Stem Cell Reports
Report
OPEN ACCESS

Activation of p38, p21, and NRF-2 Mediates Decreased Proliferation of Human Dental Pulp Stem Cells Cultured under 21% O_2

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http://dx.doi.org/10.1016/j.stemcr.2014.08.002

SUMMARY

High rates of stem cell proliferation are important in regenerative medicine and in stem cell banking for clinical use. Ambient oxygen tensions (21% O_2) are normally used for in vitro culture, but physiological levels in vivo range between 3% and 6% O_2. We compared proliferation of human dental pulp stem cells (hDPSCs) cultured under 21% versus 3% O_2. The rate of hDPSC proliferation is significantly lower at 21% O_2 compared to physiological oxygen levels due to enhanced oxidative stress. Under 21% O_2, increased p38 phosphorylation led to activation of p21. Increased generation of reactive oxygen species and p21 led to activation of the NRF-2 signaling pathway. The upregulation of NRF-2 antioxidant defense genes under 21% O_2 may interact with cell-cycle-related proteins involved in regulating cell proliferation. Activation of p38/p21/NRF-2 in hDPSCs cultured under ambient oxygen tension inhibits stem cell proliferation and upregulates NRF-2 antioxidant defenses.

INTRODUCTION

Human mesenchymal stem cells (hMSCs) have a therapeutic potential in tissue engineering and regenerative medicine (Caplan, 2007), mostly due to immunologic properties (Jones and McTaggart, 2008) and their ability to differentiate into cardiovascular or neuronal cells (Tae et al., 2006) among others.

Human dental pulp stem cells are mesenchymal cells derived from the neural crest, which have been already proved to regenerate tissue in oral inflammatory diseases (Aimetti et al., 2014; Nakashima et al., 2009). These cells can be obtained from permanent and deciduous pulp tissue, which is easily available from teeth after extraction without ethical issues. As previously mentioned, they have a potential role for clinical use either immediately after isolation, or for use in stem cell banking. Therefore, it is highly important to obtain and culture them under the best possible conditions.

The in vitro culture of MSCs has been routinely carried out under the ambient oxygen tension (18%–21% O_2) (Mohyeldin et al., 2010). However, in vivo these cells are not exposed to such a hyperoxic environment (Harrison et al., 2002; Kofoed et al., 1985; Matsumoto et al., 2005; Pasarica et al., 2009). Depending on the cell type, the local oxygen tension in MSCs niches varies between 1% and 7% O_2 in bone marrow (Harrison et al., 2002) and between 10% and 15% O_2 in the adipose tissue (Bizzarri et al., 2006). Although values of 3% to 6% O_2 (20–40 mmHg) in adult organs and tissues have been reported (Hall and Giaccia, 2005; Kozam, 1967), the actual oxygen concentration in situ depends predominantly on the vascularization of the tissue and its metabolic activity (Ward, 2008). The dental pulp has a relatively high blood flow. It is estimated to be 40–50 ml/min/100 g of pulp tissue in a mature tooth (Meyer, 1993). This flow is relatively high, compared to that of other oral tissues and skeletal muscle (Kim, 1985).

Previous studies have shown the negative impact of the ambient oxygen tension (21% O_2) on the physiology of stem cells, e.g., neuronal (Rodrigues et al., 2010), bone marrow (Dos Santos et al., 2010; Hung et al., 2012), umbilical cord (Lavrentieva et al., 2010), or adipose tissue (Efimenko et al., 2011; Kim et al., 2012). Although reduced rates of cell proliferation have been observed during culture under 21% O_2, an oxygen tension that causes oxidative stress, the underlying molecular mechanisms have not been investigated in a systematic manner.

