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ROLE OF $p16^{INK4a}$ AND BMI-1 IN OXIDATIVE STRESS-INDUCED PREMATURE SENESCENCE IN HUMAN DENTAL PULP STEM CELLS

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CERTIFICAN:

Que la Srta. **Cristina Mas Bargues**, Licenciada en Farmacia por la Universidad de Valencia, ha realizado bajo su dirección la presente Tesis Doctoral, titulada:

"ROLE OF p16^{INK4a} AND BMI-1 IN OXIDATIVE STRESS-INDUCED PREMATURE SENESCENCE IN HUMAN DENTAL PULP STEM CELLS"

para la obtención del título de Doctora.

Y para que conste a los efectos oportunos, firman la presente certificación.

Valencia, 2017

Fdo. Dr. José Viña Ribes	Fdo. Dra. Consuelo Borrás Blasco	Fdo. Dr. Juan Gambini Buchón

TO MY FAMILY

A MI FAMILIA

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ABSTRACT

ROLE OF $p16^{INK4a}$ AND BMI-1 IN OXIDATIVE STRESS-INDUCED PREMATURE SENESCENCE IN HUMAN DENTAL PULP STEM CELLS

Human mesenchymal stem cells (MSCs) have a therapeutic potential in tissue engineering and regenerative medicine. Human dental pulp stem cells (hDPSCs) have proven to be a good source for cell therapy as pulp tissue is easily available from teeth after extraction without ethical issues. Cell therapy requires a large number of cells, thus, an *in vitro* expansion step is required before implantation. Long-term *in vitro* culture entails the inconvenience of senescence following a certain number of passages, thereby losing hDPSCs stemness properties and regenerative potential. Currently, the *in vitro* culture of MSCs is carried out under ambient oxygen tension (21% pO_2). However, the local oxygen tension varies between 3-6% pO_2 within the organism depending on the vascularization of the tissue and its metabolic activity. Mimicking physiological conditions in cell culture experiments is a useful tool to gain knowledge. Thus, the aim of this study was to compare long-term *in vitro* culture of hDPSCs under ambient (21% pO_2) and physiological (3% pO_2) oxygen partial pressure, and to determine whether hyperoxia can alter the physiology and affect the senescence of normal adult stem cells.

First, we investigated if ambient oxygen tension could induce oxidative stress in hDPSCs during long-term culture. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the biological ability of a system to readily detoxify the intermediates and to repair the resulting damage. Oxidative stress is known to produce damage to biomolecules such as DNA, carbohydrates, lipids and proteins. Therefore, we assessed ROS levels, mitochondrial membrane potential, protein and lipid oxidation, as well as antioxidant gene expression during long-term hDPSCs *in vitro* culture. Our data revealed increased ROS levels, protein carbonylation and lipid oxidation, and a disruption of the mitochondrial membrane potential in the cells that were cultured under ambient oxygen tension. Furthermore, these cells showed an upregulation of

the expression of manganese superoxide dismutase, catalase and glutathione peroxidase genes, suggesting an increased antioxidant defence to withstand increasing ROS production. Thus, ambient oxygen tension induces oxidative stress in hDPSCs *in vitro* culture.

Senescence was described as an irreversible state of cell cycle arrest, which is accompanied by morphological alterations, reduced proliferation rate, apoptosis resistance and increased expression of senescence-associated β-galactosidase activity and $p16^{INK4a}$. The *CDKN2A* gene, also known as the *INK/ARF* locus, encodes both $p16^{INK4a}$ and $p14^{ARF}$, which are cell cycle regulators. The $p16^{INK4a}$ protein inhibits cyclin D-dependent CDK4 and CDK6 to prevent phosphorylation of the retinoblastoma protein (pRb). The hypophosphorylated form of pRb sequesters E2F transcription factors, preventing them from coordinately activating a suite of genes required for DNA replication. The $p14^{ARF}$ protein binds to the MDM2 E3 ubiquitin ligase to prevent p53 polyubiquitylation and to facilitate p53 activation. In turn, the p53 transcription factor regulates an extensive group of genes that are commonly induced by cellular stress leading to apoptosis. We then determined some senescence characteristics in hDPSCs cultured under both oxygen tension conditions. Cells that were cultured under ambient oxygen tension rapidly began to show enlarged and flattened phenotypes and decreased their regenerative potential as they only reached 15 passages, while those cells that were cultured under physiological oxygen tension reached 25 passages and preserved a "younger" phenotype. Accordingly, 21% pO_2 was accompanied by increased β-galactosidase activity, increased expression of $p16^{INK4a}$ and reduced expression of $p14^{ARF}$. Taken together, our results suggest that oxidative stress induces a premature senescence of hDPSCs cultured under ambient oxygen tension.

Another characteristic that is not included in the definition of stem cell senescence, but should be taken into consideration, is the loss of stemness properties. hDPSCs are adult stem cells that express pluripotency-related genes. Those genes are the four transcription factors: *OCT4*, *SOX2*, *KLF4* and *c-MYC*, abbreviated to, "OSKM", and they are involved in the induction and maintenance

of pluripotency. hDPSCs cultured at 3% pO_2 showed high levels of *OCT4* and *SOX2* at early stages, but their expression was downregulated as passages accumulated. However, at advanced passages, these cells upregulated the *KLF4* and *c-MYC* expression. On the other hand, hDPSCs cultured at 21% pO_2 showed a downregulation of all four OSKM factors along passages. Taken together, our data suggest that ambient oxygen tension entails a loss of the stemness properties in long-term *in vitro* culture.

Finally, we aimed to investigate a possible relation between $p16^{INK4a}$ and OSKM expression in hDPSCs. A well described upstream regulator of the *INK/ARF* locus is BMI-1. Through repression of target gene expression, the BMI-1 protein regulates a myriad of cellular processes critical for cell growth, cell fate decision, development, senescence, apoptosis, and self-renewal of stem cells. Thus, we tested whether BMI-1 regulates hDPSCs fate while modulating $p16^{INK4a}$ and OSKM gene expression. To this end, we first analysed BMI-1 protein expression in hDPSCs in long-term culture under either oxygen pressure percentage. Our results show that hDPSCs cultured at 3% pO_2 retained constant levels of BMI-1 along passages. However, hDPSCs cultured at 21% pO_2 showed higher BMI-1 protein levels at early stages, which rapidly plummeted as passages accumulated. These data suggest that ambient oxygen tension accelerated BMI-1 protein degradation. We next proceeded to silence *BMI-1* gene expression in hDPSCs cultured under ambient oxygen tension by siRNA transfection. Knocked-down hDPSCs exhibited the same amount of BMI-1 protein as hDPSCs cultured under physiological oxygen tension. Interestingly, following transfection, $p16^{INK4a}$ expression was not altered, but *OCT4* and *SOX2* expression levels were upregulated.

Taken together, we can conclude that *in vitro* culture carried out under ambient oxygen tension causes an oxidative stress-induced premature senescence and a loss of the stemness properties of hDPSCs. Moreover, BMI-1 levels should be kept in a balance that allows normal stem cell proliferation, while preventing stem cell senescence thereby maintaining proper stem cell homeostasis.

