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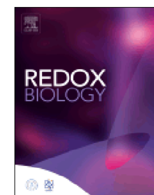

Research Paper

Role of p16[INK4a] and *BMI-1* in oxidative stress-induced premature senescence in human dental pulp stem cells

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ABSTRACT

Human dental pulp stem cells (hDPSCs) are a source for cell therapy. Before implantation, an *in vitro* expansion step is necessary, with the inconvenience that hDPSCs undergo senescence following a certain number of passages, loosing their stemness properties. Long-term *in vitro* culture of hDPSCs at 21% (ambient oxygen tension) compared with 3–6% oxygen tension (physiological oxygen tension) caused an oxidative stress-related premature senescence, as evidenced by increased β-galactosidase activity and increased lysil oxidase expression, which is mediated by p16[INK4a] pathway. Furthermore, hDPSCs cultured at 21% oxygen tension underwent a downregulation of *OCT4, SOX2, KLF4* and *c-MYC* factors, which was recued by *BMI-1* silencing. Thus, p16[INK4a] and *BMI-1* might play a role in the oxidative stress-associated premature senescence. We show that it is important for clinical applications to culture cells at physiological pO_2 to retain their stemness characteristics and to delay senescence.

1. Introduction

Organismal aging is associated with a loss of the homeostasis. One of the elements that contribute to this deterioration is increased cell senescence [1]. Hayflick originally described senescence as a permanent cell cycle arrest due to the limited replicative potential of cultured human fibroblasts [2]. Senescence after a number of cell doublings during *in vitro* culture is inevitable under current culture conditions, resulting in cellular phenotypic changes and growth arrest [3–5]. This observation of cellular senescence has been extrapolated to somatic stem cells *in vivo* and might reflect the aging process of the whole organism [4].

In vitro cellular senescence refers to both replicative and premature senescence [6]. Premature or accelerated senescence can be induced by stress signals, such as activation of oncogenes, strong mitogenic signals, and/or reactive oxygen species (ROS) levels. As we previously reported, oxidative stress is responsible for the low proliferation rate under ambient oxygen tension (21% pO_2) through p38, p21, and NRF-2 activation [7]. Cell culture-inherent oxidative stress can cause critical telomere attrition, accumulation of DNA damage and de-repression of the *INK4/ARF* locus, leading to stress-induced premature senescence (SIPS) [8]. Lysyl oxidase enzymes (*LOXL1* and *LOXL2)* have been also shown to be oxidative stress-sensitive. Among other roles, such as cell motility and cell adhesion, they have been related to cell growth control and cellular senescence [9].

To maintain their replicative and self-renewing potential stem cells have in place mechanisms to repress activation of cell death pathways. The Polycomb-group transcriptional repressor *BMI-1* has emerged as a key regulator in several cellular processes including stem cell self-renewal and cancer cell proliferation. *BMI-1* was first identified in 1991 as a frequent target of Moloney virus insertion in virally accelerated B-lymphoid tumours of E mu-myc transgenic mice [10]. Through repression of target gene expression in a lineage and context- dependent manner, *BMI-1* regulates a myriad of cellular processes critical for cell

Abbreviations: MSC, mesenchymal stem cells; hDPSCs, human dental pulp stem cells; SIPS, stress-induced premature senescence; MDA, malondialdehyde; OSKM, OCT4, SOX2, KLF4 and c-MYC

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growth, cell fate decision, development, senescence, aging, DNA damage repair, apoptosis, and self-renewal of stem cells [11]. The most studied and validated *BMI-1* target is the *INK/ARF* locus, which encodes two structurally distinct proteins, $p16^{INK4a}$ and $p14^{ARF}$, both of which restrict cellular proliferation in response to aberrant mitogenic signalling. Thus, collectively *BMI-1* regulates p53/pRb axis through repression of the *INK/ARF* locus, which has been described as the principle barrier to the initiation and maintenance of neoplastic transformation [12]. *BMI-1* is known to repress the *INK/ARF* locus expression, which encodes two structurally distinct proteins, $p16^{INK4a}$ and $p14^{ARF}$, both of which restrict cellular proliferation in response to aberrant mitogenic signalling [12]. *BMI-1* has been implicated in the modulation of self-renewal in several types of stem cells, including hematopoietic [13], neural [14], and mammary [15].

Self-renewal of stem cells is critical for their persistence through life, however the capacity to maintain this characteristic declines with age [16,17]. Pluripotency genes, *OCT4*, *SOX2*, *KLF4* and *C-MYC* (OSKM) [18], are expressed in both pluripotent and adult stem cells, such as mesenchymal stem cells (MSCs) and are down-regulated upon long-term in vitro expansion and differentiation [19].

Our main purpose was to analyse the role of $p16^{INK4a}$ and *BMI-1* in oxidative stress-induced senescence in long term human dental pulp stem cells (hDPSCs) cultures. In this study we demonstrate that non-physiological *in vitro* cell culture conditions at 21% pO_2 induces premature senescence of hDPSCs, which is mediated by downregulation of *BMI-1* leading to an activation of $p16^{INK4a}$ pathway. By restoring *BMI-1* levels, we were able to rescue *SOX2* and *OCT4* expression under oxidative stress conditions, reflecting that *BMI-1* is not only involved in stem cell self-renewal, but also in stemness maintenance. In summary, we show that oxygen tension is critical when culturing hDPSCs. Ambient oxygen tension (21% pO_2) induces premature hDPSCs senescence compared with physiological oxygen tension (3% pO_2) due to activation of $p16^{INK4a}$ pathway. Moreover, this is accompanied by a *BMI-1*-dependent stemness potential loss. This is of importance in regenerative medicine and also in stem cell banking for clinical use.