The aim of this study was to determine the growth rate of human dental pulp stem cells (hDPSCs) under physiological oxygen tension (3%), and to investigate the cell signaling pathways underlying decreased stem cell proliferation during routine culture under ambient oxygen tension (21%). We show that oxidative stress is responsible for the low proliferation rate under ambient oxygen tension and describe the signaling pathway linking oxidative stress with proliferation of hDPSCs. We demonstrate that oxidative stress leads to the sequential activation of p38 MAPK, p21, and the Nuclear factor erythroid 2-related factor 2 (NRF-2) antioxidant defense pathway (Ishii et al., 2004). A practical consequence is that incubation with

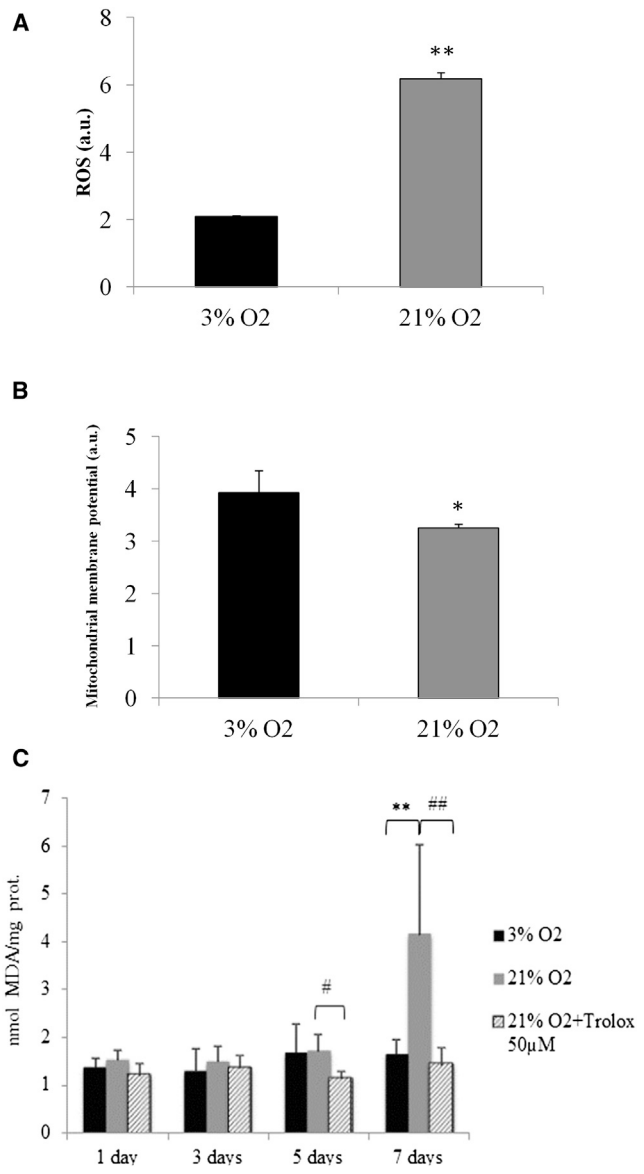

Figure 1. Oxidative Stress-Related Parameters in hDPSCs

(A) ROS levels. Results are represented as the mean ± SD for three independent experiments (three replicates each). The statistical significance is expressed as $^{**}p < 0.01$ versus 3% O_2.

(B) Membrane potential levels. Results are represented as the mean ± SD. The statistical significance is expressed as $^{*}p < 0.05$ versus 3% O_2.

(C) MDA levels. Results are represented as the mean ± SD. The statistical significance is expressed as $^{**}p < 0.01$ 3% O_2 versus 21% O_2 and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ 21% versus 21% O_2 with Trolox. See also Figure S1.

Trolox, a water-soluble analog of vitamin E (Raspor et al., 2005), or with an inhibitor of p38 (SB203580) restores high proliferation rate of hDPSCs even if cultured under 21% O_2.