RESUMEN

PAPEL DE $p16^{INK4a}$ Y BMI-1 EN LA SENESCENCIA PREMATURA INDUCIDA POR ESTRÉS OXIDATIVO EN CÉLULAS MADRE DE PULPA DENTAL HUMANA

INTRODUCCIÓN Y OBJETIVOS

Las células madre son aquellas células que se dividen asimétricamente para producir una copia de sí mismas y una segunda célula que seguirá su camino hasta convertirse en una célula diferenciada y especializada. Así pues, las células madre que podemos encontrar en el organismo adulto comparten al menos dos características. En primer lugar, se pueden hacer copias idénticas de sí mismas durante largos períodos de tiempo; esta capacidad de proliferar se refiere como auto-renovación a largo plazo. Segundo, pueden dar lugar a tipos celulares maduros que tienen morfologías características y funciones especializadas. Las células madre mesenquimales humanas (CMM) tienen pues un potencial terapéutico en ingeniería de tejidos y en medicina regenerativa. En particular, las células madre de pulpa dental humana (CMPD) han demostrado ser una buena fuente de células para la terapia celular porque el tejido pulpar es fácilmente accesible y carece de problemas éticos.

Sin embargo, las terapias celulares requieren de un elevado número de células, para lo cual es necesaria una etapa de expansión celular *in vitro* previa a la implantación. A su vez, el cultivo *in vitro* a largo plazo lleva implícito el inconveniente de la senescencia tras un cierto número de pases, momento en el cual las células pierden su capacidad de autoregeneración, característico de las células madre. Por esta razón, el estado de senescencia debe tenerse en cuenta para poder obtener células madre de buena calidad que puedan ser utilizadas en las terapias celulares.

Actualmente, el cultivo *in vitro* de las CMM se lleva a cabo bajo tensión de oxígeno ambiental (21% pO_2). Sin embargo, dentro del organismo, la tensión de oxígeno a nivel local oscila entre el 3-6% pO_2, dependiendo de la vascularización del tejido y de su actividad metabólica. El cultivo celular debe tratar de imitar las

condiciones fisiológicas del organismo, por lo tanto, el objetivo de este estudio fue el de comparar el cultivo *in vitro* a largo plazo de las CMPD bajo ambas concentraciones de oxígeno: ambiental (21% pO_2) y fisiológica (3% pO_2). Así, existen trabajos que muestran que altas concentraciones de oxígeno pueden causar estrés oxidativo a través de la producción de especies reactivas de oxígeno (ROS) que pueden dañar lípidos, proteínas y ADN, cambiando el metabolismo de la célula. En este contexto, es interesante destacar que cultivar las CMM en condiciones de hipoxia, o mejor dicho, de "normoxia" fisiológica, antes del trasplante podría mejorar el potencial regenerador de tejido. Además, la reducción de los niveles de oxígeno se acompaña de una mayor tasa de crecimiento, sugiriendo que el estrés oxidativo es un factor contribuyente en la senescencia celular.

La senescencia se describe como un estado irreversible de detención del ciclo celular, que se acompaña de alteraciones morfológicas, una menor tasa de proliferación, resistencia a la apoptosis y un aumento de la expresión de la actividad β-galactosidasa asociada a la senescencia y de $p16^{INK4a}$. El crecimiento celular está controlado por dos vías principales: una que implica la proteína retinoblastoma (pRb), la cual regula la salida del ciclo celular en la fase G1 y otra que implica la participación de la proteína p53, que induce la detención del crecimiento o apoptosis en respuesta al estrés celular. El gen *CDKN2A*, también conocido como locus *INK/ARF*, codifica los genes $p16^{INK4a}$ y $p14^{ARF}$, que son reguladores del ciclo celular. La proteína $p16^{INK4a}$ inhibe las ciclinas dependientes de quinasa CDK4 y CDK6, para prevenir la fosforilación de la proteína del retinoblastoma (pRb). La forma hipofosforilada de pRb secuestra los factores de transcripción de E2F impidiéndoles activar de forma coordinada una serie de genes que son necesarios para la replicación del ADN. La proteína $p14^{ARF}$, por su parte, se une a la ubiquitin ligasa MDM2 E3 para facilitar la activación de p53. A su vez, el factor de transcripción p53 regula un grupo extenso de genes que son comúnmente inducidos por el estrés celular que conduce a la apoptosis.

Otra característica que no está incluida en la definición de senescencia de las células madre, pero que debería de tomarse en consideración, es la pérdida de la pluripotencia o capacidad de autoregeneración. La pluripotencia va ligada a la expresión de unos factores de transcripción, conocidos como: OCT4, SOX2, KLF4 y c-MYC, y abreviados como "OSKM". Dichos genes están involucrados tanto en la inducción como en el mantenimiento de la pluripotencia en las células madre. Es decir, estos genes son capaces de borrar la huella epigenética de las células somáticas hasta convertirlas en las conocidas células madre pluripotentes inducidas (iPSC), cuyas características se equiparan a las de las células madre embrionarias.

Finalmente, se pretendió investigar una posible relación entre la senescencia y la pérdida de pluripotencia de las CMPD, ambas asociadas al cultivo *in vitro* a largo plazo. Un posible nexo de unión es la proteína BMI-1. Dicha proteína modula la compactación de la cromatina a través de la metilación de las histonas en los nucleosomas; es por tanto un regulador epigenético. Más concretamente, BMI-1 es un represor génico, cuya diana mejor descrita es el locus *INK/ARF*. También se sabe que BMI-1 juega un papel clave no sólo en la prevención de daños al ADN, sino también en el mantenimiento de la función mitocondrial y la homeostasis redox. Así pues, la proteína BMI-1 regula una amplia gama de procesos celulares críticos para el crecimiento celular, la decisión del destino celular, el desarrollo, la senescencia, la apoptosis y la auto-renovación de las células madre. Por lo tanto, nos propusimos investigar si BMI-1 regula el destino de las CMPD al modular la expresión de los genes $p16^{INK4a}$ y OSKM.

Nuestro objetivo principal fue pues el de analizar el papel de $p16^{INK4a}$ y de BMI-1 en la senescencia inducida por el estrés oxidativo en células madre de pulpa dental a largo plazo.

METODOLOGÍA

En primer lugar, analizamos si la tensión de oxígeno ambiental induce estrés oxidativo en las CMPD durante el cultivo a largo plazo. Para ello se midió la producción de especies reactivas del oxígeno (ROS) y el potencial de membrana

mitocondrial por citometría de flujo. También se analizaron los niveles de malondialdehído (MDA) como marcador de la peroxidación lipídica por cromatografía líquida de alta eficacia (HPLC) y los niveles de carbonilación proteica por western blot. Además de estos marcadores, también se determinaron los niveles de las enzimas antioxidantes: manganeso superóxido dismutasa (MnSOD), glutatión peroxidasa (GPx) y catalasa (CAT), mediante la técnica de la reacción en cadena de la polimerasa (PCR).