2. Material and methods

2.1. Dental pulp stem cells isolation and culture

Intact third molars were collected from men and women (aged from 15 to 35 years old). All patients were informed and agreed freely to participate and signed the informed consent by contributing the extracted tooth, which was always extracted for reasons independent of this study. The study was approved by the institutional review board of the University of Valencia. Cells cultured from dental pulps did not exhibit any clinical and/or radiological sign or symptom of inflammation and/or infection. To isolate the cells, the pulps were firstly fragmented by trituration, then chemically digested with 2 mg/mL EDTA in Krebs-Henseleit buffer, and finally digested with a combination of type I collagenase and type II dispase at a final concentration of 4 mg/mL during 30 min in a humid incubator at 37 °C, 5% CO_2, and 3% pO_2. Digested pulp fragments were centrifuged at 1000*g* for 2 min, and the precipitate was resuspended and seeded in culture flasks with complete DMEM (Dulbecco's Eagle Modified Medium with low glucose supplement 1*g*/L, 10% heat-inactivated FBS and 1% antibiotic) under the same conditions of temperature and oxygen pressure.

After the first passage, hDPSCs were divided in two groups: one group was moved to a humid incubator with an oxygen pressure of 21%, while the other group was kept in the same incubator used for the isolation at 3% oxygen tension. Cells were then cultured for 7 months.

All reagents were purchased from Gibco, Invitrogen.

2.2. siRNA transfection for BMI-1 knockdown

Young hDPSCs (5 passages) were seeded in a six well culture plate, at 2×10^5 cells per well in 2 mL antibiotic-free normal growth medium supplemented with 10% FBS and incubated until the cells were 60–80% confluent. For each transfection, 0.8 mL of siRNA Transfection Medium was added to each tube containing the siRNA Transfection Reagent mixture (Solution A + Solution B) following manufacturer's instructions (Santa Cruz Biotechnologies). The mixture was overlayed onto the washed cells prior to a 6 h incubation at 37 °C in a CO_2 incubator. 1 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration was added post-incubation without removing the transfection mixture. 24 h later, the medium was replaced with fresh 1x normal growth medium and cells were assayed using the appropriate protocol 24 h after the addition of fresh medium in the step above.

2.3. Reactive oxygen species and mitochondrial membrane potential determination by flow cytometry

Cells were washed twice with warm PBS and treated with trypsin (Gibco, Invitrogen) and then resuspended in serum-free DMEM containing 1 g/L glucose. To detect intracellular peroxide levels, cells were stained with DHR123 (dihydrorhodamine-123, Thermo Fisher Scientific) at a final concentration of 1 µg/mL. Cells were then incubated for 30 min at 37 °C in the dark. Mitochondrial membrane potential was measured after cell staining with 1 µg/mL TMRM (tetramethylrhodamine methyl ester), Thermo Fisher Scientific for 30 min at 37 °C in the dark. After incubation, values were read by FACS-Verse flow cytometry until 20,000 events were recorded.

2.4. Lipid peroxidation measured using high-performance liquid chromatography

hDPSCs lipid peroxidation was determined as malondialdehyde (MDA) levels, which were detected using high-performance liquid chromatography (HPLC) as an MDA-thiobarbituric acid (TBA) adduct following a method described previously [20]. This method is based on the hydrolysis of lipoperoxides and subsequent formation of an adduct between TBA and MDA (TBA-MDA_2). This adduct was detected using HPLC in reverse phase and quantified at 532 nm. The chromatographic technique was performed under isocratic conditions, the mobile phase being a mixture of monopotassium phosphate 50 mM (pH 6.8) and acetonitrile (70:30).

2.5. Protein carbonylation measured using high-performance liquid chromatography

The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone by reacting with 2,4-dinitrophenylhydrazine so that they could be detected using Western blotting using specific antibodies. Oxidative modification of total proteins was assessed by immunoblot detection of protein carbonyl groups using the OxyBlot Protein Oxidation Detection kit in accordance with the manufacturer's instructions (Merk Millipore). The procedure to quantify total protein carbonyls using the OxyBlot kit was densitometry of the Oxyblot and Ponceau staining followed by finding the ratio between the total density in the Oxyblot and the Ponceau.

2.6. RNA extraction and RT-qPCR analysis

Total RNA was isolated from hDPSCs by using TRIzol reagent (Invitrogen), according to the manufacturer's instruction. RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA preparations was assessed by the 260/280 ratio. cDNA was synthesized from 0.5 µg total RNA using a MultiScribe reverse transcriptase (RT) system kit of Applied Biosystems (High-Capacity cDNA Reverse Transcription Kits). The reaction was incubated as recommended by the manufacturer, for 10 min at 25 °C, followed by 120 min