RESULTS

Characterization of hDPSCs

The phenotype of the hDPSCs was confirmed by five positive and one negative marker of these hDPSCs after four to five cell passages. The positive markers for mesenchymal stem cells were STRO1 (Gronthos et al., 1994), OCT4 (Nichols et al., 1998), CD133 (Meregalli et al., 2010), Nestin (Honda et al., 2007), and CD34 (Wood et al., 1997). The negative marker was CD45, which is specific for hematopoietic stem cells (Barclay et al., 1988; Pittenger et al., 1999; Zhang et al., 2003). Confocal microscopy showed that the hDPSCs were positive for STRO1, OCT1, CD133, CD34, and Nestin and negative for CD45, thus confirming the mesenchymal stem cell phenotype (Figure S1 available online).

Oxidative Stress Is Higher at 21% Than at 3% O_2

We hypothesized that cells cultured at 3% O_2 would have a much lower level of oxidative stress than those cultured at 21% O_2. Figure 1A shows that this is the case. The level of ROS in dental pulp stem cells cultured under 21% oxygen at confluence was almost 3-fold higher than at 3% oxygen. We also determined mitochondrial membrane potential as a marker of the mitochondrial status and found that mitochondria were "healthier" in cells cultured under 3% compared to 21% O_2, because they exhibited a higher mitochondrial membrane potential (see Figure 1B).

Malondialdehyde (MDA) is one of the products generated during the process of lipid peroxidation especially in biological membranes. hDPSCs cultured at 3% O_2, 21% O_2, or 21% O_2 with 50 µM Trolox (water-soluble structural analog of vitamin E) after 1, 3, 5, and 7 days of culture were used to determine lipid peroxidation. Figure 1C shows that incubating stem cells at 21% O_2 results in a marked increase in lipid peroxidation (measured as MDA) that is prevented by Trolox.

Adhesion of hDPSCs to the Culture Plate Is Lower at 21%O_2 Than at 3% O_2

Cellular adhesion, an indication of the capacity of the cells to recover after a passage, is regulated by expression of adhesion proteins (Hung et al., 2012). Adhesion of hDPSCs to culture plate was significantly higher in cultures under 3% O_2 compared 21% O_2 (Figure 2A).

Proliferation of hDPSCs at 3% O_2 Is Higher Than at 21% O_2

Adhesion of hDPSCs to culture plates is ~30% lower at 21% compared to 3% O_2 (see Figure 2A). Thus, to have the same number of adhering cells from the starting point of the growth curve (6 hr), we seeded 30% more cells at 21% O_2

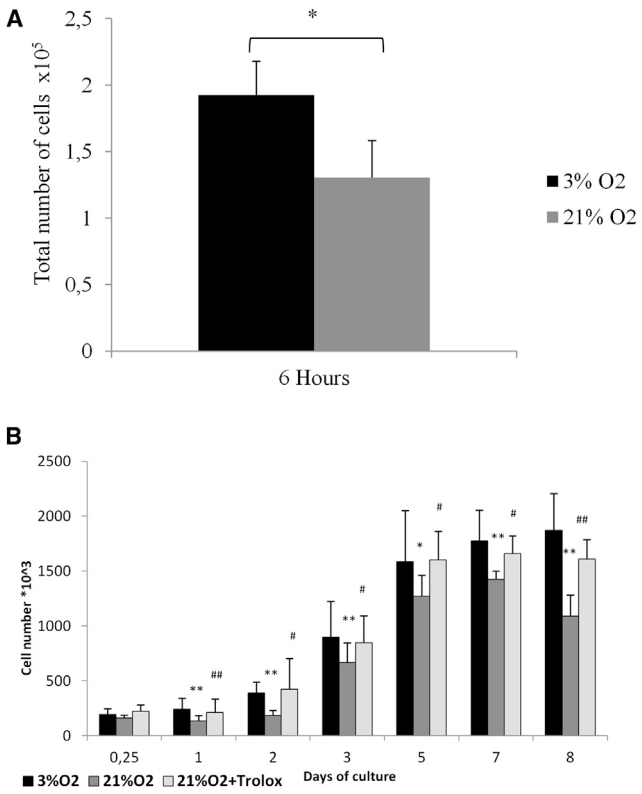

Figure 2. Higher Rate of Cell Proliferation in hDPSCs Cultured under 3% O_2

(A) Adhesion of hDPSCs cultured at 3% O_2 is higher than at 21% O_2. Results are mean ± SD for three independent experiments (three replicates in each experiment). Statistical significance is expressed as *$p < 0.05$.

