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Original Paper

Hypoxia Preconditioning of Human MSCs: a Direct Evidence of HIF-1α and Collagen Type XV Correlation

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Key Words
Hypoxia preconditioning • Mesenchymal stromal cells • Osteopotency • Collagen type XV • HIF-1α

Abstract
Background/Aims: Mesenchymal stromal cells (MSCs) hold considerable promise in bone tissue engineering, but their poor survival and potency when *in vivo* implanted limits their therapeutic potential. For this reason, the study on culture conditions and cellular signals that can influence the potential therapeutic outcomes of MSCs have received considerable attention in recent years. Cell maintenance under hypoxic conditions, in particular for a short period, is beneficial for MSCs, as low O_2 tension is similar to that present in the physiologic niche, however the precise mechanism through which hypoxia preconditioning affects these cells remains unclear. ***Methods:*** In order to explore what happens during the first 48 h of hypoxia preconditioning in human MSCs (hMSCs) from bone marrow, the cells were exposed to 1.5% O_2 tension in the X3 Hypoxia Hood and Culture Combo – Xvivo System device. The expression modulation of critical genes which could be good markers of increased osteopotency has been investigated by Western blot, immunufluorescence and ELISA. Luciferase reporter assay and Chromatin immunoprecipitation was used to investigate the regulation of the expression of Collagen type XV (ColXV) gene. ***Results:*** We identified ColXV as a new low O_2 tension sensitive gene, and provided a novel mechanistic evidence that directly HIF-1α (hypoxia-inducible factor-1 alpha) mediates ColXV expression in response to hypoxia, since it was found specifically *in vivo* recruited at ColXV promoter, in hypoxia-preconditioned hMSCs. This finding, together the evidence that also Runx2, VEGF and FGF-2 expression increased in hypoxia preconditioned hMSCs, is consistent with the possibility that increased ColXV expression in response to hypoxia is mediated by an early network that supports

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the osteogenic potential of the cells. ***Conclusion:*** These results add useful information to understand the role of a still little investigated collagen such as ColXV, and identify ColXV as a marker of successful hypoxia preconditioning. As a whole, our data give further evidence that hypoxia preconditioned hMSCs have greater osteopotency than normal hMSCs, and that the effects of hypoxic regulation of hMSCs activities should be considered before they are clinically applied.

Introduction

The processes that take place during skeletal fracture and mechanisms underlying a possible spontaneous healing have only been partially understood [1, 2]. By the use of appropriate experimental *in vivo* and *in vitro* models, it has been understood which cells, soluble factors and components of the extracellular matrix (ECM) have a key role, but the biological network and the metabolic adaptation is complex [3-6]. The knowledge in this field is certainly an essential prerequisite for developing potential cell-based regenerative therapies to obtain bone healing.

A major challenge is to accelerate bone repair/regeneration properly by restoring the functionality of damaged bone tissue, avoiding complications and maintaining the long-term reliability. This requires not only the identification of key molecules with regulatory function in the skeletal homeostasis, but also a concerted effort to try to standardize the experimental procedures and explain some conflicting results. The scenario is burdened by the fact that the ability of an organism to react to a bone insult depends on the type of damage and on many individual's inherent variables including biological factors present in the microenvironment of site of damage.

What happens during tissue regeneration at sites of bone damage is difficult to reproduce in *in vitro* experimental models, despite different approaches have been adopted to investigate both the cellular responsiveness to microenvironment perturbations and the susceptibility of certain genes to be modulated by specific cell culture conditions.

Oxygen (O_2) has been demonstrated to be a potent signaling molecule, and O_2 tension is one of the crucial factors in the skeletal homeostasis that may be adequately monitored and changed in cell culture systems [7-12]. Vascular disruption during bone fracture creates a hypoxic environment within the developing callus [7]. Accordingly, the initial stages of repair at the fracture site occur in a hypoxic zone where a heterogeneous cell population including pluripotent mesenchymal stromal cells (MSCs), osteoblast precursors, mature osteoblasts and osteocytes is present [7, 13]. Therefore, mimicking the natural microenvironment by lowering *in vitro* O_2 tension could provide results closer to what happens *in vivo*. Moreover, hypoxic cultural environment could be of value for cell expansion/propagation before *in vivo* transplantation for bone tissue regeneration therapy [8, 9]. This consideration is particularly critical for MSCs that, residing in bone marrow niches where the oxygen level is 1-2%, require appropriate culture conditions just to preserve stem cell identity and to determine fate toward self-renewal or differentiation [12, 14, 15]. Some evidences suggest that a short-term exposure to low O_2 tension can play a critical role in directing stem cell fate towards a more robust osteogenic differentiation [16, 17]. It is evident that different cellular pathways are influenced by the O_2 tension, but many aspects remain to be explored. Oxygen signaling, although is not a core osteogenic pathway by itself, affect an array of osteogenic factors that, however, still remain to be investigated in detail. In order to shed light on the mechanisms supporting this phenomenon, the present study aimed to characterize the properties of hypoxia-preconditioned hMSCs, in terms of i. e. cellular ability to respond within a short time to variation of O_2 tension, and ii. gene expression modulation. On the one hand, the rate and mode of response by the cells to *in vitro* treatments, such as low O_2 tension, may be predictive of their ability to perceive the physiological microenvironment and the effectiveness of their potential use *in vivo* [18]. On the other, the identification of low O_2 tension sensitive genes allows to recognize critical regulatory components of the osteogenic pathway, primarily