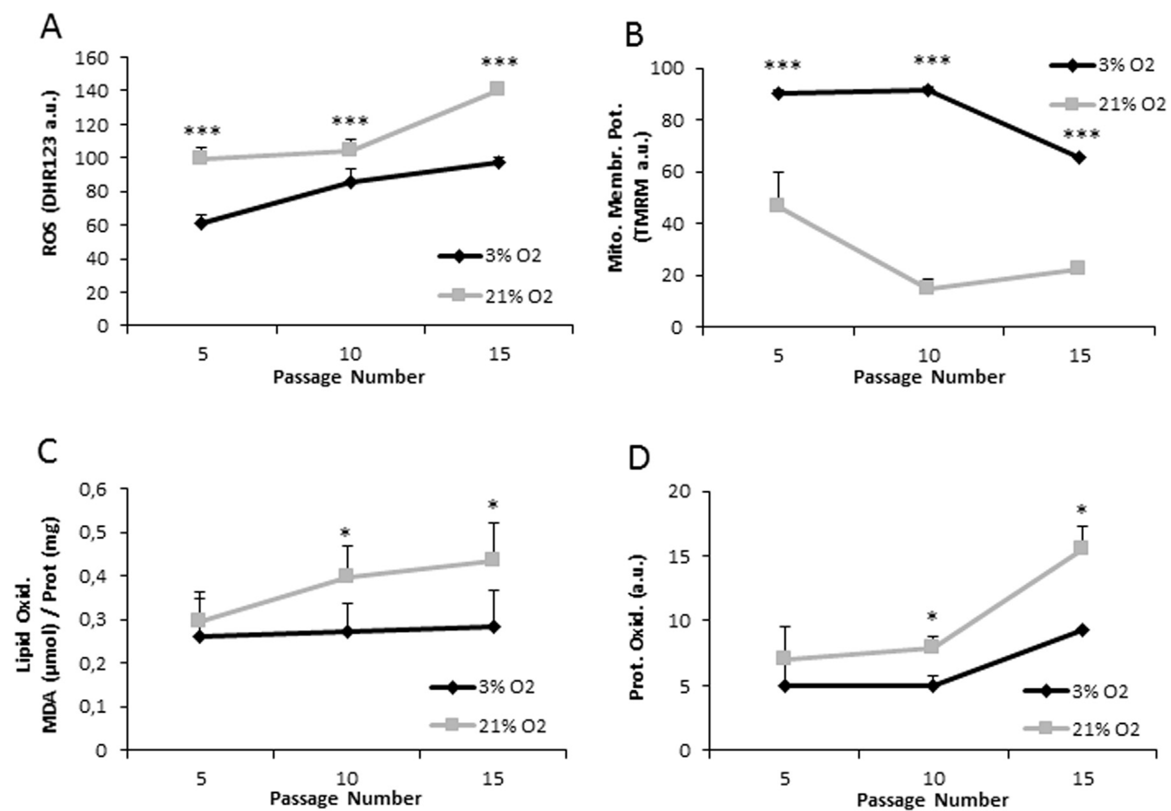

Fig. 1. Oxidative stress related parameters in hDPSCs cultured at 21% or 3% oxygen tension along passages. (A) ROS levels measured by dihydrorhodamine-123 (DHR123), (B) Mitochondrial membrane potential levels measured by tetramethylrhodamine methyl ester (TMRM), (C) Lipid oxidation levels measured by malondialdehyde (MDA) and (D) protein oxidation levels. The data are shown as means ± SD (n=5). The statistical significance is expressed as *p < 0.05; ***p < 0.001 versus 3% pO_2.

at 37 °C, and then for 5 min at 85 °C, and finally cooled to 4 °C to collect the cDNA and then stored at −20 °C prior to the real-time PCR assay.

The quantitative PCR was performed using the detection system 7900HT Fast Real-Time PCR System (Applied Biosystems). Target and control were run in separate wells following procedure: 10 min at 95 °C and then 35 cycles of denaturation at 95 °C for 15 s and annealing and extension at 62 °C for 1 min per cycle. All experiments were repeated at least three times for each sample.

Gene-specific primer pairs and probes for *BMI-1* (3'-CCAGGGCTTTTCAAAAATGA-5' and 5'-GCATCACAGTCA TTGCTGCT-3'), *OCT4* (3'-GATCCTCGGACCTGGCTAAG-5' and 5'-GACTCCTGCTTCACCCTCAG-3'), *SOX2* (3'-AAAACAGCCCG-GACCGCGTC-5' and 5'-CTCGTCGATGAACGGCCGCT-3'), *KLF4* (3'-CCCACATGAAGCGACTTCCC−5' and 5'-CAGGTCCAGGAG-ATCGTTGAA−3'), *C-MYC* (3'-CGCCCTCCTACGTTGCGGTC-5' and 5'-CGTCGTCCGGGTCGCAGATG-3'), $p16^{INK4a}$ (3'-GGGGGCAC-CAGAGGCAGT-5' and 5'-GGTTGTGGCGGGGGCAGTT-3') and $p14^{ARF}$ (3'-CCCTCGTGCTGATGCTACTG-5' and 5'-CATCATGACCTGG-TCTTCTAGGAA-3') were assayed together with Maxima SYBR Green/ ROX qPCR Master Mix (2X) (Fermentas) and normalized against *GAPDH* (3'-TGAACGGGAAGCTCACTGG-5' and 5'-TCCACCA-CCCTGTTGCTGTA-3') housekeeping gene. Relative expression was analysed using the standard curve method.

Gene-specific primer pairs and probes for *LOXL1* (Hs00935937_m1), *LOXL2* (Hs00158757_m1), and *TET1* (Hs04189344_g1), were used together with 1x TaqMan® Universal PCR Master Mix (Applied Biosystems) and normalized against GAPDH (Hs00375015_m1). In this case, the expression was calculated according to the 2−ΔΔCt method.

2.7. Senescence-associated β-galactosidase staining by flow cytometry

SA-β-Gal staining was performed using FluoReporter® LacZ Kit (Molecular Probes) following manufacturer's instructions. 100 uL of resuspended cells (10^7 cells/mL) in staining medium were placed into an appropriate flow cytometer tube and treated with 100 uL of prewarmed fluorescein di-β-$_D$-galactopyranoside (FDG) 2 mM working solution for exactly one minute at 37 °C. FDG loading was stopped by adding 1.8 mL ice-cold staining medium containing 1.5 µM propidium iodide. FDG values were read by flow cytometry until 20,000 events were recorded.

2.8. Protein analysis using western blotting

Total protein was harvested by lysing the cells in a lysis buffer containing a protease inhibitor cocktail (Roche Products). Protein content was determined by a modified Lowry method [21]. 30 µg of protein from each sample was separated on SDS-12.5% polyacrylamide gels and transferred onto a PVDF membrane (BioRad). Membranes were blocked with 0.01 g/mL BSA in TBS-0.05% Tween 20 (TBS-T) and incubated with the following antibodies: anti-*BMI-1* (1:200), anti-Tubulin (1:1000) and anti-Mouse (1:10,000). The protein bands were detected by chemiluminiscence.

2.9. Statistical analysis

Quantitative variables are expressed as means and SD. Qualitative data are expressed as total number and percentage. Statistical analysis consisted of Student's *t*-test for 2 means and ANOVA to compare 2 means with one variation factor. If the n is not the same in all the groups, the comparison of Scheffé was used. All values are means ± SD of measurements in at least three different cultures (three replicates each). Significance was defined as *p < 0.05, **p < 0.01, and ***p < 0.001.