(B) Growth curve of hDPSCs. Results are means ± SD for six independent experiments (three replicates each). The statistical significance is expressed as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ 3% O_2 versus 21% O_2, $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ 21% O_2 versus 21% O_2 with Trolox, $^{\&}p < 0.05$, and $^{\&\&}p < 0.013$% O_2 versus 21% O_2 with Trolox.

than at 3% O_2. Where indicated, incubation with Trolox (50 μM) began 6 hr after seeding and during the rest of the experiment. Proliferation, evaluated by direct counting of cells at 1, 2, 3, 5, 7, and 8 days after seeding revealed a significantly higher cell number at 3% compared to 21% O_2 after all time points. Trolox reversed the effect of increased oxygen tension on cell proliferation after 3, 5, 7, and 8 days of culture (Figure 2B). Cell viability was >90% in all conditions.

Cell-Cycle Regulators of hDPSCs at 3% and 21% O_2

Activation of p38MAPK is an indicator of oxidative stress in proliferating cells (Ito et al., 2006). Thus, we examined p38 MAPK expression in hDPSCs cultured under 3% O_2, 21% O_2, or 21% O_2 + Trolox (50 μM) at 3, 5, and 7 days after seeding under the conditions described previously (30% more cells seeded at 21% O_2). p38MAPK phosphorylation was significantly higher in hDPSCs cultured at 21% O_2 compared to 3% O_2, and this difference was prevented by treatment with 50 μM Trolox (Figure 3A); therefore, this activation is dependent on oxidative stress.

p21 is a downstream target of p38, and notably incubation of hDPSCs under 21% O_2 resulted in an expression of p21 significantly higher than in cells cultured at 3%. Trolox also prevented this effect (Figure 3B).

NRF-2-Mediated Antioxidant Defenses against Oxidative Stress in hDPSCs

In order to study NRF-2-associated antioxidant defenses in hDPSCs under 3% O_2 or 21% O_2, we examined protein expression of its downstream antioxidant enzymes: heme oxygenase 1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1). We treated confluent hDPSCs with 100 μM diethylmaleate (DEM), a GSH depleting agent that renders cells more prone to oxidative stress (García-Ruiz et al., 1995). We found a significantly higher basal and DEM stimulated expression of HO-1 in hDPSCs cultured under 21% O_2 compared to 3% O_2 (Figure 3C). Similar differences were observed for NQO1, another antioxidant enzyme regulated by NRF-2 (Figure 3D). Thus, incubation under 21% O_2 elicits an NRF-2 mediated antioxidant response due to the reported activation of NRF-2 under conditions of 21% O_2 (Cho et al., 2002).

Decreased Cell Proliferation under 21% O_2 Is Determined by p38 and NRF-2 Activation

In order to demonstrate that p38 and NRF-2 activation are responsible for the lower cell proliferation under 21% O_2, we performed two cell proliferation curves. In one, we compared the cell proliferation under 3% O_2 versus 3% O_2 plus 10 μM resveratrol, an activator of NRF-2 (see Figure S2). As shown in Figure 4A, activation of NRF-2 resulted in a decrease of cell proliferation. We also incubated cells under 21% O_2 versus 21% O_2 plus 10 μM SB203580 (an inhibitor of p38 phosphorylation, see Figure S3), and, as shown in Figure 4B, we found an increase of cell proliferation by inhibiting p38 activation, thus showing the role of p38 activation on the decreased rate of cell proliferation.

