensions, but the amount was significantly higher in hypoxia than in normoxia ($P = 0.05$). After the addition of OM, bFGF was measurable

TABLE IV. Mean Fluorescence Intensity (MFI)

	Standard medium			Osteogenic medium		
Cluster differentiation or antigen	Hypoxia	Normoxia	*P*	Hypoxia	Normoxia	*P*
CD49e	3 ± 0	4 ± 0	0.255	4 ± 0	4 ± 1	0.838
CD105	17 ± 6	23 ± 5	0.417	8 ± 1	17 ± 4	0.05
CD73	14 ± 3	14 ± 2	0.958	12 ± 2	11 ± 1	0.642
CD90	195 ± 27	281 ± 45	0.143	237 ± 45	153 ± 17	0.101

MFI (mean value ± SEM) of different markers in cells cultivated with standard and osteogenic medium under hypoxic or normoxic condition.

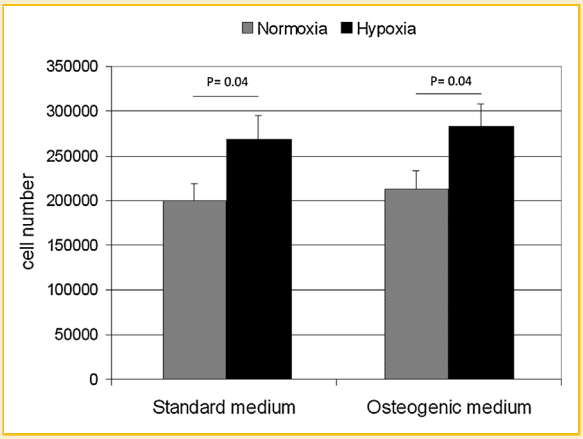

Fig. 3. Effect of hypoxia on ASC proliferation (n = 6). Cells were cultivated in SM or OM under hypoxic and normoxic conditions until T1. Numbers of viable cells were counted using erythrosine B staining. Hypoxia induced a significant increase in the proliferation rate of ASC in both culture conditions. Mean ± SEM.

only in hypoxia. The difference between hypoxia and normoxia disappeared in the presence of mineralization medium (Fig. 7B).

On the contrary, TGF-β was detectable in all the culture conditions, but the release was significantly lower in hypoxic condition, either when ASC were cultured with SM ($P = 0.006$) or OM ($P = 0.006$). At T3 time point, hypoxia has no longer any effect, because the release of TGF-β from cells cultivated with MM was significantly increased in comparison to that measured in OM cultures, but only in hypoxic condition ($P = 0.027$; Fig. 7C).

DISCUSSION

ASC represent a plentiful source of adult multipotent stem cells that could provide wide possibilities for applications in orthopedics when the physiological capability of bone regeneration is not sufficient or compromised. Essential requirements for an effective ASC-based cell therapy are not only the good yield of stem cells, but also the assurance that they preserve the multipotentiality, the ability to proliferate and to differentiate towards the osteogenic lineage. A main goal is to verify that the microenvironment in which the osteoprogenitors will be transplanted allow to accomplish their function. An effective method for supporting the use of ASC into clinical setting is to apply culture conditions that mimic the damaged bone. Pathological lesions of bone tissue that could take advantage by regenerative approaches, for instance osteonecrosis and nonunion, are characterized by hypoxic microenvironment. In this study we investigated the influence of low oxygen tension on cell growth and osteogenic differentiation of human ASC. Our results show that hypoxia acts dually, favoring the cell proliferation and the maintenance of the stemness in the absence of osteogenic stimuli, but inducing the differentiation when the bone-like microenvironment is mimicked. Indeed, when cells were cultured without osteogenic stimuli the cell proliferation and the expression of stemness genes were increased, thus testifying that hypoxia favors the maintenance of the undifferentiated state. A high proliferation rate was observed also in the presence of osteogenic medium, but the most remarkable result concerned the enhancement of the osteogenic potential induced by low oxygen concentrations, as the bone nodule formation was more rapid, and of considerably higher extent than the normoxic counterpart. This finding was confirmed by molecular analysis, since the low oxygen tension promoted the expression of noncollagenous and collagenous proteins which are related to the MSC differentiation and mineralization [Granchi et al., 2010]. In addition, the up-regulation of *ALPL* transcripts suggests that the mineralization process is facilitated by hypoxic conditions [Schäck et al., 2013].