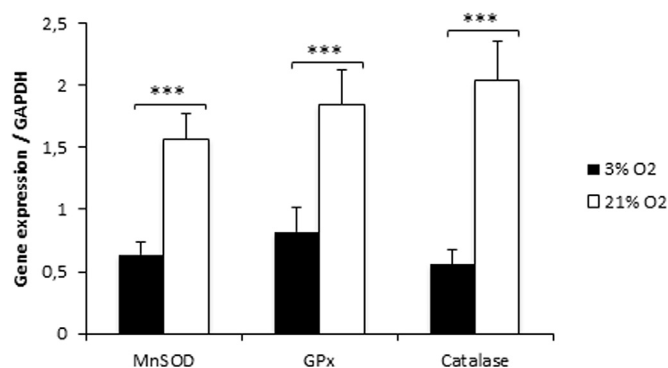

Fig. 2. Antioxidant genes expression in hDPSCs cultured at 21% or 3% oxygen tension. Manganese superoxide dismutase (MnSOD) levels, glutathione peroxidase (GPx) levels and catalase levels. The data are shown as means ± SD (n=5). The statistical significance is expressed as ***$p < 0.001$ versus 3% pO_2.

3. Results

3.1. Ambient oxygen tension induces oxidative stress in hDPSCs long term culture

hDPSCs cultured under ambient oxygen tension showed significantly higher levels of ROS, lipid oxidation and protein carbonylation than those cultured under physiological oxygen tension, as well as a loss of their mitochondrial membrane potential. These differences were found from early passages and were maintained during long term culture, showing that hDPSCs at passage 15 at 3% pO_2 were less damaged than their counterparts at 21% pO_2 (Fig. 1A–D). According to this, we found that hDPSCs cultured at 21% pO_2 showed significantly increased expression of the antioxidant enzymes manganese superoxide dismutase (MnSOD), catalase and glutathione peroxidase (GPx) (Fig. 2). This increase of antioxidant shield might be due to an attempt to hormetically adapt the metabolism to the increased oxidative stress at 21% pO_2.

3.2. Oxidative stress induces premature senescence (SIPS) under ambient oxygen tension during long term culture of hDPSCs

hDPSCs were cultured under ambient (21% pO_2) or physiological oxygen tension (3% pO_2) serially until the cells were exhausted. hDPSCs cultured under ambient oxygen tension only reached 15 passages, while those cultured under physiological oxygen tension, were able to achieve 25 passages (Fig. 3A). Moreover, we could observe that hDPSCs cultured at 21% pO_2 began to show enlarged and flattened shapes around passage 15, while hDPSCs cultured at 3% pO_2 at passage 25 were still morphologically thinner (Fig. S1).

β-Galactosidase staining is one of the most commonly used markers of senescence. hDPSCs cultured at 21% pO_2 had significantly higher

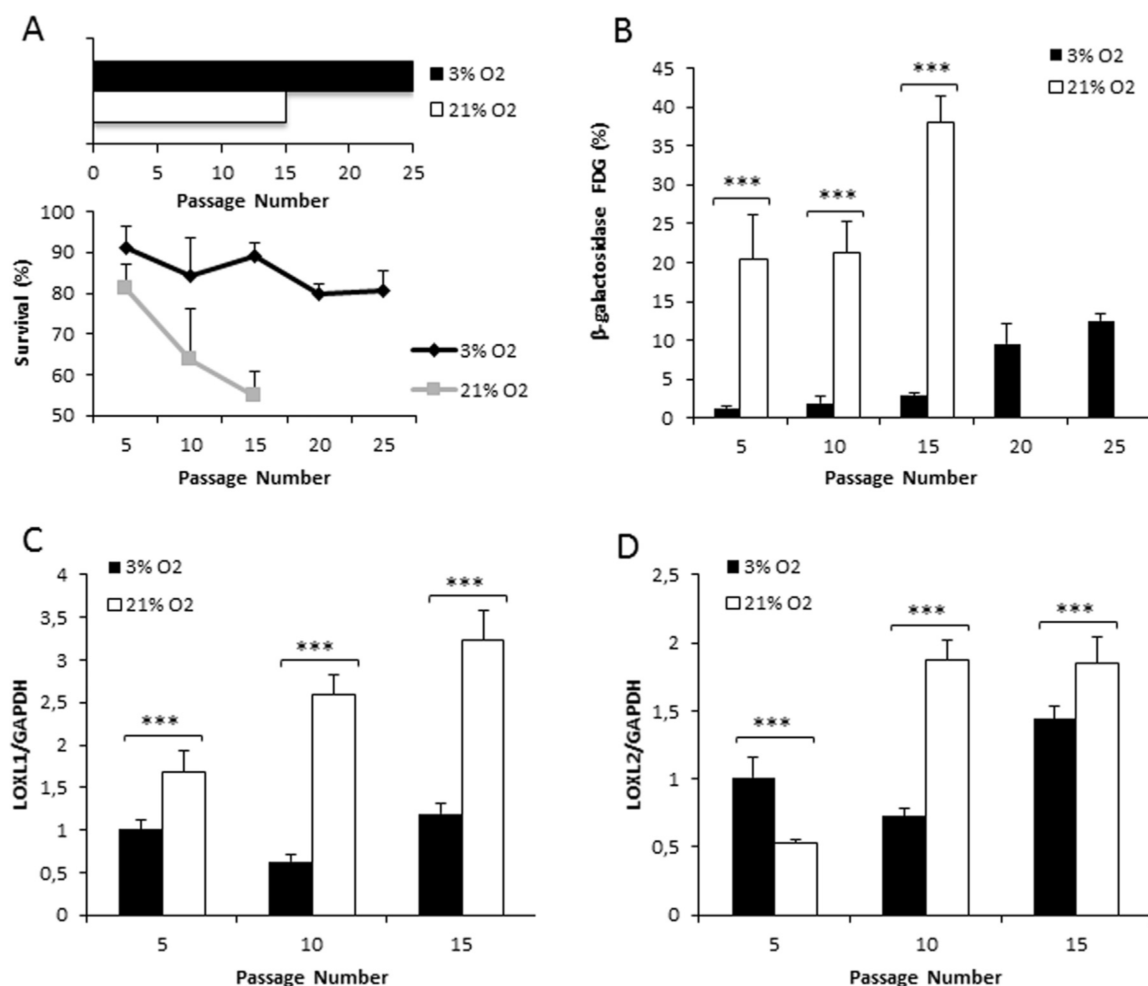

Fig. 3. Oxidative stress induces premature senescence in hDPSCs during long term culture at 21% oxygen tension. (A) Number of passages reached (upper pannel) and survival curve (lower pannel), (B) β-galactosidase activity measured by fluorescein di-β-D-galactopyranoside (FDG) load, (C) *LOXL1* and (D) *LOXL2* relative mRNA expression levels. The data are shown as means ± SD (n=5). The statistical significance is expressed as ***$p < 0.001$ versus 3% pO_2.