A continuación, analizamos si la tensión de oxígeno ambiental provoca una senescencia prematura inducida por estrés oxidativo en las CMPD durante el cultivo a largo plazo. Para ello, analizamos la morfología celular por microscopía y determinamos la actividad de la enzima β-galactosidasa por citometría de flujo. Paralelamente, comparamos la expresión de los genes $p14^{ARF}$ y $p16^{INK4a}$ por PCR para determinar la vía de señalización implicada en la senescencia prematura de las CMPD cultivadas al 21% pO_2.

Seguidamente, analizamos si la tensión de oxígeno ambiental produce una pérdida de pluripotencia en las CMPD durante el cultivo a largo plazo, para lo cual medimos la expresión de los factores OCT4, SOX2, KLF4 y c-MYC por PCR.

Por último, comparamos la evolución de la expresión de BMI-1 tanto a nivel génico como proteico, en las CMPD durante el cultivo a largo plazo bajo ambas presiones de oxígeno. Finalmente, mediante la introducción de pequeños ARN de interferencia (siRNA), se silenció la expresión de BMI-1 para ver si tenía algún efecto sobre la expresión de los factores de pluripotencia.

RESULTADOS Y DISCUSIÓN

Las CMPD normalmente residen a bajas concentraciones de oxígeno. En los mamíferos, incluidos los humanos, en el momento en que el oxígeno llega a los órganos y tejidos, la concentración de oxígeno cae a 2-9%, con una media de 3% en la pulpa dental. A pesar de este hecho, todavía es común cultivar células al 21% pO_2. Sin embargo, la función celular normal requiere un entorno estable de

oxidación-reducción. El exceso en la tensión de oxígeno se ha descrito como un factor importante que podría desestabilizar la homeostasis redox celular.

El estrés oxidativo refleja un desequilibrio entre la producción de especies reactivas del oxígeno (ROS) y la capacidad de detoxificación de los productos intermedios o de reparar el daño resultante. En el presente estudio, mostramos que la reducción del nivel de oxígeno ambiental condujo a una disminución del estrés oxidativo intracelular y del daño a biomoléculas durante el cultivo a largo plazo. Nuestros datos revelaron un aumento de los niveles de ROS, de la carbonilación de proteínas y de la oxidación de lípidos, así como una caída del potencial de membrana mitocondrial de las CMPD que se cultivaron a tensión de oxígeno ambiental. Además, estas células mostraron una sobreexpresión de las enzimas MnSOD, CAT y GPx, lo cual sugiere un aumento de la defensa antioxidante para hacer frente a la creciente producción de ROS. Por lo tanto, nuestros datos sugieren que el cultivo *in vitro* al 21% pO_2 conlleva un estrés oxidativo.

En este estudio, demostramos que las CMPD cultivadas a tensión de oxígeno ambiental cambiaron su morfología mostrando formas más aplanadas o alargadas, los cual se acompañó de un incremento de residuos en el medio de cultivo. Además, la tasa de proliferación de las CMPD se redujo significativamente a tensión de oxígeno ambiental, puesto que las células cultivadas al 3% pO_2 alcanzaron 25 pases mientras que las cultivadas al 21% pO_2 sólo alcanzaron 15 pases en el mismo periodo de tiempo. Estas observaciones se acompañaron de una mayor actividad de la enzima β-galactosidasa asociada a la senescencia a lo largo de los pases, lo que sugiere que estas células estaban entrando en un estado senescente. De hecho, aquí se demuestra que las CMPD se pueden mantener en cultivo durante al menos 25 pases manteniendo un fenotipo "más joven" y conservando la cinética de proliferación cuando éstas se cultivan a presión de oxígeno fisiológica. Es decir, durante el cultivo a largo plazo bajo tensión de oxígeno ambiental, las CMPD pierden gradualmente su potencial proliferativo y muestran signos cada vez mayores de senescencia tales como fenotipos más grandes y niveles incrementados de SA-β-Gal.

En conjunto, nuestros resultados sugieren que las CMPD cultivadas a tensión de oxígeno ambiental se someten a una senescencia prematura, que se evidencia por el agrandamiento del fenotipo, la reducción del potencial proliferativo y el aumento de la actividad SA-β-Gal. Este fenómeno parece ser causado por la acumulación de ROS, dando lugar a la senescencia prematura inducida por estrés.

De hecho, durante mucho tiempo se ha sabido que una tensión reducida de oxígeno promueve el crecimiento y prolonga la vida replicativa de las células humanas mantenidas en cultivo. Los ROS tienen importantes funciones en la señalización celular, pero su papel en la regulación de la progresión del ciclo celular es poco conocido. Los niveles de ROS aumentan significativamente a medida que las células pasan de la fase G1 a la fase S del ciclo celular y son necesarios para la entrada en fase S. Sin embargo, los puntos de control del ciclo celular también se activan por aumento de ROS, lo que indica que la proliferación celular se basa en mantener los niveles de ROS dentro de un rango funcional.

La senescencia es una característica normal de las células, por la cual pierden su capacidad replicativa tras un número finito de divisiones. Las vías inhibitorias del ciclo celular $p16^{INK4a}$/pRb y $p14^{ARF}$/p53 representan dos importantes vías que controlan la proliferación, y su inactivación puede extender el número límite de divisiones de células mitóticas en mantenidas cultivo. Dado el papel de $p16^{INK4a}$ en la regulación del ciclo celular y la reciente implicación del estrés oxidativo en la senescencia de las células madre, se investigó un posible vínculo entre la producción de ROS y la expresión de $p16^{INK4a}$. Nuestros resultados muestran que las CMPD cultivadas a largo plazo al 21% pO_2 presentaban signos de senescencia, que se acompañaban de un aumento en los niveles de expresión de $p16^{INK4a}$ y una reducción en los niveles de expresión de $p14^{ARF}$. De acuerdo con esto, las células humanas generalmente expresan cantidades crecientes de $p16^{INK4a}$ cuando se aproximan a su límite de vida útil *in vitro*. De hecho, las señales de estrés como los ROS estimulan la activación de la transcripción de $p16^{INK4a}$ y desempeñan funciones importantes en la iniciación, así como el mantenimiento, de

la senescencia celular. Por esta razón, añadimos un agente antioxidante para ver si podíamos restablecer los niveles de expresión de $p16^{INK4a}$. El tratamiento con Trolox 50 μM pudo rescatar los niveles de expresión de $p16^{INK4a}$ en el cultivo a largo plazo de CMPD al 21% pO_2. En conjunto, podríamos decir que una baja tensión de oxígeno podría retrasar la senescencia de las CMPD mediante la regulación negativa de la expresión de $p16^{INK4a}$.

Las CMPD cultivadas al 3% pO_2 mostraron elevados niveles de expresión de los genes *OCT4* y *SOX2* en los primeros pases, pero su expresión disminuyó conforme se acumulaban pases, lo cual sugiere que estos dos factores estarían implicados en la inducción de la pluripotencia. Sin embargo, en pases avanzados, estas células mostraron elevados niveles de expresión de los genes *KLF4* y *c-MYC*, sugiriendo el papel de mantenimiento de la pluripotencia de estos dos factores. Por su parte, las CMPD cultivadas al 21% pO_2, mostraron menores niveles de expresión de los cuatro factores OSKM a lo largo de los pases. En conjunto, nuestros datos sugieren que la tensión de oxígeno ambiental acelera la pérdida de la pluripotencia durante el cultivo a largo plazo. Este resultado fue consistente con otros hallazgos, lo cual sugiere que un microambiente con bajo contenido de oxígeno proporciona una condición óptima para el mantenimiento de las propiedades de las células madre.