We also found some differences in the expression of membrane antigens typical of ASC [Bourin et al., 2013]. Hypoxia without osteogenic stimuli decreased the expression of CXCR4 (CD184), an alpha-chemokine receptor specific for stromal-derived-factor-1

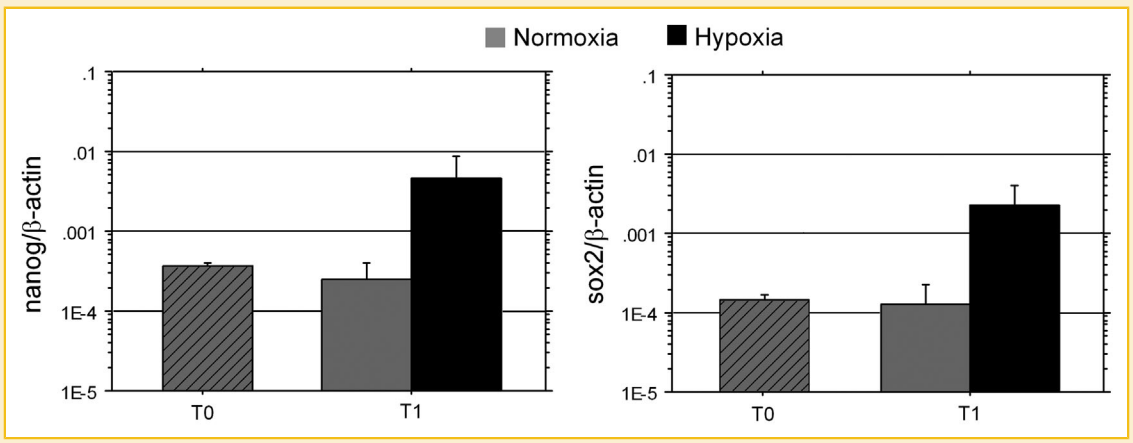

Fig. 4. Effect of hypoxia on stemness gene expression in ASC (n = 6). The mRNA expression levels of *NANOG* and *SOX-2* were evaluated by Real-time PCR on cells cultivated in SM until T1 time point and compared to the basal expression, the T0 time point. Hypoxia favored the expression of both stemness genes. Results are expressed as mean ± SEM of the ratios between "gene of interest" and "β-actin" (Log10 scale).