involving HIF-1α (hypoxia-inducible factor-1 alpha) [19]. As potential good candidate to analyze in this context we focused on non-fibrillar collagen (Col) XV, a chondroitin sulphate modified glycoprotein belonging to the multiplexin subfamily (multiple triple helix domains with interruptions), recently identified as a novel human osteoblast extracellular matrix (ECM) protein [20-22]. In particular, our previous evidence suggests that ColXV may act as an ECM organizer in the early-phases of the osteogenic process and that its expression in hMSCs is a prerequisite to promote the subsequent deposition of mineral matrix [23]. In this study, we further evaluate the role of ColXV testing the hypothesis that hypoxic environment may influence its expression in hMSCs.

Materials and Methods

Isolation and growth of hMSCs

Frozen hMSCs isolated from bone marrow aspirates of 12 healthy donors using Ficoll-Hypaque density gradient (d=1.077 g/ml), as previously described [23], were anonymously selected from the Laboratory repository. hMSCs were extensively characterized as previously reported [23, 24]. The cells were grown in α-MEM medium supplemented with 15% fetal bovine serum (FBS) (Euroclone S.p.A., Milan, Italy), penicillin-streptomycin (100 U/ml-100μg/ml) (Sigma-Aldrich, St. Louis, MO, USA). At subconfluence, cells were trypsinized and expanded or used immediately for *in vitro* experiments.

Cell culture under different oxygen tension

hMSCs were plated at 20.000 cells/cm^2 and cultured in normoxic conditions at 95% air (20% O_2) – 5% CO_2 in a conventional incubator for 24h at 37 °C, allowing the cells to attach. Plates were then divided into two groups and incubated under either normoxic or hypoxic conditions for 48h. For the hypoxic cultures, cells were incubated under full-time hypoxic preconditioning in the Xvivo System model X3 (BioSpherix Ltd., Parish, NY, USA) maintaining a hypoxic gas mixture composed of a 1.5% O_2, 5% CO_2, and balanced N_2 up to 48h at 37 °C. The equipment consists of a modular set of closed incubator and closed hood, and allows high optimal solution for cell incubation and handling under hypoxic conditions, preventing the effect of brief interruption of culture condition that can compromise the outcome of the experiments.

Scratch wound healing assay

Scratch wounds were created in confluent hMSCs after 24h of normoxia or hypoxia exposure by using a sterile 20 μl pipette tip. After washing away suspended cells, cultures were refed with medium and cultured in the same experimental condition (normoxia and hypoxia) for further 24h. Cell migration into the wound space was estimated at 0 and 24h after wounding with image analysis, using an inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with a digital camera, and the NIH ImageJ software. Wound closure was determined as the difference between wound width at 0 and 24h.

Actin staining

For analysis of F-actin organization, cells were fixed with 4% paraformaldehyde for 10 min. Subsequently, cells were washed with PBS 1X, permeabilized with 0.1% Triton X-100 for 15 min and stained with Phalloidin CruzFluor-633 conjugated (cat. no. sc-363796, Santa Cruz Biotechnology, Dallas, USA) for 30 min at room temperature. After washing with PBS 1X, the cells were mounted and observed using fluorescence microscopy (Nikon Eclipse 50i).

Assessment of apoptosis

Quantification of apoptotic cells were assessed with the Annexin V binding assay. Briefly, cells were collected and stained with Annexin V-FITC conjugated (Life Technologies, Carlsbad, CA, USA) (100 ng/ml) and propidium iodide (Sigma-Aldrich) (10 μg/ml) at room temperature in the dark for 15 min. Flow cytometric analysis was performed with BD FACSCalibur (Becton-Dickinson Biosciences, San Jose, CA, USA), for each sample 20.000 cells were counted. Data analysis was performed with Kaluza Flow Analysis Software (Beckman Coulter, Brea, CA, USA).

Reactive oxygen species (ROS) measurement

The generation of intracellular ROS was examined by flow cytometry. $1x10^6$ cells were incubated with 10 μM dichlorodihydrofluorescein diacetate (DCFH-DA) (Thermo Fisher Scientific, Waltham, MA, USA) as substrate, in complete medium for 30 min at 37°C in dark to allow cellular incorporation. Cells were then washed, resuspended in PBS 1X, and kept on ice for an immediate detection by flow cytometry. ROS production was assessed based on the DCF fluorescence intensity from 15.000 cells by BD FACSCalibur (Becton-Dickinson). Data analysis was performed with Kaluza Flow Analysis Software (Beckman).