Nuestros resultados muestran que las CMPD cultivadas al 3% pO_2 conservaban unos niveles constantes de la proteína BMI-1 a lo largo de los pases. Sin embargo, los niveles de BMI-1 en las CMPD cultivadas al 21% pO_2 no se mantuvieron constantes, sino que se desplomaron conforme se acumulaban los pases. Se sabe muy poco sobre la regulación post-transcripcional de BMI-1. Recientemente, se ha sugerido que BMI-1 es una proteína de corta duración. Algunos investigadores han publicado que BMI-1 podría ser un sustrato de AKT; la activación de la vía de señalización de AKT coincide con la fosforilación de BMI-1 lo cual le confiere mayor estabilidad. Por el contrario, la vía de señalización de p38 dependiente de estrés oxidativo hace que BMI-1 se degrade y pierda su capacidad modificadora de la cromatina. Además, también se ha sugerido que los

niveles de BMI-1 están regulados por degradación proteasomal. Colectivamente, estas observaciones apoyan la noción de que los niveles de BMI-1 están regulados por las vías de señalización dependientes de estrés oxidativo en las células madre.

Curiosamente y a pesar de ello, las CMPD de pase 5, cultivadas al 21% pO_2 mostraron mayores niveles de BMI-1, tanto a nivel génico como proteico. Así pues, se procedió a silenciar la expresión del gen *BMI-1* en dichas células, hasta equiparar los niveles de proteína en las CMPD cultivadas bajo ambas presiones de oxígeno.

A continuación, analizamos los efectos de dicho silenciamiento sobre la senescencia y la pluripotencia de las CMPD. Los resultados obtenidos demuestran que los niveles de expresión de $p16^{INK4a}$ no se vieron afectados, sin embargo, los de *OCT4* y *SOX2* sí. Tras reducir los niveles de BMI-1 en las CMPD jóvenes cultivadas al 21% pO_2, los factores *OCT4* y *SOX2* incrementaron su expresión hasta igualarse a la de las CMPD jóvenes cultivadas al 3% pO_2. Es decir, este resultado sugiere, que el silenciamiento parcial de BMI-1 es capaz de rescatar la expresión de *OCT4* y *SOX2* en las CMPD cultivadas bajo tensión de oxígeno ambiental.

CONCLUSIONES

En conjunto, podemos concluir que el cultivo *in vitro* mantenido a tensión de oxígeno ambiental provoca una senescencia prematura inducida por estrés oxidativo y una pérdida de la expresión de los factores de pluripotencia en las CMPD.

Además, los niveles de BMI-1 deben mantenerse en un equilibrio que permita la proliferación normal de las células madre, al tiempo que previene la senescencia de las células madre, y por tanto, el mantenimiento de la homeostasis de las células madre.

1 INTRODUCTION

1.1 STEM CELLS

1.1.1 Definition

In its canonical view, a stem cell is presented as one that divides asymmetrically to produce a copy of itself and a second cell that is on its path to differentiate (Figure 1.1). This second cell may have one of many characteristics depending on the context of its development. In certain instances, this cell would be a precursor type that undergoes terminal differentiation. Alternatively, a cell generated from asymmetric division could itself be a progenitor that amplifies itself and then differentiates into one of many cell types. These cells have a finite proliferative potential, and eventually exhaust themselves once they give rise to differentiated progeny. Finally, an asymmetric cell division from a stem cell could generate a copy of itself and a second stem cell with a more limited potential for self-renewal.

Stem cells are generated in the embryo. They persist in specific niches where they can remain mitotically quiescent for long periods of time. Stem cells expand their numbers as they self-renew by symmetric division. They can also maintain their numbers and produce rapidly dividing progenitors by asymmetric division (Martinez-Agosto et al., 2007).

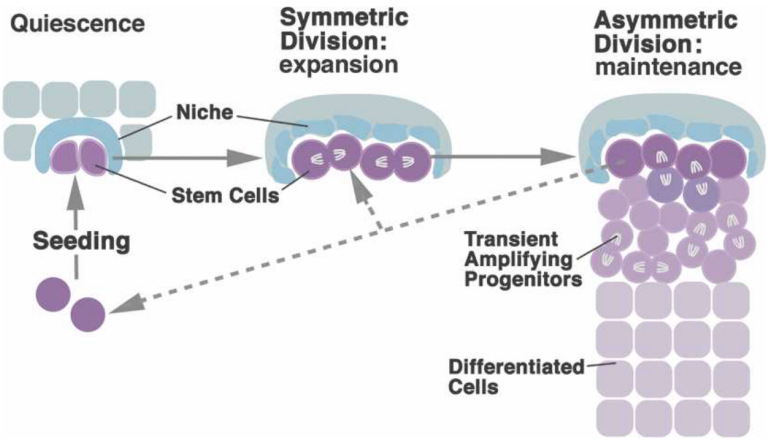

Figure 1.1 Stem cells dynamics.

Taken from: Martinez-Agosto et al. (2007).

1.1.2 Classification

The definition of a stem cell inevitably requires an assessment of its potential to give rise to a number of differentiated progeny. This potential can vary among different tissue types, and by the origin and requirement of a particular type of stem cell (Jaenisch and Young, 2008).

1.1.2.1 Potency

According to their potency, we can divide stem cells in four groups:

- Totipotent stem cells are defined as those that can give rise to all tissues in an organism, including germ line, embryonic and extra-embryonic tissues. The zygote is the only totipotent stem cell.

- Pluripotent stem cells originate from the inner cell mass of the embryo and give rise to all tissues *in vivo*, except trophoblasts. In this group we include embryonic stem cells (ESCs).

- Multipotent stem cells are more limited in their potency. Adult stem cells (ASCs) such as hemaetopoietic stem cells constitute the classic example of a multipotent stem cell, which can give rise to a large repertoire of differentiated cell types belonging to the lymphoid and myeloid lineages.

- Stem cells can also be unipotent if they are only capable of giving rise to a single cell type that is constantly produced throughout the life span of the organism. The best described example is the germ line stem cell (GSC), which can generate a single differentiated cell fate: either an egg (ovum) or a sperm.

1.1.2.2 Origin

According to their origin, stem cells can be classified into ESCs and ASCs. Several strategies have been employed to induce the conversion of differentiated cells into an embryonic state in order to supply the ethical problems associated with ESCs. All these approaches led to the generation of a new cell type, commonly referred to as "induced pluripotent stem cells" or iPSCs.

1.1.2.2.1 Embryonic stem cells (ESCs)

As stated before, an embryonic stem cell (ESC) is defined by its origin. It is derived from the blastocyst stage of the embryo. The blastocyst is the stage of embryonic development prior to implantation in the uterine wall. At this stage, the preimplantation embryo is made up of 150 cells and consists of a sphere made up of an outer layer of cells (the trophectoderm), a fluid-filled cavity (the blastocoel), and a cluster of cells on the interior (the inner cell mass).