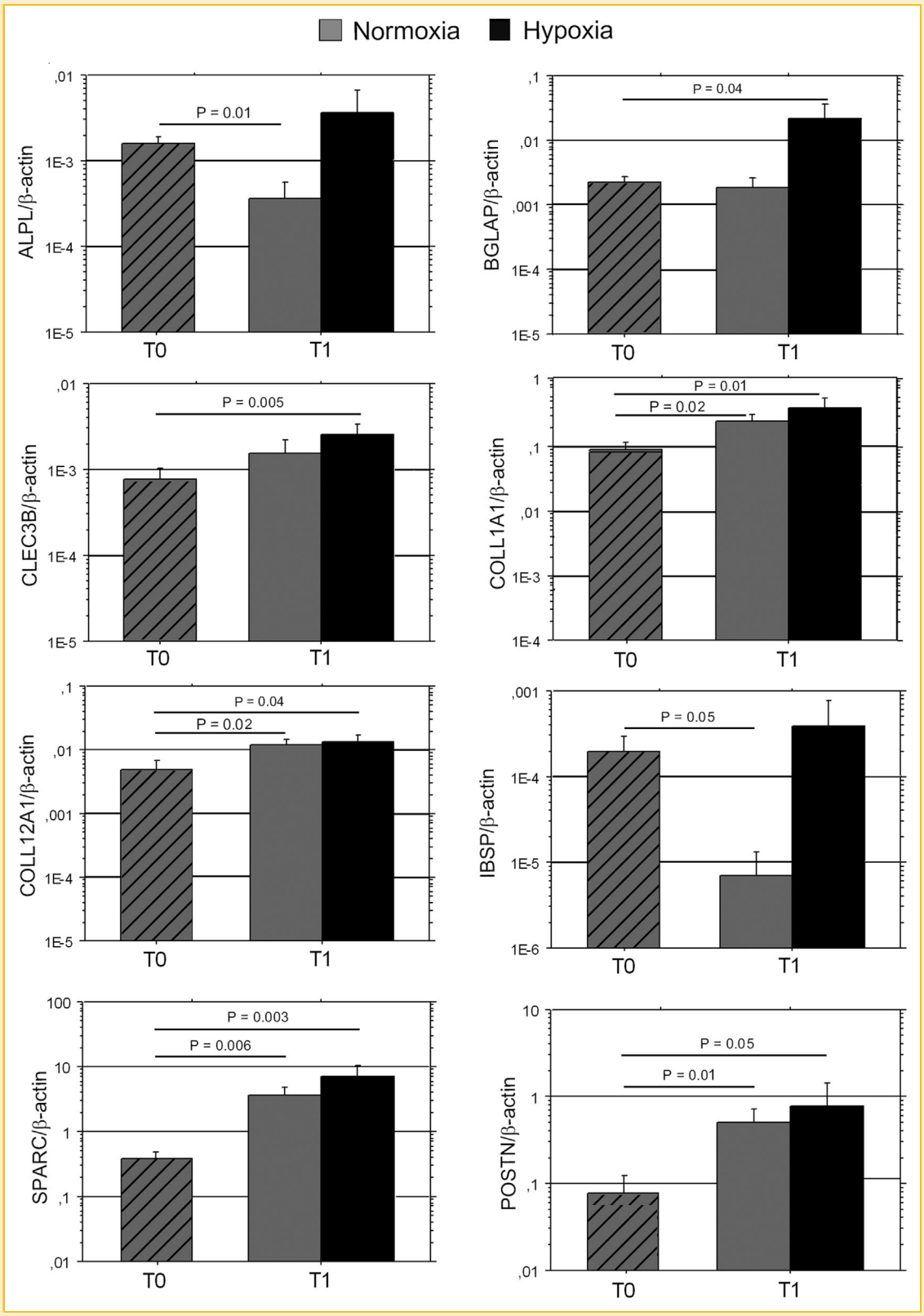

Fig. 5. Effect of hypoxia on the expression of bone related genes in ASC ($n = 6$). The bone markers expression was evaluated after 6 days of culture in OM under hypoxia or normoxia (T1) and compared to the basal expression (T0). Hypoxic culture enhanced the osteogenic potential of ASC compared to normoxic culture. Results are expressed as mean ± SEM of the ratios between "gene of interest" and "β-actin" (Log10 scale).