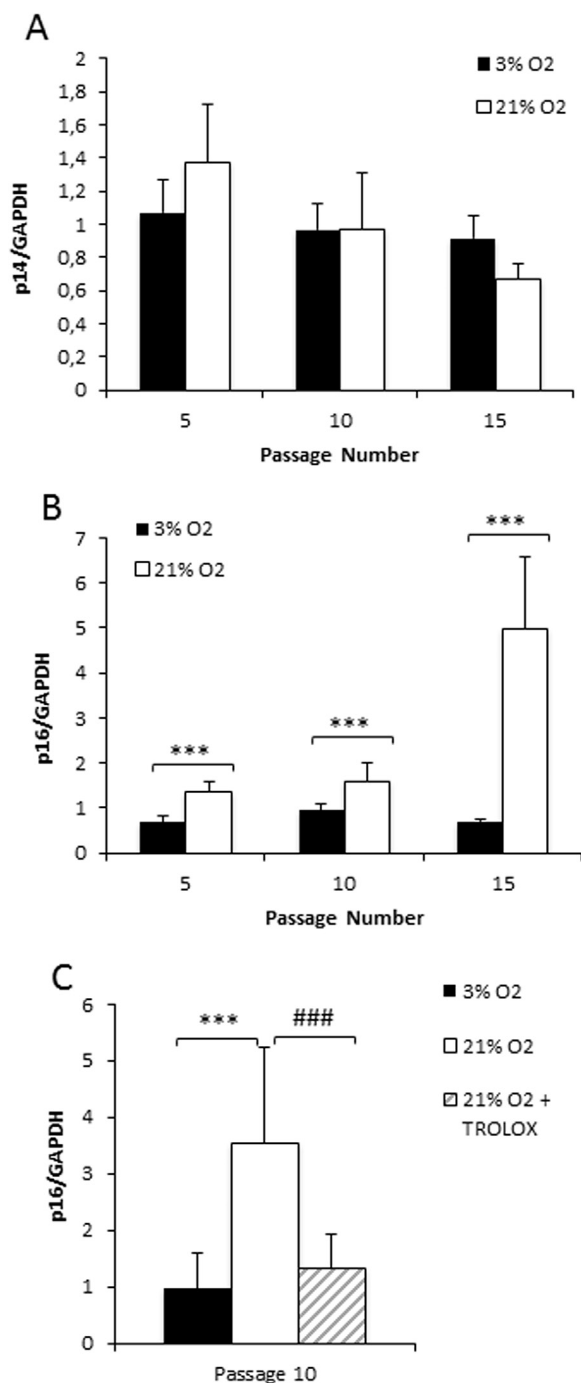

Fig. 4. Oxidative stress-induced premature senescence. Correlation with p16^{INK4a} and p14^{ARF}. (A) p14^{ARF} mRNA levels, (B) p16^{INK4a} mRNA levels and (C) p16^{INK4a} mRNA levels when treatment with 50 µM Trolox. The data are shown as means ± SD (n = 5). The statistical significance is expressed as $^{***}p < 0.001$ versus 3% pO_2 and $^{\#\#\#}p < 0.001$ versus 21% pO_2 + Trolox.

levels of β-galactosidase activity at any passage analysed (5, 10, 15) when compared with 3% pO_2. This difference increased along passages. Moreover, hDPSCs cultured at 3% pO_2 were even less senescent at passage 25 than hDPSCs cultured at 21% pO_2 at passage 15 (Fig. 3B).

LOXL1 and *LOXL2* are lysyl oxidase enzymes involved in cell cycle regulation. We show in Fig. 3C and D, that hDPSCs cultured at 3% pO_2 had lower mRNA levels of both enzymes in comparison to those cultured at 21% pO_2. Furthermore, as passaging number increased, so did *LOXL1* and *LOXL2* mRNA levels when cultured at 21% pO_2 but not at 3% pO_2.

Therefore, hDPSCs cultured at 21% pO_2 undergo premature senescence compared to those cells cultured at 3% pO_2.

3.3. Oxidative stress-induced premature senescence (SIPS) is mediated by p16^{INK4a} pathway

We analysed the mRNA expression pattern of both p14^{ARF} and p16^{INK4a} in hDPSCs long term culture at 3% and 21% pO_2. p14^{ARF} mRNA expression levels were not affected by long-term culture or by oxygen concentration. However, p16^{INK4a} mRNA levels revealed an expression pattern very similar to β-galactosidase activity levels, i.e., there was an increase of its mRNA expression along the passages and it was always higher at 21% pO_2 (Fig. 4A and B).

In order to demonstrate that oxidative stress was mediating the p16^{INK4a} induced premature senescence at 21% pO_2, we cultured hDPSCs at this oxygen tension with 50 µM Trolox, a hydrosoluble antioxidant analogue of Vitamin E. We found that Trolox reversed the effect of ambient oxygen tension on p16^{INK4a} mRNA expression (Fig. 4C). Therefore, oxidative stress increases p16^{INK4a} expression, which in turn accelerates senescence in hDPSCs cultured under ambient oxygen tension.