CFU assay

Colony-forming unit (CFU-F) assay was performed on hMSCs. Briefly, cells were plated in six-well plates at 250 cells/cm^2, incubated for 48h in normoxic or hypoxic condition and then cultured over 7 days in normoxia. Thereafter, colonies were fixed with 100% cold methanol, stained with 1% crystal violet dye (Sigma-Aldrich) at room temperature, and washed twice. Colonies containing more than 30 nucleated cells were counted under Eclipse Ni-U microscope (Nikon Corporation).

Western blot analysis

Total cell extracts were prepared from cells exposed to normoxia or hypoxia condition as previously reported [25]. 20 μg of each sample were electrophoresed through a 4-15% SDS-polyacrylamide gradient gel. The proteins were then transferred onto an Immobilon-P PVDF membrane (Millipore, Billerica, MA). After blocking with TBS-0.1% Tween-20 and 5% nonfat dried milk (Sigma-Aldrich), the membrane was probed with the following antibodies: collagen type 15 (1:200; clone C-20), HIF-1α (1:200; clone H1α67), Runx2 (1:500; clone M-70), Oct4 (1:500; clone C-10), Sox2 (1:500; clone E-4) (Santa Cruz Biotechnology), Sox9 (1:1000, #AB5535, Merck Millipore, Germany), and VEGF (1:1000; clone VG-1, Abcam, Cambridge, UK). After washing with TBS-0.1% Tween 20, the membranes were incubated with the appropriate horseradish peroxidase conjugated secondary antibodies (Dako, Glostrup, Denmark). Immunocomplexes were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck-Millipore). A mouse monoclonal anti-β-actin antibody (Sigma-Aldrich) was used for normalization. Densitometric analysis was performed by ImageJ software (NIH, USA, public domain available at: http://rsb.info.nih.gov/nih-image/).

Immunofluorescence analysis

For detection of ColXV, Runx2 and Sox9, hMSCs were grown on glass cover slips, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min, and permeabilized for 5 min at room temperature in 0.2% Triton X-100 in PBS 1X. Cells were then treated for 20 min with blocking solution (PBS 1X/ 1% BSA/ 10 % FBS), incubated over night at 4 °C with the anti-human collagen type15 (ColXV) (1:200, clone C-20), Runx2 (1:100, clone M-70) (Santa Cruz Biotechnologies) and Sox9 (1:100, #AB5535, Merck Millipore) then stained for 45 min at room temperature with appropriate Alexa-488 fluorophore-conjugated secondary antibodies (1:200, Thermo Fisher). Nuclei were stained using DAPI solution (0.5 μg/ml, Sigma-Aldrich). The cells were mounted and observed using fluorescence microscopy (NikonEclipse 50i).

Preparation of conditioned media and analysis of soluble factors

Culture media from hMSCs incubated either in normoxia or hypoxia for 48h, was collected in 15 ml tubes, centrifuged at 5.000 g at 4°C for 10 min to remove cell debris and stored at -80°C. The levels of FGF2 were detected by multiplex bead-based sandwich immunoassay kit (BioRad Laboratories Inc., Segrate, Italy), and ColXV was measured by the enzyme-linked immunosorbent assays (ELISA) using commercial Kit (FineTest, Wuhan, China), following the manifactures'indication. Each sample was analyzed in duplicate.

Identification of HRE (hypoxia-response element) in the human COL15A1 gene promoter

The prediction of hypoxia response elements (HRE) in the COL15A1 gene 5' flanking region spanning +56 to -539, previously identified as core promoter [26], was performed using Patch 1.0 and AliBaba 2.1 public software. The analysis identified four canonical HREs, 5'-RCGTG-3'.

Plasmid construction and luciferase reporter assay

A 593 bp fragment containing the 5'-flanking region of the human COL15A1 gene (-539 to +56) was generated by PCR using human genomic DNA of human HEK-293 cells as template. The PCR product was digested with *MluI* and *HindIII* (Promega, Madison, USA) and subsequently cloned into the promoterless pGL3 Basic vector containing a firefly luciferase cDNA (Promega), generating the pGL3-ColXV-luc construct. For luciferase experiments, human cervical carcinoma HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Euroclone) and transfected with 0.25 μg or 0.5 μg of the reporter construct using Lipofectamine 2000 Reagent (Thermo Fisher), according to the manufacturer's instructions. 24 hours after transfection the medium was replaced and the cells were cultured, where required, in presence of 0.5 mM of $CoCl_2$ for another 24 hours. Cells were then washed once with PBS 1X and lysed in 120 μL of 1X passive lysis buffer (Promega). The luciferase activity was measured using the Luciferase Assay System (Promega) in a GloMax 20/20 single tube Luminometer (Promega) and corrected for total protein content.

Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays were performed as described previously [27, 28]. Briefly, $7x10^6$ cells were incubated for 48h under hypoxia or normoxia. Crosslinking of DNA-bound protein was achieved by 10 min incubation of the cells in a 1% formaldehyde solution at room temperature. Next, cell lysates were sonicated, yielding an average DNA fragment size of 200–1000 bp. Immunoprecipitations were performed overnight (4°C) using 5 μg ChIP-grade rabbit monoclonal anti–HIF-1α antibody (cat. no. NB100-134 Novus Biologicals, CO, USA). Incubation with pre-immune rabbit IgG served as negative controls. The antibody-bound proteins were precipitated for 2 h at 4°C using 60 μl of Protein A-conjugated agarose beads (Merck Millipore). After several washes in low-salt buffer, high-salt buffer, LiCl buffer, DNA was eluted from agarose beads with elution buffer and DNA samples were purified by PCR purification kit (Promega) following the kit instructions. The DNA was eluted in 50 μl of RNase- and DNase-free water. Quantitative real-time PCR (40 cycles) was performed on a CFX 96 thermocycler (Bio-Rad, CA, USA) using iTaq Universal SYBR Green SuperMix (BioRad) and primer pairs spanned the 314 bp region (-537/-223) on the COL15A1 core promoter: Forward 5'-TCCACTCCTGGGCATTCAAGT-3', Reverse 5'-CTGCCTTATGTAACCTGCGA-3'. The Input fractions were used as the internal control. Quantification of HIF-1α–DNA binding was calculated as a fold enrichment using the $2^{(-\Delta\Delta Ct)}$ method and normalized against the pre-immune IgG sample.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) or as median with interquartile range. Comparison between two groups were assessed by non-parametric tests (Wilcoxon matched-pair signed rank test) because the data did not have a normal distribution. The statistical analysis was performed with GraphPad Prism 7.04 (La Jolla, CA, USA), data were considered significant when $p<0.05$.

Results

Culturing and characterization of hMSCs in hypoxia

Bone marrow derived hMSCs were obtained from 12 donors. Considering that, *in vivo*, these cells reside in the niche areas of bone marrow where the standard oxygen level is 1–2% [29], short-term *hypoxic* exposure was performed at 1.5% O_2 and compared with standard cell culture condition (normoxia). A first series of experiments, as shown below, demonstrated the beneficial effects of hypoxia preconditioning.

Firstly, the cells were tested for their migratory capabilities that is one of the inherent traits of all MSCs [30]. As shown in Fig. 1, after 48 h of culture a significant increase of hMSC migration in hypoxic condition was observed. In addition, hypoxia preconditioning did not significantly affect the actin cytoskeletal organization, reactive oxygen species (ROS) production and cell viability, as demonstrated by the TRITC-Phalloidin binding (Fig. 2A), DCF-DA assay (Fig. 2B) and Annexin-propidium iodide staining (Fig. 2C), respectively.

These evidences suggest that the exposure of hMSCs to hypoxic priming didn't activate in the first 48 hours those undesired/adverse effects such as ROS accumulation which can lead to apoptosis, resulting in lethal cell injuries.

Detection and confirmation of cellular hypoxia preconditioning

To confirm that the hMSCs metabolically responded to hypoxia preconditioning, we assessed whether the cells exposed to 1.5% O_2 activated the HIF-1α, a central regulator of the cellular response to hypoxia ubiquitously expressed in mammalian cells [31]. Western blot analysis revealed a significant increase of HIF-1α protein in the hypoxic hMSCs respect to the normoxic control hMSCs (Fig. 3A). Consistently, the expression of vascular endothelial growth factor (VEGF), a primary transcriptional target of HIF-1α [32], significantly increased in the hypoxic samples (Fig. 3A), as well as the secretion of fibroblast growth factor 2 (FGF-2) [33] whose induction required HIF-1α (Fig. 3B).

Stemness properties after hypoxia preconditioning have been also evaluated in terms of clonogenicity and expression of stemness-related genes such as pluripotent factors OCT-4 and SOX-2 which are transcription factors essential for self-renewal and survival of MSCs [34, 35]. As shown in Fig. 3C, the cells did not show significant change of OCT-4 and SOX-2 protein expression level suggesting that hypoxia-preconditioned hMSCs retained a multipotency similar to those cultured in normoxia. Likewise, the CFU-F assay, useful to define the number of progenitor cells

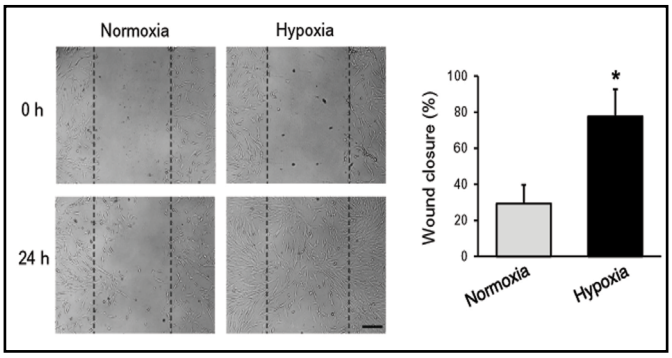

Fig. 1. Effect of hypoxia preconditioning on the wound closure ability of hMSCs. Confluent hMSCs were maintained for 24h in normoxia or hypoxia (1.5% O_2) prior to wounding. Scratch wounds were created by using a sterile 20 µl pipette tip (0 h) and observed over the indicated time periods, 0 and 24 hours. A representative wounds scratch both in normoxia and hypoxia is shown in the pictures (magnification, 4X). Scale bar = 50 µm. The percentage of wound closure was quantified as the difference between wound width at 0 and 24h, in three separate experiments. *p<0.05.