DISCUSSION

Oxidative Stress as a Signaling Mechanism to Explain Low Rates of Proliferation Rate in hDPSCs

Hyperoxia has been described as an important factor to destabilize cellular redox homeostasis (Fan et al., 2008). High oxygen concentrations can cause oxidative damage via production of reactive oxygen species (ROS), leading to damage of lipids, proteins, and DNA (Wiseman and Halliwell, 1996). Nevertheless, ambient oxygen tension

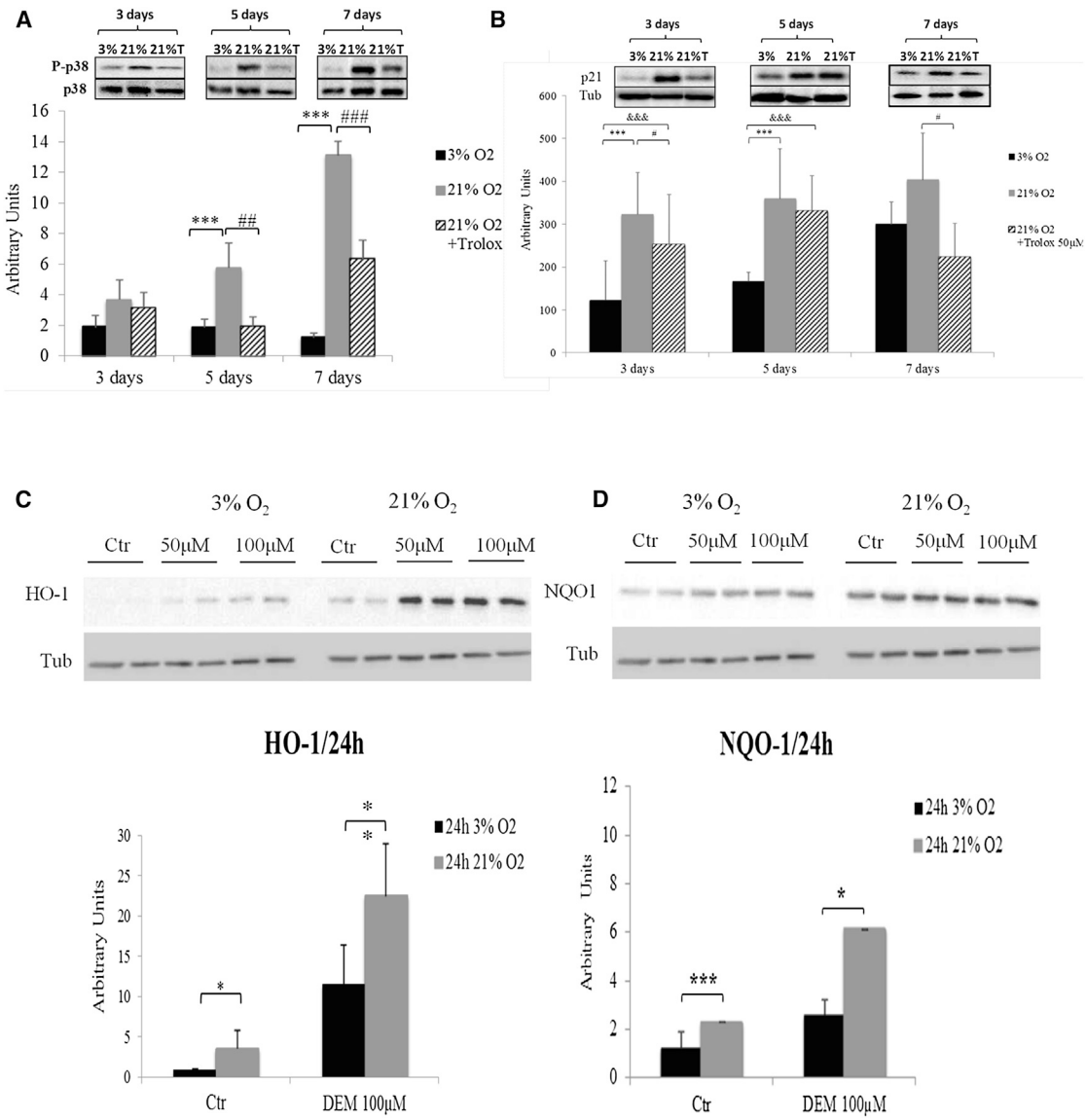

Figure 3. Molecular Pathways Involved in the higher Proliferatio of hDPSCs Cultured under 3% O_2