The first documentation of the isolation of embryonic stem cells from human blastocysts was in 1994 (Bongso et al., 1994). In 1998, James Thomson and his colleagues reported methods for deriving and maintaining human ESCs from the inner cell mass of human blastocysts that were produced through *in vitro* fertilization (IVF) and donated for research purposes (Thomson et al., 1995; Thomson et al., 1998). Since then, techniques for deriving and culturing human ESCs have been refined. The ability to isolate human ESCs from blastocysts and grow them in culture seems to depend in large part on the integrity and condition of the blastocyst from which the cells are derived. In general, blastocysts with a large and distinct inner cell mass tend to yield ESCs cultures most efficiently.

As we have previously said, ESCs are pluripotent cells that can give rise to cells from the three germ layers: endoderm, mesoderm and ectoderm (Amit et al., 2000; Itskovitz-Eldor et al., 2000; Reubinoff et al., 2000; Schuldiner et al., 2000).

1.1.2.2.2 Induced pluripotent stem cells (iPSCs)

Great advances were expected in the field of regenerative medicine; however, there were some problems with medical treatments involving human ESCs. First, there is a bioethical issue because a fertilized egg is used to generate ESCs. In addition, when ESCs are applied to an organ in regenerative medicine, the recipients must be given an immunosuppressant drug to prevent transplant rejection (Miyazaki et al., 2012).

The use of induced pluripotent stem cells (iPSCs) solved the problems mentioned above. In 2006, iPSCs were generated from mouse embryonic fibroblasts (MEF) (Takahashi and Yamanaka, 2006). They were then generated from human dermal fibroblasts the following year (Takahashi et al., 2007). The iPSCs are reprogrammed from differentiated somatic cells by going back to an undifferentiated state similar to ESCs. Both ESCs and iPSCs are defined as pluripotent and are able to differentiate into three germ layers (endoderm, mesoderm and ectoderm) as well as self-renew. Thus, iPSCs may solve the problems related to clinical applications.

Some of the reprogramming methods reported include: nuclear transfer, cell fusion, reprogramming using cell extracts and direct reprogramming (Figure 1.2).

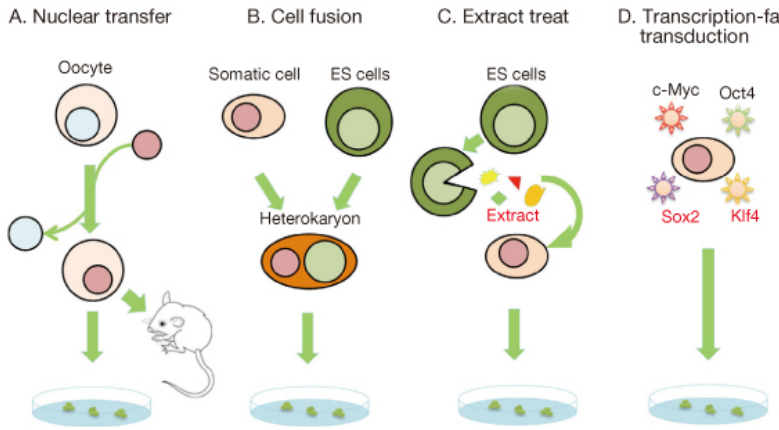

Figure 1.2 Strategies to induce reprograming of somatic cells.
Taken from: Yoon and You (2011).

Strategies for reprogramming somatic cells

- Nuclear transfer

In 1952, Briggs and King succeeded in producing the tadpole, the first cloned animal, from the cell nucleus of a frog in the blastocyst stage (Briggs and King, 1952). For mammals, the first nuclear transfer-derived cloned sheep was generated in 1986 by transferring a blastomere nucleus from a four- to eight-cell sheep embryo to an enucleated unfertilized egg; in the following year, the cloned mouse was produced by transferring nuclei from eight-cell mouse embryos to enucleated two-cell embryos (Robl et al., 1986; Tsunoda et al., 1987).

In addition, cloned animals were produced successfully using nuclear transfer techniques with many types of somatic cells, including cumulus cells, leukocytes, hepatocytes, neuronal cells, myocytes, lymphocytes and germ cells (Brem and Kuhholzer, 2002; Hochedlinger and Jaenisch, 2002; Jaenisch et al., 2004). Nuclear transfer reprograms the cell nuclei of differentiated somatic cells by transplanting them into an enucleated oocyte. In order to create a cloned embryo without using sperm after nuclear transfer, the egg was activated by electrical or chemical stimulation.

However, an unfertilized egg must be procured for clinical applications and immunological rejection may occur due to genetic factors from the nuclei isolated from somatic cells and the oocyte. In 2007, it was reported that only 2 cells out of a total of 304 oocytes were successfully created as ESCs by nuclear transfer (Byrne et al., 2007), suggesting a low induction efficiency.

- Cell fusion

Cell fusion is another reprogramming method. The somatic cell genome is highly reprogrammed in a syncytium where cell division occurs repeatedly with the nuclei of ESCs and somatic cells mixed (Tada et al., 2001; Tada et al., 2003). Therefore, it is supposed that ESCs are able to reprogram the somatic cells by

deleting their properties and overwriting the somatic cell genome with the properties of ESCs.

ESC hybrids formed teratomas in immunodeficient mice and they generated chimera embryos after blastocyst injection, which demonstrates the pluripotency of ESC hybrids. It has been reported that the pluripotent cells can also be generated from mice and human beings through reprogramming using cell fusion (Surani, 2005; Yu et al., 2006).

It is currently unknown whether the somatic cell nuclei can be reprogrammed by cell fusion with only the cytoplasmic elements of ESCs or if the nuclear elements are also needed. It is desirable to selectively remove only the chromosomes of ESCs from the fused nuclei, but it is technically difficult (Pralong et al., 2005). It will be a long time before this advanced technique is used in clinical applications.

- Reprogramming using cell explants

Another reprogramming method inserts cell extracts, which are obtained from pluripotent stem cells such as ESCs, into somatic cells. As described in the previous section on "Cell fusion", ESCs have a specific characteristic that reprograms the somatic cells. The cell extract is chemically isolated from ESCs and consists of reprogramming factors that enter into the somatic cells and induces their reprogramming.

It was reported in 2005 that culturing HEK293 and NIH3T3 cells with the factors isolated from human embryonic carcinoma NCCIT cells increased the expression levels of undifferentiation markers, including OCT4, and that the cells could be induced to differentiate into neurogenic, adipogenic, osteogenic and endothelial lineages (Taranger et al., 2005). However, they could not be differentiated into three germ layers *in vivo*. Thus, this reprogramming method may be incomplete.

- Direct reprogramming

In 2006, it was reported that four transcription factors (OCT4, SOX2, KLF4 and c-MYC), named OSKM, were introduced into MEFs to generate iPSCs (Takahashi and Yamanaka, 2006).