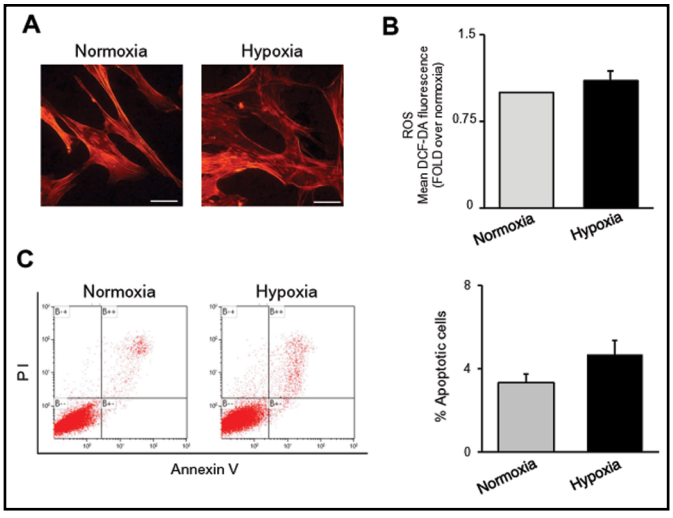

Fig. 2. Effect of hypoxia preconditioning on actin cytoskeletal organization, ROS production and cell viability of hMSCs. (A) The distribution of F-actin filaments was revealed by Phalloidin CruzFluor-633 conjugated staining and detected by fluorescence microscope at 40X magnification, Scale bar = 20 µm. (B) Intracellular levels of ROS was measured by DCF-DA assay; data are expressed as mean ± SD of three independent experiments each one performed in triplicate. (C) hMSCs were harvested and stained with Annexin V-propidium iodide (PI) before analyzed by flow cytometry. Representative Annexin V-PI plots both in normoxia and hypoxia are shown. The histogram represents the percentage of apoptotic cells (ratio of Annexin V-positive cells/total cells). Data are expressed as mean ±S.D. of three independent experiments.

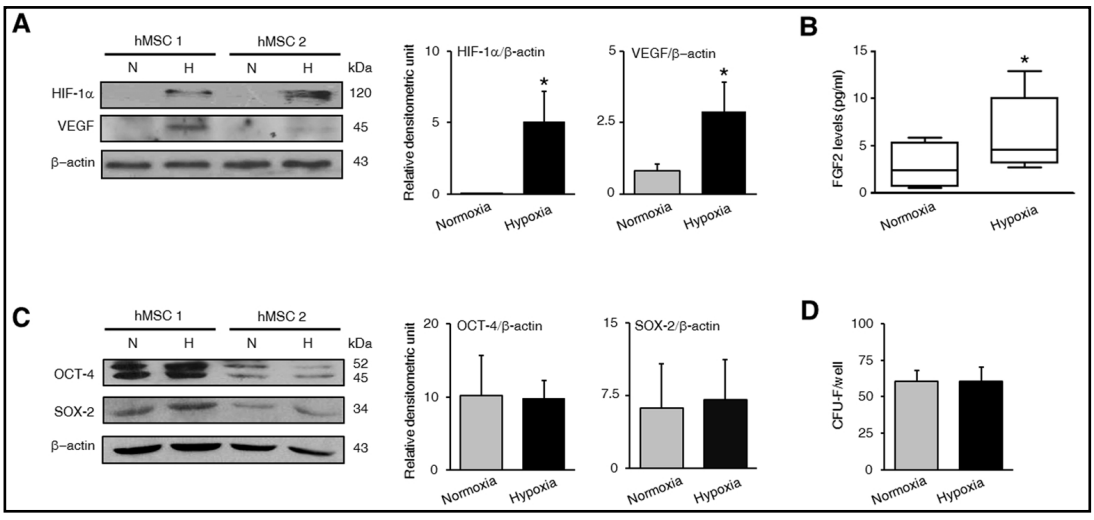

Fig. 3. Hypoxia preconditioning induced the expression of HIF-1α protein and its targets in hMSCs. (A) Expression of HIF-1α and VEGF were evaluated by Western blot in hMSCs exposed to hypoxia (H) or normoxia (N). Representative Western blots are reported with densitometric analysis of all samples analyzed (n=6). Data are expressed as mean ± SD of HIF-1α/β-actin or VEGF/β-actin ratio, of four independent experiments. *p<0.05 versus normoxia. (B) Conditioned media were collected from hMSCs and FGF2 protein level was determined by ELISA. Data are expressed as median with interquartile range of four independent experiments in duplicate. *p<0.05, compared with normoxia. (C) Expression of OCT-4 and SOX-2 were evaluated by Western blot in hMSCs exposed to hypoxia (H) or normoxia (N). Representative Western blots are reported with densitometric analysis of all samples analyzed (n=4). Data are expressed as mean ± SD of OCT-4/β-actin or SOX-2/β-actin ratio, of four independent experiments. (D) The effect of hypoxia preconditioning on colony forming ability of hMSCs was determined by CFU-F assay. Cells were plated in six-well plates at 250 cells/cm^2, incubated for 48h in normoxic or hypoxic condition and then cultured over 7 days. The plates were stained with cristal violet and the colonies (CFU-F) were manually counted. Data are expressed as the average number of colonies per well ± SD of three independent experiments.

and to predict the performance of any cell based therapeutic [36], demonstrated that short-term hypoxia exposure did not affect colony forming ability of the cells (Fig. 3D).