3.4. Loss of stemness under ambient oxygen tension during long term culture of hDPSCs

Oxidative stress can affect stemness, therefore we measured the mRNA expression levels of OSKM transcription factors. *SOX2* and *OCT4* are implicated in pluripotency induction, while *KLF4* and *C-MYC* are involved in pluripotency maintenance. Our results show that, comparing hDPSCs cultured at 3% pO_2 vs 21% pO_2, *SOX2* and *OCT4* mRNA expression was significantly higher at passage 5, and *KLF4* and *C-MYC* mRNA expression was significantly higher at passage 15 (Fig. 5A). *TET1* is one member of a family of enzymes that alter the methylation status of DNA. They are involved in stem cell self-renewal, proliferation and differentiation. We observed that *TET1* mRNA levels were downregulated in hDPSCs cultured at 21% pO_2, in comparison to 3% pO_2 (Fig. 5B).

Therefore, we show that culturing hDPSCs at 3% pO_2 increases OSKM transcription factors compared to 21% pO_2.

3.5. BMI-1 can rescue SOX2 and OCT4 expression under ambient oxygen tension

BMI-1 protein levels were significantly higher in hDPSCs cultured at 21% pO_2 at passage 5 when compared to 3% pO_2. However, they decreased very rapidly with passages and were significantly lower at passage 15, in comparison to those cultured at 3% pO_2 (Fig. 6A and B).

We used siRNA transfection in order to obtain a mild *BMI-1* knockdown so that hDPSCs at passage 5 cultured at 3% or 21% pO_2 had the same *BMI-1* protein expression level (Fig. S2). *BMI-1* knockdown did not have any effect on p16^{INK4a} expression (data not shown), however it restored *SOX2* and *OCT4* mRNA levels in hDPSCs cultured at 21% pO_2 (Fig. 7). This reflects a relationship between *BMI-1* and pluripotency transcription factors.

4. Discussion

hDPSCs normally reside in low oxygen concentrations. In mammals including humans, hDPSCs are located in perivascular niches close to the vascular structure in almost all tissues [22–24]. By the time oxygen reaches the organs and tissues, oxygen concentration drops to 2–9%, with a mean of 3% in the dental pulp tissue [25,26]. Despite this fact, it is still common to culture cells at high non- physiological 21% pO_2. Here we demonstrate that long term culture of hDPSCs in a physiological oxygen tension (3% pO_2) has beneficial effects on both cellular senescence and stemness potential maintenance in comparison to

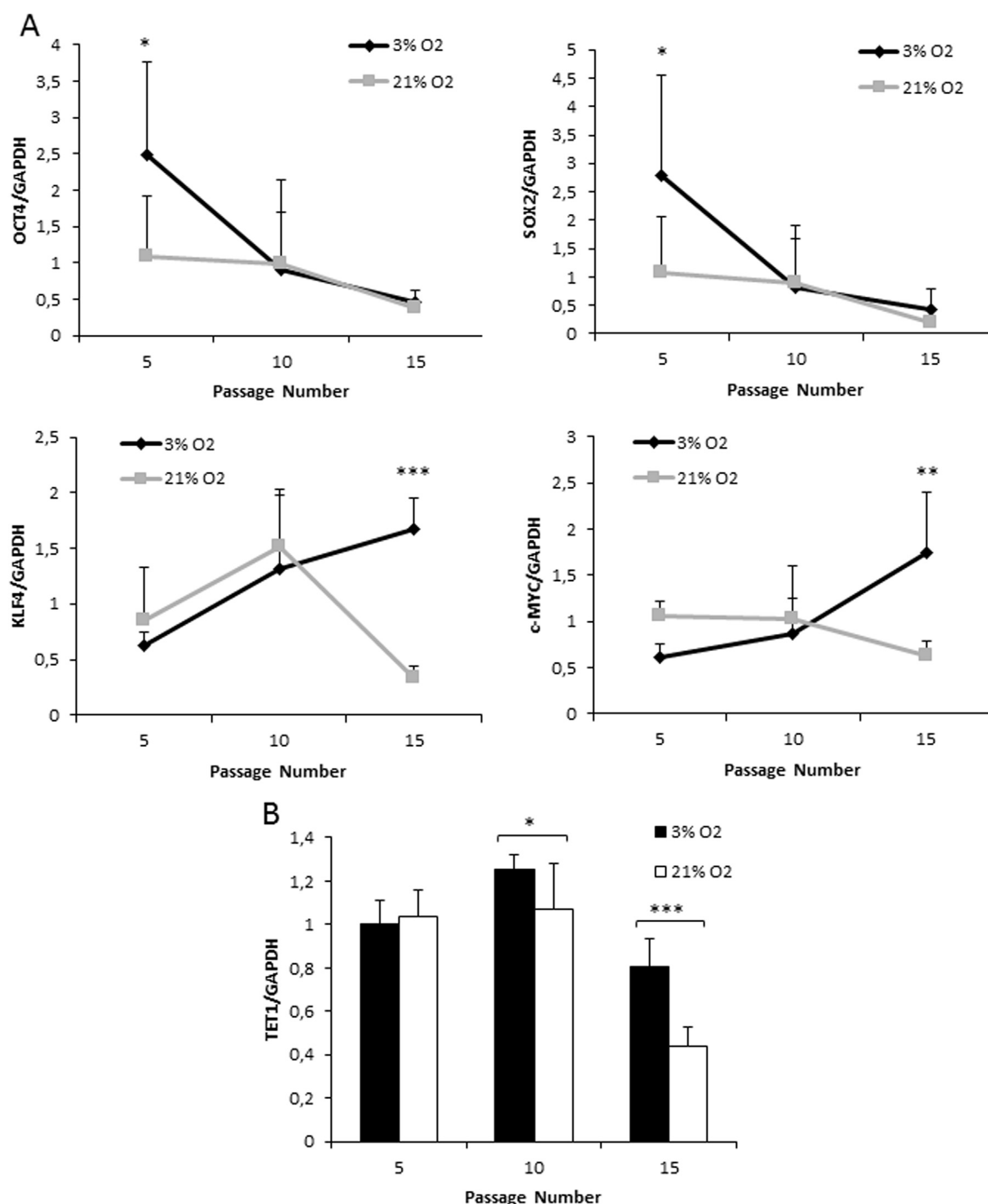

Fig. 5. Pluripotency markers in hDPSCs cultured at 21% vs 3% oxygen tension along passages. (A) *OCT4*, *SOX2*, *KLF4* and *C-MYC* mRNA levels and (B) *TET1* mRNA levels relative expressions. The data are shown as means ± SD (n=5). The statistical significance is expressed as *p < 0.05; **p < 0.01; ***p < 0.001 versus 3% pO_2.

culturing cells under ambient oxygen tension.