Transcription factors are one of the groups of proteins that read and interpret the genetic print in the DNA. They bind to the DNA and help initiate a program of increased or decreased gene transcription. As such, they are vital for many important cellular processes. The octamer-binding transcription factor OCT4 belongs to the POU transcription factor family and is crucial to sustain a pluripotent state of ESCs. It is known that the level of OCT4 decreases with the stage of cell differentiation and OCT4 does not occur in mature somatic cells (Loh et al., 2006). The Kruppel-like factor 4 (KLF4) functions as a transcriptional repressor in opposite to other members of the KLF family (they are transcriptional activators). KLF4 may regulate the expression of genes, which have a pivotal role in crucial cell processes, including cell proliferation/ differentiation and stem cells programming (Yet et al., 1998). SOX family consists of twenty genes encoding transcription factors characterized by conserved high mobility group domain which is involved in DNA-binding. SOX genes are essential in sex determination, embryo development, and maintenance of stem cell status (Sarkar and Hochedlinger, 2013). In normal conditions, c-MYC transcription factor plays an important role in cell proliferation regulation, differentiation, and apoptosis, as well as cell transformation (Sodir et al., 2011).

In addition, a teratoma was formed by transferring iPSCs into the subcutaneous tissue of a nude mouse and cells were able to differentiate into the tissues of three germ layers (Takahashi and Yamanaka, 2006). Later, Abad and colleagues demonstrated that full reprogramming can also occur *in vivo* by transitory induction of the four factors OCT4, SOX2, KLF4 and c-MYC in mice which resulted in teratomas emerging from multiple organs (Abad et al., 2013). Furthermore, microinjection of these factors into a mouse blastocyst led to the

production of a chimera mouse, indicating these were germ line factors that were maintained in the next generation. These findings suggested that the pluripotency of these cells was equivalent to that of ESCs (Okita et al., 2007). Since then, direct reprogramming has been widely studied by introducing these transcription factors or various other factors. The pluripotent stem cells are induced by various somatic cells, including fibroblasts, blood cells and keratinocytes (Loh et al., 2006; Grinnell et al., 2007; Duinsbergen et al., 2008; Huangfu et al., 2008; Kim et al., 2008b; Ebert et al., 2009; Kim et al., 2009).

It is unclear how the introduction of these transcription factors generates iPSCs equipped with the same functions of ESCs. Recent reports suggest that OCT4, SOX2 and NANOG may downregulate the expression of genes involved in the induction of differentiation of cells to maintain their undifferentiated properties and all four factors (including c-MYC) cause the epigenetic changes, such as the chromatin modification and DNA methylation, that generate iPSCs. The promoter regions of *NANOG* and *OCT4* genes were reported to be demethylated in iPSCs as well as in ESCs (Takahashi and Yamanaka, 2006).

1.1.2.2.3 Adult stem cells (ASCs)

Adult stem cells (ASCs), like all stem cells, share at least two characteristics. First, they can make identical copies of themselves for long periods of time; this ability to proliferate is referred to as long-term self-renewal. Second, they can give rise to mature cell types that have characteristic morphologies (shapes) and specialized functions. Typically, stem cells generate an intermediate cell type or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell.

ASCs can be organised into three groups regarding their original layer: ectoderm, mesoderm and endoderm. Ectodermal cells will give rise to skin and neurons; mesodermal cells will generate cardiac, muscle, blood and bone cells; and

endodermal cells will produce visceral cells, such as pancreatic, lung, kidney or thyroid cells.

Friedenstein and colleagues were the first in successfully isolate mesenchymal stem cells (MSCs) which come from the mesoderm (Friedenstein et al., 1970). MSCs can be isolated from dental pulp, periodontal ligament (Gronthos et al., 2000), deciduous teeth (Miura et al., 2003), periosteal (Nakahara et al., 1991), synovial membrane (De Bari et al., 2001), muscle (Bosch et al., 2000), adipose tissue (Zuk et al., 2002) and trabecular bone (Tuli et al., 2003). MSCs are able to produce cells from the mesodermal layer, such as chondrocytes, osteoblasts, adipocytes (Pittenger MF et al., 1999), myoblasts (Wakitani et al., 1995), tendon cells (Altman et al., 2002), odontoblasts (Gronthos et al., 2000; Miura et al., 2003) and cementoblasts (Kemoun et al., 2007).

Alternatively, ASCs may differentiate into a tissue that would arise from a different germ layer. In this case, the cells would be deemed to show plasticity or pluripotency (Song and Tuan, 2004). For example, MSCs may differentiate into ectoderm-derived neural cells (Azizi et al., 1998; Kopen et al., 1999).

1.1.3 Human dental pulp stem cells (hDPSCs)

In the present study, we used dental pulp stem cells from healthy human donors (hDPSCs). Teeth are originated between both ectoderm and mesoderm layers (Lumsden, 1988). The ectodermal portion will give rise to the enamel, while mesodermal portion will produce dentin, pulp and cement tissues (Ruch, 1985; Thesleff and Sharpe, 1997). The dental pulp, a soft tissue found within the core of the tooth, consists primarily of connective tissue, an extensive blood capillary system, nerves, fibroblasts, odontoblasts, and immune cells. The primary function of the pulp is to form dentin through the actions of odontoblasts, although nutritional (Vongsavan and Matthews, 1992), immune defence (Jontell et al., 1998), and sensory roles (Holland, 1994) are also apparent.

hDPSCs express multiple stem cell markers including mesenchymal, and embryonic markers (Patel et al., 2009; Govindasamy et al., 2010; Guimaraes et al., 2011; Osathanon et al., 2011). *In vitro*, hDPSCs can be readily induced to differentiate into mesenchymal lineage cell types of osteoblasts/odontoblasts, chondrocytes and adipocytes (Gronthos et al., 2002; Zuk et al., 2002; Zhang et al., 2006; Grottkau et al., 2010). Additional studies have also indicated the potential for myogenic (Zhang et al., 2006; Kerkis et al., 2008; Nakatsuka et al., 2010), melanocytic (Stevens et al., 2008), neuronal (Woodbury et al., 2000; Gronthos et al., 2002; Zuk et al., 2002; Zhang et al., 2006), and hepatocytic (Ishkitiev et al., 2012) differentiation.

1.1.4 hDPSCs isolation

hDPSCs isolation can be achieved by enzymatic digestion of the dental pulp tissue. Digestion enzymes are proteins that brake intercellular junctions of the connective tissue that support tissues, thus releasing cells. Pulp tissue consists of a heterogeneous population of cells: stromal cells, vascular cells, endothelial and perivascular cells, nerve cells, mastoid cells, T lymphocytes and macrophages, all within an extracellular matrix rich in collagen, fibronectin and laminin which are the main substrate of matrix metalloproteinases (MMPs), especially interstitial collagenases (Goldberg and Lasfargues, 1995; Nakata et al., 2000). MMPs participate in the degradation and replacement of the extracellular matrix of all body tissues including bone, enamel and dentin.

Interstitial collagenases are one of the most used groups for pulp tissue digestion. Collagenase MMP-1, 8 and 13 initiate the degradation of collagen I, II and III, which continue their degradation by other MMPs and non-MMPs proteolytic enzymes. These enzymes depend on cofactors such as calcium and zinc to exert their action; collagen degradation. The resulting collagen fragments are easily denatured and transformed into gelatine. Once gelatine is formed, MMP-2 and MMP-9 degrade it in thousands of tiny fragments.