(A) Phosphorylated p38 MAPK levels in hDPSCs. Values are mean ± SD for three independent experiments (three replicates each). The statistical significance is expressed as ***$p < 0.001$ 3% O_2 versus 21% O_2, $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ 21% O_2 versus 21% O_2 with Trolox.

(B) Protein expression of p21 in hDPSCs. Results are means ± SD for three independent experiments (three replicates each). The statistical significance is expressed as ***$p < 0.001$ 3% O_2 versus 21% O_2, $^{\#}p < 0.05$ 21% O_2 versus 21% O_2 plus Trolox, $^{\&\&\&}p < 0.001$ 3% O_2 versus 21% O_2 plus Trolox.

(C and D) Protein expression of HO-1 (C) and NQO1 (D) in hDPSCs. Data represent the average ±SD. Every point is the mean of three independent experiments (three replicates each). The statistical significance versus controls is expressed as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. HO-1, heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase 1.

(18%–21% O_2 equivalent to 160 mmHg) is routinely used to culture hDPSCs in vitro despite the fact that physiological oxygen tension in the organism is markedly lower (i.e., 3%–6% O_2 equivalent to 20–40 mmHg) (Hall and Giaccia, 2005). Recently, the negative impact of the ambient oxygen tension on physiological function of different stem cells has been reported. This includes proliferation (Rodrigues et al., 2010; Kim et al., 2012), reduced stem cell migration (Fuchs and Weber, 1994), and reduced osteogenic differentiation (Hung et al., 2012). However, to our knowledge the underlying molecular mechanisms have not been elucidated.

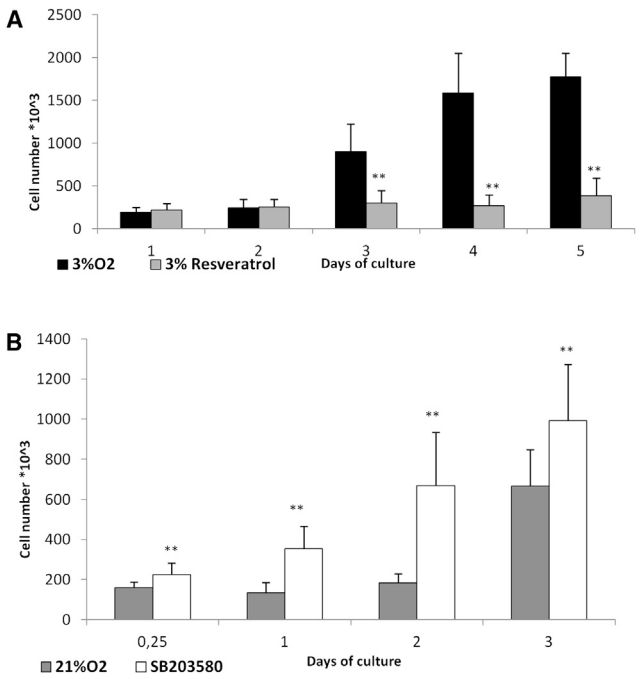

Figure 4. Growth Curve of hDPSCs

(A) At 3% O_2 or 3% O_2 + resveratrol (10 μM) Results are means ± SD for three to six experiments. The statistical significance is expressed as **p < 0.01 versus 3% O_2.

(B) At 21% O_2 or incubated with an inhibitor of Pp38 (10 μM SB203580). Results are means ± SD for three to six experiments. The statistical significance is expressed as **p < 0.01 versus 3% O_2. See also Figures S2 and S3.