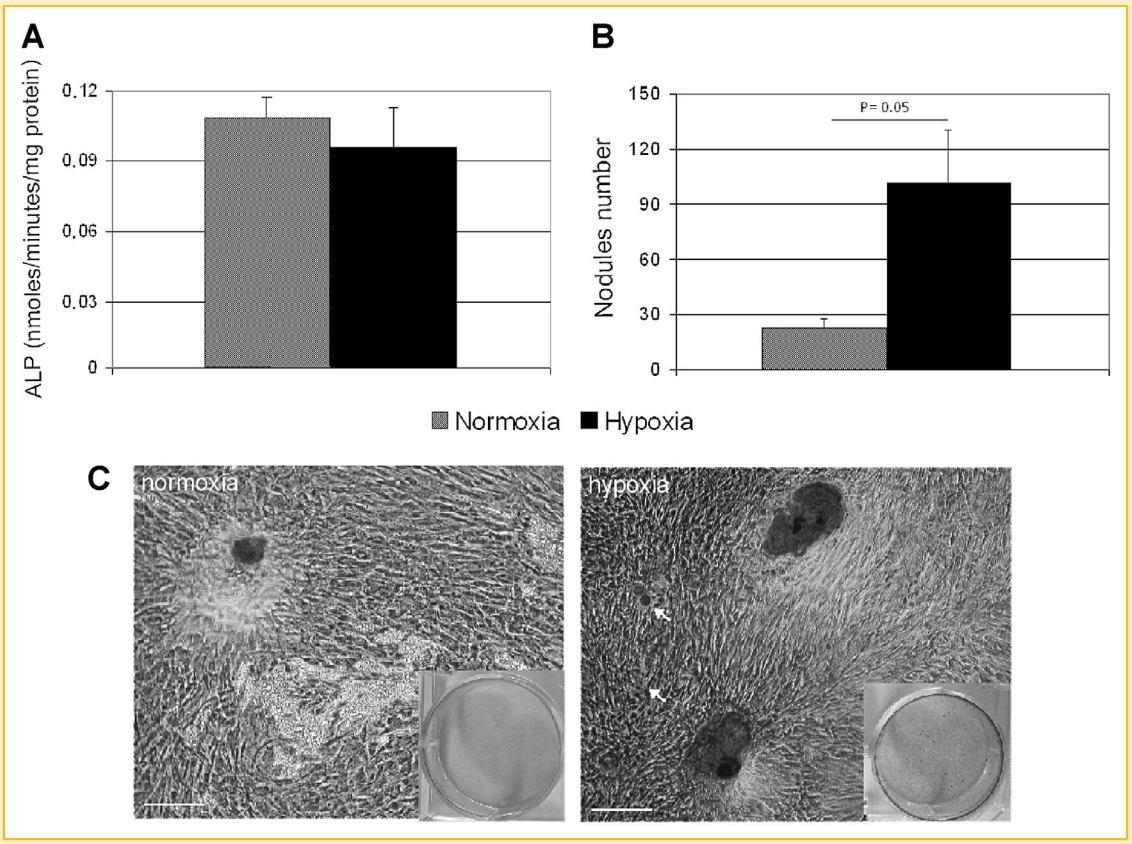

Fig. 6. Effect of hypoxia on ALP activity and mineral nodules deposition in ASC ($n = 6$). A: Biochemical ALP activity. B: Number of calcium phosphate nodules deposited by cells. C: Representative pictures of mineral nodules stained with alizarin red (bar 100 μm). At low oxygen tension ASC formed a number of nodules more than threefold higher. Moreover, the nodules deposited under hypoxia were of different dimensions with very large nodules and numerous nodules of small size (C, arrows). Results are expressed as mean ± SEM.

(SDF-1). SDF-1/CXCR4 axis is involved in mobilization and recruitment of bone-marrow derived MSC and sustains the fracture repair process [Granero-Moltó et al., 2009]. Recent studies have shown that the down-regulation of CXCR4 expression correlates with the loss of MSC homing but does not affect the proliferative ability of the cells [Li et al., 2013]. In addition, we found a significant decrease in the number of CD34-positive cells. Consistent with this result, Valorani et al. observed that the expression of CD34 was reduced in human ASC cultured in hypoxic environment [Valorani et al., 2012]. Even though the role of CD34 in the biology of ASC has not been completely clarified, some authors suggest that the CD34-negative ASC are more responsive to osteogenic commitment [Suga et al., 2009]. Taken together, the down regulation of CD184 and CD34 let to hypothesize that cells cultured in a "niche-like" microenvironment preserve their undifferentiated state but could have a higher propensity to differentiate towards the osteogenic lineage.

In order to evaluate the growth factor production following the hypoxic conditioning, we analyzed some of the growth factors released from ASC. We focused on bFGF, PDGF-BB, and TGF-β, as their role in bone pathophysiology is well recognized [Lieberman et al., 2002]. PDGF-BB and bFGF were detectable only in some samples and their release was favored by low oxygen tension. This finding may explain the higher proliferation rate of cells cultivated in hypoxic condition. In fact, PDGF-BB and bFGF are well known for their mitogenic properties, and their inhibition by neutralizing antibodies reduces the MSC growth [Fekete et al., 2012]. In addition, MSC cultivated with medium supplemented with PDGF-BB and bFGF increased the proliferation rate and the number of cell doublings before reaching senescence [Gharibi and Hughes, 2012].

Hypoxia induced a significant decrease in TGF-β release, either in the presence or the absence of differentiating factors. At the same time, we observed the significant decrease of CD105 (Endoglin), which is recognized as an accessory receptor for TGF-β that may be affected by the hypoxic conditioning [Roemeling-van Rhijn et al., 2013]. TGF-β exhibits bidirectional effects on osteoblast activity. On one hand, it stimulates the expression of bone matrix proteins, but, on the other hand, it inhibits osteoblast differentiation, especially during the early stage of the osteogenic commitment [Alliston et al., 2001]. Our data suggest that hypoxia drives the TGF-β effect towards the latter direction.