Other types of metalloproteinases can be used, such as dispase II (Gronthos et al., 2000; Laino et al., 2005; Papaccio et al., 2006; Koyama et al., 2009; Waddington et al., 2009) and thermolysin (Perry et al., 2008; Woods et al., 2009). Dispase is a neutral metalloproteinase from *Bacillus polymyxa* which requires calcium for its activity. This enzyme cuts fibronectin and collagen IV, both components of the dense lamina and anchoring fibrils of the basement membrane.

Thermolysin is an extracellular metalloproteinase, isolated from gram-positive bacteria *Bacillus thermoproteolyticus* (Klopman and Bendale, 1989). It requires a zinc ion for its catalytic activity and four calcium ions for its thermal stability. In comparison with other proteins that are denatured against heat, thermolysin does not undergo any conformational change to at least 70°C (Matthews et al., 1974). This enzyme is specific for cleaving peptide bonds containing hydrophobic amino acid residues such as L-leucine, L-isoleucine, valine and L-phenylalanine, and furthermore it is especially useful for the partial hydrolysis of polypeptides which do not contain arginine or lysine. The mechanism of action for thermolysin was proposed by Matthews in 1988, which suggests, from a series of structural studies, that the zinc ion of the native thermolysin forms a coordination complex with three ligands provided by the protein and a fourth ligand provided by a water molecule in which the zinc ion has two functions: polarizing the carbonyl group of the substrate and facilitating the deprotonation of the nucleophilic water.

Another complementary method consists of a chemical disaggregation of the pulp tissue with ethylene diamine tetra acetic acid (EDTA) at a concentration of 0.02% (w/v). It is a divalent cation chelator in phosphate buffered saline or Krebs buffer without calcium and magnesium and is also used together with trypsin to increase disintegration.

1.1.5 *In vitro* stem cell culture

Dental hDPSCs have become a vital tool in medicine thanks to their easy retrieval and the possibility of maintaining them in culture to expand them. In order to be able to carry out any therapy with stem cells, it is necessary to have a very high number of cells. Most mesenchymal stem cells have a limited life-span *in vitro*, which means that they can only expand to a certain limit of cell divisions before they enter a senescence state and cease to proliferate.

Many scientists have reported that the long-term culture of stem cells is accompanied by a morphology known as "fried egg", and is characterized by a reduction in the ability to differentiate. For this reason, the senescent state must be taken into account in order to obtain good quality stem cells that can be used in cell therapies.

1.1.5.1 Oxygen tension influence on stem cell culture

Most conventional *in vitro* cell cultures are performed under ambient oxygen concentration (20-21% pO_2/160 mm Hg), which is often referred to as “normoxia”. In contrast, *in vivo* mesenchymal stem cells (MSCs) are not exposed to such a high concentration of oxygen. MSCs are developed in environments with low oxygen tension, that ranges between 1 and 7% pO_2 in the bone marrow (Chow et al., 2001; Harrison et al., 2002), 10-15% pO_2 in the adipose tissue (Bizzarri et al., 2006) and 13-18% pO_2 in blood and lungs (Steurer et al., 1997).

Mean values of 3-6% pO_2 (20-40 mm Hg) are commonly accepted in most tissues, including dental pulp, although the actual concentration of oxygen *in situ* depends on tissue vascularization and its metabolic activity (Ward, 2008). This fact suggests that *in vitro* cell culture should be performed under low oxygen tension conditions to mimic their natural physiological environment. El Alami and colleagues determined the growth rate of hDPSCs under physiological oxygen tension, and investigated the cell signalling pathways underlying the decreased stem cell proliferation during routine culture under ambient oxygen tension (El

Alami et al., 2014). Moreover, culturing hDPSCs under ambient oxygen tension conditions entails an oxidative stress-induced premature senescence (Mas-Bargues et al., 2017).

It is interesting to note that cultivating MSCs under conditions of hypoxia, or rather, physiological "normoxia", prior to transplantation improves tissue regenerative potential (Rosova et al., 2008).

1.2 OXIDATIVE STRESS

1.2.1 Definition

Oxidative stress is defined as an alteration of the balance between prooxidant species and antioxidants, in favour of the former (Sies, 1985).

Despite the physiological role of some reactive oxygen species, they can also lead to undesired oxidation reactions, against which organisms have had to develop antioxidant defences (Halliwell, 1996). The formation of a certain number of free radicals is a normal and inevitable process since they are the product of an infinity of chemical reactions essential for the cellular life. These reactive species do not cause oxidative damage under normal conditions because the cell is provided with a large amount of antioxidant mechanisms (Slater, 1984).

When there is an imbalance between prooxidant and antioxidant substances in favour of the former, the result is oxidative damage, which can affect various molecules, and which can be reflected in their physiological functions. Thus, oxidative stress can be caused by an excess of pro-oxidants, a deficiency of antioxidant agents, or by both factors at the same time.

1.2.2 Free radicals

Free radicals are chemical species that contain one or more unpaired electrons in its valence layer, which makes these species to have a high reactivity (Fridovich, 1978). They are characterized by their great oxidizing power and their

very short life (Simic and Taylor, 1988).

These chemical species are abundant in living systems. Moreover, some authors in their study on the origin and evolution of life propose free radicals as one of the causes of the origin of life in our planet (Harman, 2001). Thus, on the one hand oxygen gives rise to life and on the other hand, because of its ability to form different free radicals, it is capable of damaging essential structures for its development, such as DNA, proteins, carbohydrates and lipids (Sies, 1983). In addition, free radicals participate in physiological processes such as aging (Pacifici and Davies, 1991) and exhausting physical exercise (Davies et al., 1982; Sastre et al., 1992).

In nature, free radicals are mostly compounds derived from oxygen and are called reactive oxygen species (ROS). These species are either highly reactive or capable of giving rise to reactive species. Some of them are real free radicals derived from oxygen, such as the hydroxyl radical. Others, like H_2O_2, are not really radical in the strict sense of their definition. In addition, there are other non-oxygen-derived radical species that are taking interest in recent years, such as those derived from nitrogen (RNS).

ROS may have an exogenous or endogenous origin (Finkel and Holbrook, 2000). Endogenous ROS are produced inside the cell. Many biological processes release ROS as waste products, via cellular signalling pathway or as a defence mechanism (Morgan and Liu, 2011). Among them, we can include the Fenton-Haber-Weiss reaction, the nicotinamide adenine dinucleotide phosphate reduced family (NADPH oxidases), peroxisomes, cytochrome p450, cytokines, growth factors, lipooxygenase, cyclooxygenase and, above all, mitochondrial electron transport chain (Finkel and Holbrook, 2000; Balaban et al., 2005) It is composed of a series of proteins with redox capacity that reduce molecular oxygen until the formation of a molecule of water. This reaction is coupled to oxidative phosphorylation, in which energy is produced in the form of adenosine triphosphate (ATP).