In the present study, we show that reduction of the pO_2 level led to decreased intracellular oxidative stress and cellular components damage during hDPSCs long term culture. This was accompanied by reduced ROS levels, less protein and lipid damage, as well as a better conservation of the mitochondrial membrane potential. In accordance to this, it has been demonstrated that high concentrations of oxygen can cause oxidative stress via production of reactive oxygen species (ROS) and free radicals that damage lipids, proteins and DNA [27]. In this work, we also linked oxygen tension to altered mRNA expression of MnSOD, CAT and GPx. The increment of antioxidant enzyme activities indicated that cellular anti-oxidative system was triggered to resist oxidative damage. Fan and colleagues already demonstrated that higher oxygen concentrations resulted in more H_2O_2 generation in human cells, which implied that the increase of H_2O_2 levels could enhance transcription of MnSOD, CAT and GPx [28].

As passage number increased, cells cultured under ambient oxygen tension began to show flattened or lengthened shapes, and debris in the culture medium increased. These morphological changes, have already been described as a characteristic of senescence [29,30]. In addition to this, hDPSCs cultured at 21% oxygen tension showed higher levels of senescence related β-galactosidase activity as well as $p16^{INK4a}$ expression. This behaviour in long term *in vitro* culture leads to senescence and is collectively referred to as oxidative stress induced premature

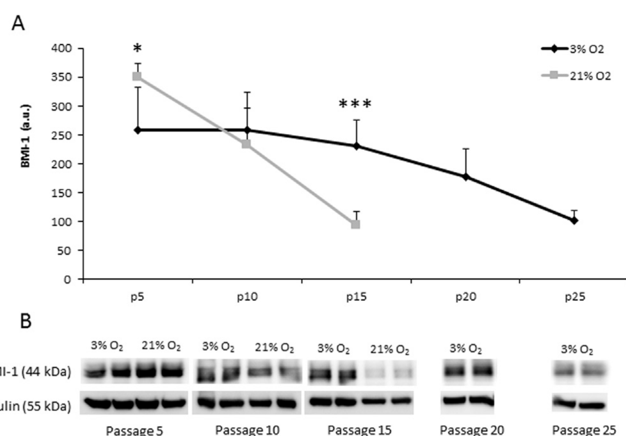

Fig. 6. *BMI-1* expression level in hDPSCs cultured at 21% vs 3% oxygen tension along passages. (A) *BMI-1* protein levels, and (B) representative western-blot images of *BMI-1* protein levels. The data are shown as means ± SD (n=5). The statistical significance is expressed as *p < 0.05; ***p < 0.001 versus 3% pO_2.

senescence (SIPS) [31]. Lysyl oxidase activity has been shown to increase under oxidative stress conditions [32]. hDPSCs cultured at 21% pO_2 have higher mRNA expression levels of both *LOXL1* and *LOXL2*. Moreover, it has been described that an increased level of these enzymes may contribute to escape from cellular senescence [9]. This could be a defensive mechanism of hDPSCs cultured at 21% pO_2 to escape from oxidative stress-induced senescence. In addition, lysyl oxidases participate in the carbonylation of several proteins, and further increase the H_2O_2 levels as subproduct of this reaction [33]. These results reinforce the observations of Fan and colleagues [28] and also may contribute to the induction of the antioxidant shield in hDPSCs incubated at 21% pO_2, as we described in this investigation.

The $p16^{INK4a}$/pRb and $p14^{ARF}$/p21/p53 cell cycle inhibitory pathways represent two important pathways controlling proliferation, and their inactivation can extend the limited division number of mitotic cells in culture [34]. Given the role of $p16^{INK4a}$ in cell cycle regulation and the recent implication of oxidative stress in stem cell senescence, we observed a potential link between ROS and $p16^{INK4a}$ regulation. Our results show that hDPSCs cultured at 21% pO_2 have higher levels of ROS, as well as increasing $p16^{INK4a}$ expression, suggesting that they are approaching senescence. In fact, stress signals such as ROS stimulate the activation of $p16^{INK4a}$ transcription and play important roles in initiation, as well as maintenance, of cellular senescence [35–37]. Ito and colleagues described, both *in vitro* and *in vivo*, that activation of $p16^{INK4a}$/pRb gene product pathway in response to elevated ROS led to the failure of hematopoietic stem cells (HSCs) function, and that treatment with antioxidant agents restored the constitutive capacity of HSCs, resulting in the prevention of bone marrow failure [38,39]. These results support our data that treatment with 50 μM Trolox can rescue $p16^{INK4a}$ levels in hDPSCs long term culture at 21% pO_2.

Interestingly, $p16^{INK4a}$ and $p14^{ARF}$ are both encoded by a single locus; however, ROS specifically affects $p16^{INK4a}$ but not $p14^{ARF}$. It may be that the $p16^{INK4a}$ pathway is of particular importance in the senescence of stem cells [40,41] as it is considered to be a robust biomarker for cellular senescence, and at the forefront of cell cycle inhibition as it binds specifically to the CDKs, displacing cyclin-D and thereby arresting cells in G1 phase [42].

Taken together, the fact that hDPSCs cultured at 21% pO_2 show increased $p16^{INK4a}$ expression, higher β-galactosidase activity, overexpressed *LOXL1* and *LOXL2*, and senescent like morphology, earlier than those cultured at 3% pO_2, means that 21% oxygen induced-oxidative stress causes premature senescence.