The cell culture with low oxygen tension is considered an interesting operating method to increase efficiency of MSC-based regenerative therapies [Haque et al., 2013]. It has been widely proved that the key event in the cellular adaptation towards constant low oxygen tension is the induction/stabilization of the transcription factor hypoxia-inducible factor (HIF)-1, which is composed of

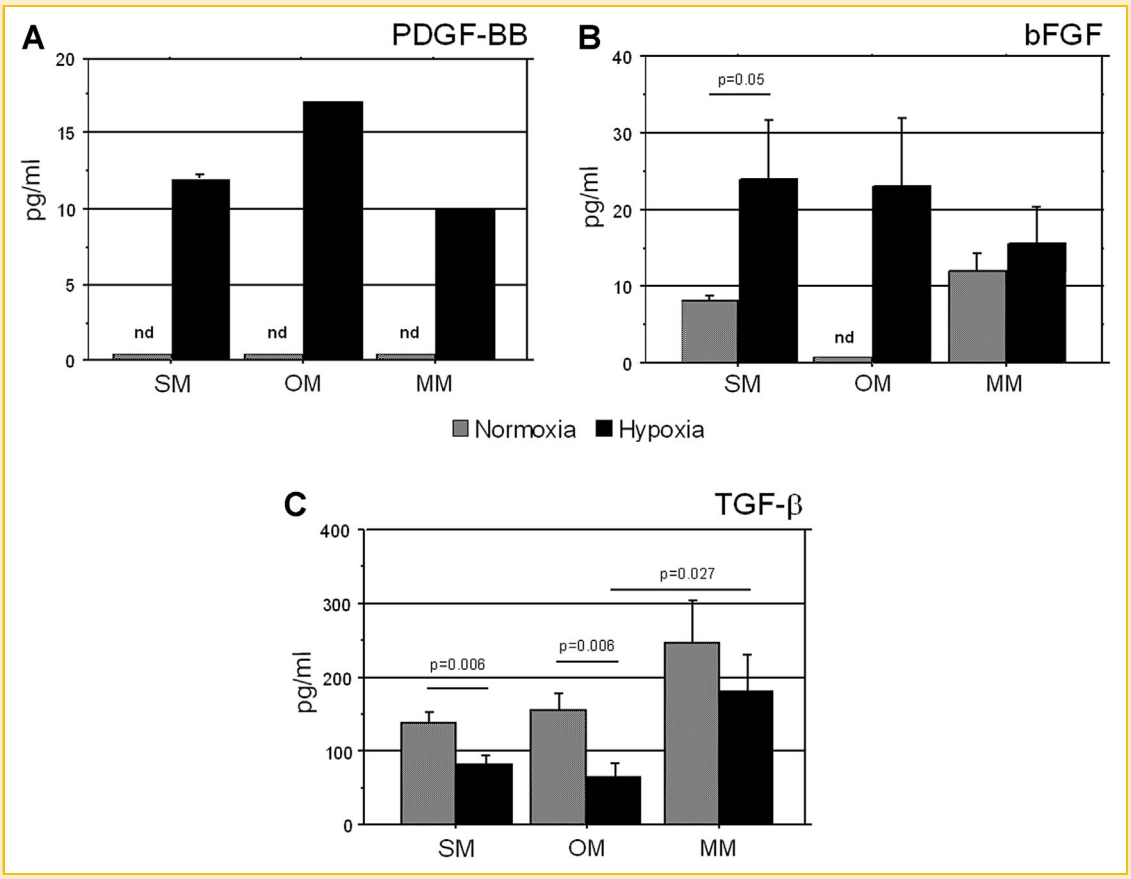

Fig. 7. bFGF, TGFβ, and PDGF-BB secretion by ASC ($n = 6$). Growth factor release was evaluated on cells cultivated for 6 days in SM or OM under hypoxia and normoxia (T1) and after the induction of the mineralization, in MM (T3). Hypoxia induced the release of mitogenic factors and limited the production of TGF-β. Mean ± SEM.