ColXV expression in hypoxia-preconditioned hMSCs

In order to investigate if hypoxia preconditioning has beneficial effects in making cells more prone to osteo-differentiation, the expression of ColXV, previously identified as predictive marker for selecting hMSCs with high osteo-differentiation potential [23], has been assessed at different levels. As reported in Fig. 4A, Western blot analysis showed that ColXV protein levels, which were detectable under normoxia, greatly increased in hypoxia-preconditioned hMSCs. These data were confirmed by immunofluorescent analysis carried out on fixed cells (Fig. 4A). As expected from the lack of ascorbate in the culture medium [37], hMSCs secreted only low levels of ColXV both in normoxia and hypoxia as revealed by ELISA (Fig. 4A). To strengthen the hypothesis that hypoxia-mediated increase of ColXV positively affects the osteogenic potential of the cells, the expression levels of Runx2, the master regulator required for osteoblast cell-fate determination, and Sox9, the key regulator for chondrogenic lineage determination, were assessed (Fig. 4B). It is known that the balance between Runx2 and Sox9 activity is critical for the process of cartilage and bone development, and that an high ratio of Runx2/Sox9 gene expression is associated with the successfull osteogenic differentiation of hMSCs osteo-chondroprogenitors [38]. Interestingly and according to our hypothesis, Western blot and immunofluorescent analysis showed that these two transcription factors are co-expressed in the hMSCs, and that hypoxia preconditioning is effective in increasing Runx2, and in decreasing Sox9 expression levels (Fig. 4B).

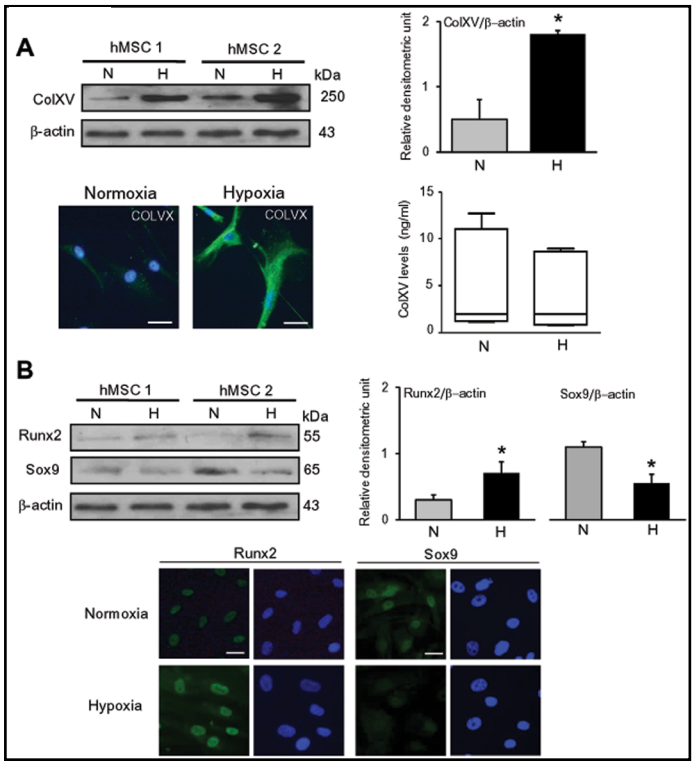

Fig. 4. Hypoxia preconditioning induced the expression of ColXV protein in hMSCs. (A) Expression of ColXV was evaluated by Western blot and immunofluorescence in hMSCs exposed to hypoxia (H) or normoxia (N). Representative Western blots are reported with densitometric analysis of all samples analyzed. Data are expressed as mean ± SD of ColXV/β-actin ratio, of six independent experiments. Each experiment was repeated in duplicate. A representative immunofluorescence staining of hMSC for ColXV (green) is showed. The nuclei counterstained are blue (merged image). Conditioned media were collected from hMSCs and ColXV protein level was determined by ELISA. Data are expressed as median with interquartile range of four independent experiments in duplicate. (B) Runx2 and Sox9 expression level was evaluated by Western blot and immunofluorescence in hMSCs exposed to hypoxia (H) or normoxia (N). Representative Western blots are reported with densitometric analysis of all samples analyzed. Data are expressed as mean ± SD of Runx2/β-actin or Sox9/β-actin ratio, of six independent experiments. Each experiment was repeated in duplicate. A representative immunofluorescence staining of hMSCs for Runx2 or Sox9 (green) is showed. The nuclei were counterstained with DAPI (blue). Images shown are representative of four independent experiments. Magnification, 40X; scale bars = 20 μm. *p<0.05, compared with normoxia.