It is well known that one of the *BMI-1* downstream targets are $p16^{INK4a}$ and $p14^{ARF}$ [43,44]. $p16^{INK4a}$ contributes to the regulation of cell cycle progression by inhibiting the S phase [45]. *BMI-1* is a repressor, so, high levels of *BMI-1* should then be followed by a decrease in $p16^{INK4a}$ expression, and subsequently by a hyper proliferation rate like in cancer cells [44]. However, we found that *BMI-1* overexpression in hDPSC cultured at 21% pO_2 at early passages was not followed by a $p16^{INK4a}$ downregulation. ROS and *BMI-1* play an opposite role on $p16^{INK4a}$ regulation. In fact, oxidative stress induced by 21% oxygen tension would be strong enough to counteract *BMI-1* downstream effects on the *INK/ARF* locus, as it has been shown that it can up-regulate $p16^{INK4a}$ expression [46]. As passages succeed, *BMI-1* levels plummet because hDPSCs cultured under ambient oxygen tension suffer an accelerated ageing accompanied by a loss of their capacity to face oxidative stress effects.

Although $p16^{INK4a}$ and $p14^{ARF}$ have been shown to be *BMI-1* targets in the context of stem cell self-renewal, they do not account for all *BMI-1* actions, and other downstream effectors are being sought [47,48]. Recently, a relationship between *BMI-1* and *SOX2* has been described [49]. *SOX2*, as well as *OCT4*, are transcription factors that are key players in the induction of pluripotency and stemness [18]. In the present study, we confirmed a loss in all OSKM transcription factors expression, as well as a deregulation in *TET1* expression, during hDPSCs *in vitro* long term culture at 21% pO_2. TET proteins are dioxygenases that regulate 5 hydroxyl-methylcytosine (5-hmC) levels in genes implicated in self-renewal, proliferation and differentiation [50,51]. Our results show that *TET1* expression levels are influenced by culture oxygen pressure and passaging number. In fact, when hDPSCs undergo long passages, *TET1* levels are downregulated, so decreasing the

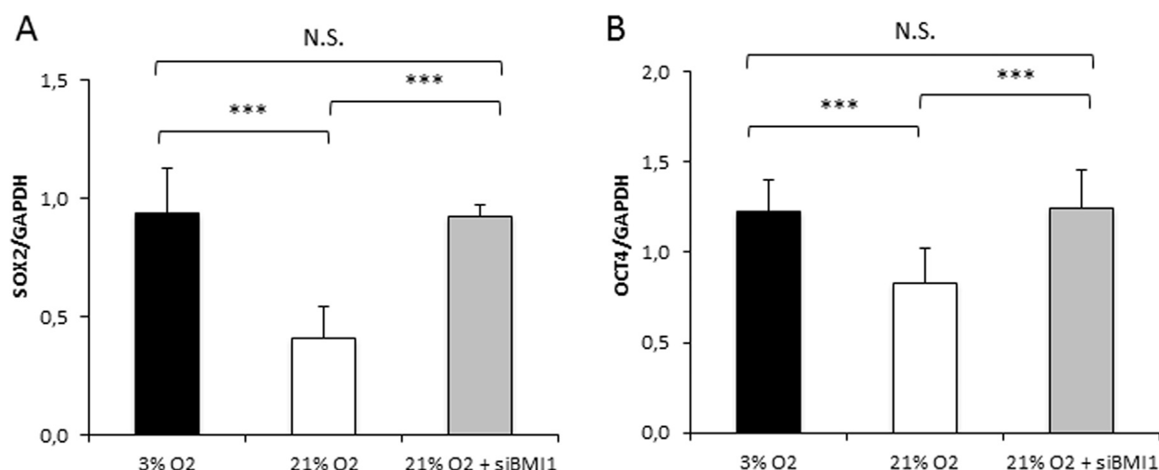

Fig. 7. *BMI-1* knockdown effect on *SOX2* and *OCT4* expression in hDPSCs cultured at 21% vs 3% oxygen tension. (A) *SOX2* mRNA expression levels and (B) *OCT4* mRNA expression levels. The data are shown as means ± SD (n=3). *p < 0.05; **p < 0,01; ***p < 0.001.

maintenance of stemness in hDPSCs [51,52]. Koh and colleagues also proposed that *OCT4* and *SOX2* regulate the expression of *TET1* [51]. Interestingly, our results show that the effect of cell passage is greater than oxygen pressure effect, suggesting that *OCT4*, *SOX2* and *NANOG* are more relevant in stem cell maintenance than *TET1*. In accordance with this, it has been shown that cells maintained at 21% pO_2 expressed significantly less *OCT4*, *SOX2* and *NANOG* than those cultured at 5% pO_2 [53]. Furthermore, it has also been described that physiological oxygen tension inhibits senescence and maintains stem cell properties [54,55].

Our results show that *BMI-1* downregulation can rescue *SOX2* and *OCT4* levels without affecting $p16^{INK4a}$ expression levels in hDPSCs cultured at 21% pO_2. Again, the opposite effect of *BMI-1* and oxidative stress plays a role in maintaining $p16^{INK4a}$ expression levels.

As it has also recently been described by Izpisúa and colleagues [56], the in vitro induction of OSKM can ameliorate some cellular markers of ageing, such as cellular senescence. hDPSCs cultured at 3% pO_2 are able to maintain the expression of this factors during a larger number of passages, so the preservation of the OSKM factors could play an important role in the delayed onset of cellular phenotypes associated with ageing observed in this cells.

In conclusion, the present study suggests that $p16^{INK4a}$ and *BMI-1* are involved in the cellular premature senescence of hDPSCs triggered by oxidative stress. It is important considering this fact when culturing primary culture cells to improve the extrinsic culture environment, in order to retain their stemness properties and to delay the process of senescence prior to clinical application.

Author contributions

CMB, JVA, MI, JSR and JSIC performed experimental work; JG and JLGG, JV and CB directed experimental work, CMB, JV and CB wrote the paper and CB designed research and directed the project.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2016.12.009.

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