oxygen-sensitive and constitutively expressed subunits, α and β, respectively. O_2 tension > 5% induce the proteosomal degradation of HIF-1α protein, while in hypoxic conditions HIF-1α protein is stabilized, dimerizes with HIF-1β and transactivates a number of genes whose products participate in a variety of cellular processes [Wan et al., 2008]. Some authors state that activation of the HIF-1α pathway accelerates bone regeneration in vivo, suggesting that hypoxia is an important cofactor in bone healing [Wagegg et al., 2012], while others show that inhibition of the HIF-1 activity did not have a significant impact on the expression of the osteogenic markers [Sahai et al., 2013]. It has been proposed that activation of the HIF-1α leads to a better healing of large bone defects, either using HIF-1α transgenic MSC [Ding et al., 2013] or a pharmacological induction [Ding et al., 2014]. In addition, before exposing MSC to the severe ischemia at the site of injury (from 0.4% to 2.3% O_2), the ex vivo preconditioning of the cells in 1–3% oxygen tension may be a valid strategy for preventing apoptosis of transplanted cells [Rosová et al., 2008]. By using in vivo experimental models or bone marrow derived cells, other authors demonstrated that the long exposure to low oxygen tension improves the osteogenic and angiogenic potential of MSC [Rosová et al., 2008; Volkmer et al., 2010; Sheehy et al., 2012; Kim et al., 2014].

On the contrary, while a large part of the literature on ASC state that hypoxia favors cell expansion and stemness, the effect on osteogenic differentiation is still debated. As shown in Table I, the contrasting results could be due to the different conditions used to simulate the hypoxic environment, for example, the timing of exposure to the low O_2 concentration. Indeed, the most part of the authors expanded the cells in normoxia, and then performed the osteogenic differentiation in hypoxia for 3 weeks, at least. In all these cases, the osteogenic potential of human ASC was inhibited, while the adipogenic or chondrogenic differentiation was enhanced. On the contrary, cells pre-cultured in hypoxia and then transferred in air oxygen tension increased their ability to differentiate into osteoblasts, thus suggesting that hypoxic preconditioning could be a valid tool to maximize the regenerative properties of ASC [Valorani et al., 2012]. Nevertheless, the preconditioning approach did not take into account that to restore a bone loss ASC will be transplanted in a hypoxic environment, and the combination of hypoxia and osteogenic stimuli could impair the osteogenic differentiation, as proved by the above mentioned authors (Table I). Actually, Choi et al. [2014] showed that the prolonged expansion of ASC under low oxygen tension (until the third passage) followed by the osteogenic differentiation in hypoxic condition did not favor the mineralization process.

In our study we investigated the effect of a continuous exposure to low oxygen tension, but the osteogenic differentiation (OM) was performed timely and the mineralization has been induced as soon ASC reached the first confluence. This experimental plan is closer to what occurs in vivo. In cell-therapy approaches aimed to repair large bone defects it is expected that MSC populate the lesion site where they encounter severe hypoxic conditions [Rosová et al., 2008]. We simulated in vitro a "bone-like" milieu, thus using additives recognized to promote the osteogenic differentiation, that is, dexamethasone to activate Wnt/beta-catenin signaling, ascorbic acid to permit the assembly of collagen type I fibril, and beta-glycerophosphate as external source of inorganic phosphate to facilitate the mineralization of the extracellular matrix [Schäck et al., 2013].

Our findings suggest that the transplantation of ASC in a hypoxic milieu, as required for regenerative medicine applications in orthopedics, does not compromise but increases their osteogenic potential. We proved in vitro that a prolonged exposure to low oxygen concentrations shows dual beneficial effects. On one hand, hypoxia promotes proliferation and favors the stemness maintenance, thus enriching the pool of cells potentially able to differentiate into osteogenic lineage. On other hand, the most considerable benefit is that the combination of hypoxia and osteogenic stimuli accelerates the cell differentiation and the mineralization process. This study provides a biological basis for the use of ASC in orthopedics, when the physiological capability of bone regeneration is not sufficient or the vascularization is highly compromised. The possibility of modulating the proliferative and differentiative state by simply changing the culture conditions of ASC may be exploited also for the ex vivo cell expansion or for the production of tissue-engineered constructs, resulting also in a significant advantage in terms of time, costs, and cell manipulating. Further studies will be conducted to better understand the molecular mechanism that underlies the HIF-1α activation and the modulation of the genes involved in the osteogenic differentiation. Finally, clinical investigations are needed to demonstrate that the novel approach may really impact the outcome of a regenerative intervention.

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