In the present study, we confirmed a lower proliferation of the stem cells at ambient oxygen tension. Moreover, we found that the adhesion of these cells at 21% O_2 is 30% lower than at 3% O_2. We show that oxidative stress is responsible for these differences, because Trolox is able to reverse the lower proliferation under 21% O_2. To clarify the mechanisms by which oxygen tension affects hDPSCs proliferation, we studied p38 MAPK and p21, two cell-cycle regulators, which are activated by stress stimuli.

Oxidative-stress-induced $p21^{Waf1/Cip1}$ expression is generally mediated via a p53-dependent mechanism (el-Deiry et al., 1995; Giono and Manfredi, 2006). Nevertheless, there is also a p53-independent pathway (O'Reilly, 2005) that involves p38 MAPK. Indeed, p38 phosphorylates $p21^{Waf1/Cip1}$ in response to oxidative stress by inducing phosphorylation at Ser130 in vitro and in vivo (Kim et al., 2002).

We found that levels of P-p53 were not correlated with levels of $p21^{Waf1/Cip1}$ (data not shown). On the other hand, increased P-p38 protein expression under 21% O_2 compared to 3% O_2 was correlated with higher p21 protein expression. We conclude that regulation of the p21 protein expression in hDPSCs under 3% O_2 or 21% O_2 is p53 independent but probably dependent on p38 MAPK activation. p21 is involved in many different cellular processes, including cell-cycle arrest, cell differentiation, senescence, and apoptosis (Dotto, 2000; Gartel and Tyner, 2002; O'Reilly, 2005). Furthermore, a recent study showed a direct interaction between p21 and NRF-2 (Chen and Carmichael, 2009). It was shown that p21 is able to interact with the DLG motif within NRF-2, thereby attenuating Keap1-mediated ubiquitination and subsequent proteasomal degradation. The antioxidant properties of p21 rely on Nfr2 activity. Because NRF-2 upregulates HO-1 and NQO1, we measured the expression of these proteins and found that their expression is upregulated in hDPSCs under ambient oxygen levels. Thus, there is an antioxidant response of hDPSCs to oxidative stress caused by high oxygen tension that is mediated by NRF-2, confirming previous studies in vivo (Cho et al., 2002) and in vitro (Visner et al., 1996).

Culture of hDPSCs under Ambient and Physiological Oxygen Concentrations: Practical Issues

A major practical aim of the study of the biology of stem cells is that they may serve as important tools in regenerative medicine. To this end, high yields of stem cells are required when they are obtained from tissues from patients. We used dental pulp stem cells, but these considerations apply to other tissues, such as fat, hair follicles, etc.

For instance, in the dental setting, it is critically important to obtain a high yield of cells so that they can be used in the same clinical act after obtaining them, to implant them in the alveoli. Minimizing time to obtain large amounts of viable cells is also critically important.

Moreover, hDPSCs have been proposed as potential sources for stem cell banking for clinical use (Perry et al., 2008), and for this purpose it is critically important to culture them under the best conditions.

Our results show that the usual high oxygen concentration (21%, atmospheric) used to obtain and culture cells is far worse than the physiological O_2 (3%), to obtain high yields of viable stem cells. In summary, we have shown that physiological O_2 tension is better than ambient O_2 for propagation of stem cells and have identified a signaling pathway involving p38 MAPK, p21, and NRF-2 activated by ambient O_2.

EXPERIMENTAL PROCEDURES

Cell Culture

Intact third molars were collected from men (aged from 15 to 20 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. The dental pulp was cut into very small pieces and disaggregated in a solution of 2 mg/ml collagenase type I for 90 min at 37°C in an oxygen-regulated cell-culture incubator at the appropriate oxygen tension (3% or 21%) and 5% CO_2. Cell suspensions were centrifuged at 1,000 × *g* at 20°C for 2 min, the medium was removed, and the cell pellet resuspended and cultured in DMEM with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen). Cells used for experiments were between passages 3 and 6.