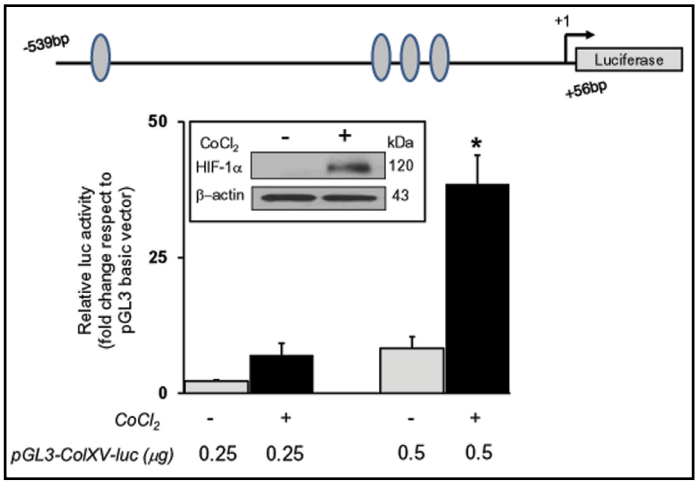

Fig. 5. Regulation of the COL15A1 promoter activity under hypoxia. Transcriptional activity of COL15A1 promoter under hypoxia was evaluated by luciferase assay using the plasmidic construct pGL3-ColXV-Luc containing the minimal core promoter of ColXV gene (-539/+56) which is represented at the top with the positioning of HIF-1α putative responsive elements (HRE) as ovals. HeLa cells were transfected with 0.25 or 0.5 μg of pGL3-ColXV-Luc or promoterless pGL3-Basic vector, in the presence (+) or in absence (-) of the hypoxia mimicking agent $CoCl_2$ for 24 hours.The transfection efficiency was normalized by measuring the total protein in the cell lysates. For fold change, the luciferase output was normalized against the promoterless pGL3-Basic vector, arbitrarily defined as 1. Results are expressed as mean + SD of three independent experiments. Each experiment was repeated in triplicate. *p<0.05. The efficacy of $CoCl_2$ in inducing HIF-1α protein was monitored by Western blot analysis and reported in the inset.

ColXV is a direct target of HIF-1α

Relatively little is known about the regulation of the expression of COLXV gene. By using Patch 1.0 and AliBaba 2.1 public software for transcription binding sites search, we identified four potential HIF-1α binding sites (HREs) in the ColXV gene regulatory region. To determine whether HIF-1α activity plays a role in ColXV transcriptional activation, HeLa cells were transfected with the pGL3-ColXV-luc reporter construct containing the human ColXV core promoter [26]. As shown in Fig. 5, in the presence of the hypoxia mimicking agent $CoCl_2$ the expression of HIF-1α significantly increased. Likewise the activity of ColXV promoter was positively affected by $CoCl_2$ in a dose-dependent manner.

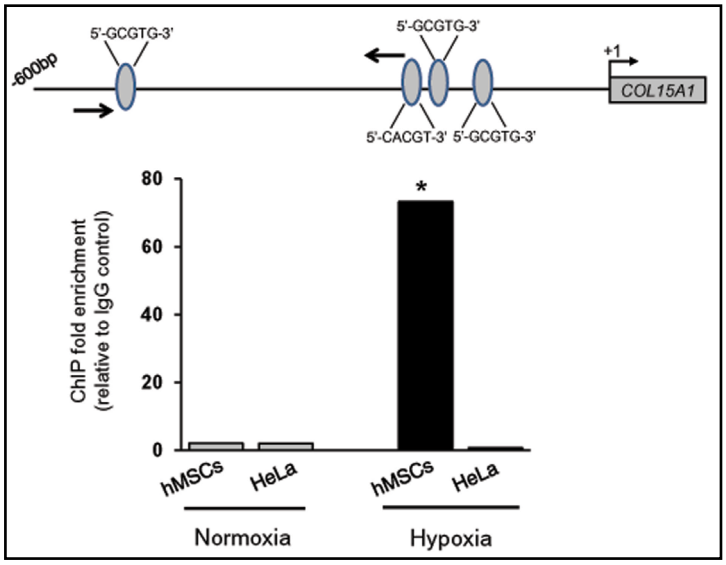

Fig. 6. Hypoxia-inducible factor-1 (HIF-1α) is in vivo recruited to the COL15A1 promoter in hMSCs. The positioning and sequences (5'-RCGTG-3') of HIF-1α potential binding sites within the COL15A1 core promoter together with the position of the specific primers used for qPCR amplifications of anti–HIF-1α immunoprecipitated chromatin are reported. The graph shows the results of ChIP-qPCR analysis performed on DNA templates obtained from hMSCs and HeLa cells exposed to hypoxia or normoxia. Results of qPCR were analyzed by the $2^{(-\Delta\Delta Ct)}$ method after normalization to the IgG negative control and data expressed as mean ± SD of four independent experiments. * $p<0.05$.