Immunofluorescence Staining

We tested CD133 (Prominin-1) (Sigma, Ref: C9493), STRO-1 (MILLIPORE, Ref: MAB4315), POU5F1 (Oct4) (Sigma, Ref: P0082), Nestin (Sigma, Ref: N5413), CD34 (Cell Signaling Technology Ref: #3569), and CD45 (Cell Signaling Technology Ref: #3575). We used Lab-Tek plates (Nalge Nunk International) treated with poly-L-lysine (Chemical Sigma-Aldrich). Dental pulp stem cells were seeded at 20,000 cells/cm^3 and cultured under physiological oxygen conditions (3% O_2) for 3 days. Cells were fixed with 3.7% paraformaldehyde, neutralized with 50 mM glycine in PBS for 10 min, and permeabilized with 0.25% Triton X-100 in PBS for 10 min. Cells were then incubated with 3% BSA for 10 min, followed by the primary and second antibody incubation. The secondary antibodies were anti-rabbit immunoglobulin (Ig) G TRITC conjugated (Sigma) and anti-mouse IgG FITC conjugated (Sigma). For immunofluorescence staining, we used laser confocal microscopy (Leica, TCS-SP2 Leica) with an argon laser and neon-helium Leica reversed microscope (Leica, DM1RB).

Reactive Oxygen Species Determination

Cells were washed with warm PBS and treated with trypsin and then resuspended in DMEM containing 1 g/l glucose. We used dihydrorhodamine-123 at a final concentration of 1 μg/ml. Cells were incubated for 30 min at 37°C in the dark. Values were read by flow cytometry until 20,000 events were recorded.

Mitochondrial Membrane Potential Determination

We used JC-1 staining to assess the mitochondrial membrane potential. The procedure was similar to dihydrorhodamine-123.

Lipid Peroxidation

Lipid peroxidation was estimated as malondialdehyde (MDA), which was detected by high-performance liquid chromatography (HPLC) as an MDA-thiobarbituric acid adduct following the method described by Wong et al. (1987).

Cell Proliferation Assays

The cells were counted after 6 hr, 1, 2, 3, 5, 7, and 8 days of seeding using a Neubauer plate.

Trypan Blue Dye Exclusion Method

Viability of hDPSCs was determined using the vital dye trypan blue, which is excluded by living cells but accumulates in dead cells. Cells were counted after they were trypsinized and incubated 1:1 with 0.01% trypan blue.

Immunoblot Analysis

Aliquots of cell lysates (40 μg) were immediately boiled for 10 min, electrophoresed in SDS-10 or 12.5% polyacrylamide gels, and electroblotted (Bio-Rad) ontopolyvinylidene difluoride (PVDF) membranes (Bio-Rad). Protein content was determined by a modified Lowry method (Lowry et al., 1951). Membranes were blocked with 0.05 g/ml BSA in TBS-0.2% Tween 20 (TBST) and incubated with primary antibodies and further incubated with a secondary horseradish peroxidase-linked anti-rabbit IgG antibody. Blots were then developed by using the "ECL Prime Western Blotting Detection Reagent" as specified by the manufacturer (Amersham Pharmacia).

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 $p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.08.002.

ACKNOWLEDGMENTS

We want to acknowledge all the individuals who have accepted to donate their teeth for the extraction of dental pulps. This work was supported by grants SAF2010-19498, from the Spanish Ministry of Education and Science (MEC); ISCIII2012-RED-43-029 from the "Red Tematica de investigacion cooperativa en envejecimiento y fragilidad" (RETICEF); PROMETEO2010/074 from "Conselleria d'Educació, Cultura i Esport de la Generalitat Valenciana"; RS2012-609 Intramural Grant from INCLIVA and EU Funded CM1001 and FRAILOMIC-HEALTH.2012.2.1.1-2. The study has been cofinanced by FEDER funds from the European Union.

Received: November 15, 2013
Revised: August 4, 2014
Accepted: August 5, 2014
Published: September 18, 2014

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