The possibility that ColXV is a direct transcriptional target of HIF-1α was examined by ChIP assay in hMSCs, performing for the first time an "*in vivo*" HRE occupancy analysis. As reported in Fig. 6, HIF-1α was specifically recruited at ColXV promoter in hypoxia-preconditioned hMSCs.

Discussion

Culture conditions and cellular signals that can influence the potential therapeutic outcomes of hMSCs are of great interest. For this reason, the ability of hMSCs to respond to a potent signaling molecule such as oxygen needs to be studied, also in view of optimizing stem cell-based therapies in the context of bone regenerative medicine and tissue engineering [39].

Most studies involving progenitor/stem cells are conventionally performed under ambient O_2 tension, without considering the hypoxic physiological niche in which most stem cells normally grow or reside. In the recent years the impact of hypoxic culture systems on the behavior of hMSCs from different sources has been investigated, and it has been demonstrated that hypoxia preconditioning increases the therapeutic potential of MSC in the treatment of ischemic diseases [40-42], traumatic brain injury [43], and in liver regeneration [44].

Although the role of hypoxia in positively conditioning hMSC multipotency, longevity, expansion, secretion and migration ability is widely accepted [8, 15, 29], the effects of a low oxygen microenvironment on hMSC osteogenic potential are often contradictory [16, 45-

48]. In fact, both the up- or down-regulation of proliferation or differentiation of hMSCs by hypoxic culture conditions have been described. The discrepancies reported in literature are likely due to the different protocols, culture media composition, oxygen tension applied, and timing of hypoxia treatment, as well as the heterogeneity of donors. Another important aspect to consider is that cellular adaptation to oxygen tension is a quick event, and hypoxia responses are primarily mediated by signaling pathways involving the hypoxia-inducible factor HIF-1α which is only transiently maintained under hypoxic culture conditions [49]. Consequently, the limited stability of HIF-1α could mask its effective role in hMSCs during a prolonged biological process, such as osteogenic terminal differentiation. Moreover, even if the interplay between glucocorticoid and HIF-1α in hypoxic areas remains unclear, many evidences demonstrated that dexamethasone reduces protein level of HIF-1α as well as its activity affecting transcription of HIF-1α target genes [50, 51]. Therefore, we cannot exclude that long-term hypoxic treatment *in vitro* can lead to results that are difficult to explain when hMSCs are induced to differentiate toward the osteogenic lineage by the conventional dexamethasone-added osteogenic medium. These observations lead us to formulate the hypothesis that the first hours of exposure to the hypoxic microenvironment may play a critical role in producing the benefit of hypoxic conditioning, as found in many different experimental models, and that the precise mechanism through which low oxygen tension affects the cells should be investigated at the early stage.

Therefore, we focused our attention on what happens during the 48 hours of hypoxia preconditioning without adding osteogenic medium, by investigating the expression modulation of critical genes which could be good candidates as markers of increased osteopotency.

In the present paper, we described the efficient production of hypoxia preconditioned hMSCs by using the X3 Hypoxia Hood and Culture Combo – Xvivo System device, able to maintain a stable oxygen tension during the entire period of culture, observation and cellular manipulation. In these conditions the cells showed a significant increase of migration without significant signs of apoptosis, cytoskeleton disorganization or ROS accumulation.

We recently demonstrated that a chondroitin sulphate modified glycoprotein, the ColXV [20, 21] is a critical regulatory component of the osteogenic pathway and that its expression in hMSCs is a prerequisite to promote the subsequent deposition of mineral matrix [23]. For the first time we correlated the increase of HIF-1α expression after hypoxia preconditioning with the increase of ColXV. We identified ColXV as low oxygen tension sensitive gene, providing a novel mechanistic evidence that HIF-1α mediates ColXV expression in response to hypoxia. Importantly, this finding, together with the evidence that also VEGF and Runx2 expression increased, as well as Runx2/Sox9 gene expression ratio, in hypoxia preconditioned hMSCs, is consistent with the hypothesis that increased ColXV expression in response to hypoxia is mediated by a network of early regulators that support the osteogenic potential of the cells. Moreover, these data help to understand the role of collagens, identifying ColXV not only as a good marker of increased osteopotency, but also as a new marker of successful hypoxia preconditioning.

Conclusion

In this perspective it is important to emphasize that: i. the ability of cells to respond within a short time to *in vitro* treatments should be predictive of their ability to perceive the physiological microenvironment and the effectiveness of their *in vivo* potential use; and ii. identifying good markers that give a measure of cell responsiveness allows us to both have important information on the adequacy of the cells we are using, and speculate the importance of a specific pre-treatment as step before clinical cells transplantation.

In conclusions, our data give further evidence that hypoxia-preconditioned hMSCs have greater osteopotency than normal hMSCs, and that the effects of hypoxic regulation of hMSCs activities should be an important parameter to consider before they are clinically applied.

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Disclosure Statement

The authors declare no conflict of interest.

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