Exogenous ROS come from sources outside the body, such as diet, xenobiotics, tobacco smoke, pollution, ionizing radiation and ultraviolet light. Regarding *in vitro* stem cell culture, the main exogenous ROS are those generated due to "hyperoxia". In fact, high oxygen tension can cause oxidative stress through the production of ROS that can damage lipids, proteins and DNA (Wiseman and Halliwell, 1996). Therefore, moderate hypoxia may lower the intracellular generation of ROS as well as its accumulation (Miller et al., 1987) (See section 1.2.5).

1.2.2.1 The "Free Radical Theory of Aging"

The "Free Radical Theory of Aging" was formulated by Harman in 1956 and it proposes that free radicals derived from oxygen are responsible for the oxidative damage that occurs with age at the cellular and tissue level (Harman, 1956). Antioxidant systems are not able to cope with all the ROS that are generated continuously throughout the life of the cell, which ends up causing oxidative damage in it, and by extension, on the tissues.

There are many experimental evidences in favour of this theory. Old animals have higher oxidation rates than young ones, and have oxidized proteins, oxidized forms of DNA and lipids accumulation (Stadtman, 1992; Sohal et al., 1993; Hamilton et al., 2001; Bokov et al., 2004). In principle, this could be attributed to a higher rate of free radicals generation by old organisms.

Also, in favour of this theory, there are numerous studies carried out in various organisms, which suggest that the reduction of oxidative stress or the increase of resistance to it, is related to the prolongation of life (Miquel and Economos, 1979; Harman, 1982; Harrington and Harley, 1988; Phillips et al., 1989; Orr and Sohal, 1994; Parkes et al., 1998; Finkel and Holbrook, 2000; Melov et al., 2000; Ruan et al., 2002; Ishii et al., 2004; Huang et al., 2006; Zou et al., 2007; Kim et al., 2008a; Quick et al., 2008; Dai et al., 2009; Shibamura et al., 2009).

Although the free radical theory of aging is among the most studied and accepted of all the hypotheses of the aging mechanism, several studies have generated ambiguity and controversy of the same (Muller et al., 2007; Perez et al., 2009a; Perez et al., 2009b; Lapointe and Hekimi, 2010; Salmon et al., 2010). For example, the vast majority of studies in mice show no change in life-span after the increase or reduction of antioxidant enzyme activity (Huang et al., 2000). It has also not yet been shown to extend life in clinical trials in humans, who were given antioxidant substances (Howes, 2006; Bjelakovic et al., 2007).

1.2.2.2 Defence mechanisms: physiological antioxidants

Since humans have evolved in the presence of oxidizing substances, evolution has provided these organisms with systems capable of dealing with this type of reactive substances. An "antioxidant" is any substance that, when present in low concentrations compared to the oxidizable substrate, significantly decreases or inhibits the oxidation of this substrate (Sies, 1993; Halliwell and Gutteridge, 1995). From the point of view of cellular physiology, we can divide them into primary, secondary and tertiary antioxidants:

Primary antioxidants prevent the formation of new species of free radicals. These antioxidants act by converting existing free radicals into less harmful molecules, or by preventing their formation. This group includes superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase and metal binding proteins that limit the availability of iron necessary for the formation of the OH^- (Halliwell and Gutteridge, 1986).

Secondary antioxidants are non-enzymatic scavengers that act when there is a free radical overproduction and enzyme systems are overflowing, thus preventing chain reactions. It includes glutathione, vitamin E, vitamin C, uric acid, bilirubin and albumin (Halliwell and Gutteridge, 1990).

Tertiary antioxidants repair biomolecules damaged by free radicals. Among them, intracellular proteolytic systems act to degrade oxidatively damaged proteins, thus avoiding their accumulation (Davies et al., 1987b; Pacifici and Davies, 1991). We can also highlight the DNA repair enzymes, methionine sulfoxide reductase and phospholipase-A2 that cuts the oxidized phospholipids of the membrane (Sevanian et al., 1985).

1.2.3 Oxidative stress and biomolecules damage

1.2.3.1 Oxidative damage to lipids

Among the main types of biomolecules, lipids, and especially polyunsaturated fatty acids, are the most susceptible of being attacked by free radicals (Cheeseman and Slater, 1993), such as the hydroxyl ($HO^{\cdot}$), peroxide (ROO•), alkoxy (RO•) and alkyl (R•) radicals, which are the main generators of oxidative lipid damage.

The process of oxidative damage to lipids, termed lipid peroxidation, begins when a free radical attacks a carbon in the aliphatic chain of a fatty acid, a hydrogen atom is released, and an alkyl radical is formed (Halliwell, 1994; Krinsky, 2012). This reaction is preferably produced in the carbons contiguous to double bonds of the polyunsaturated fatty acids, since the formed radicals can be stabilized by resonance with the double bond. Peroxide radicals can react with side chains of other polyunsaturated fatty acids, thereby spreading the radical chain reaction (Halliwell, 1994).

Thus, a single attack by a free radical results in the formation of a large number of oxidation products, in particular aldehydes, mainly malondialdehyde (MDA), and isoprostanes, 4-hydroxynonenal, and short chain hydrocarbons such as ethane and pentane (Freeman and Crapo, 1982; Cheeseman and Slater, 1993). Many of the aldehydes formed react rapidly with the cellular components causing DNA mutations, and produce structural and functional damage by reacting with proteins (Krinsky, 2012).

Lipid peroxidation is considered a very important factor in the aging of aerobic cells (Lippman, 1985), because oxidative damage to lipids of the membrane is an important factor in the decrease of the fluidity of the membranes, and therefore will affect its functionality (Shigenaga et al., 1994). MDA is a very reliable marker of the lipid peroxidation profile.

1.2.3.2 Oxidative damage to proteins

All the amino acids present in the proteins have residues susceptible of being attacked by the free radicals, mainly by the hydroxyl radical (Stadtman, 1992). Within the physiological amino acids, tyrosine, phenylalanine, tryptophan, histidine, methionine and cysteine are the most oxidative processes that suffer (Davies et al., 1987a).

In the process of oxidative damage to proteins, some amino acids such as lysine, proline and arginine are oxidized to carbonyl groups, so that the carbonyl content of proteins can be used as an indicator of oxidative damage to them. Other amino acids such as histidine, cysteine and methionine, also undergo oxidative damage, but do not form carbonyl derivatives (Stadtman, 1992).

As a consequence, protein exposure to free radicals can lead to a conformational change of the protein, thus producing modifications in its primary, secondary and ultimately tertiary structure, which, in turn, can lead to a loss or modification of its biological function, which is usually irreversible and may cause the protein denaturation (Dean et al., 1993). In addition, if such dysfunctional proteins are not degraded, the hydrophobic residues may form protein aggregates which give rise to the protein complex known as lipofuscin (Hohn et al., 2013).

1.2.3.3 Oxidative damage to DNA

We can find more than twenty by-products after an oxidative attack to DNA. Among them, the oxidation of 2-deoxyguanosine to 8-hydroxy-2-deoxyguanosine (8-oxo-dG) is one of the most frequent lesions, and is of great importance because

of its high mutagenic effect, since during replication it will produce purine transformations (Kasai and Nishimura, 1984).

Oxidative damage associated with proteins and DNA should not be considered independently. The accumulation of inactive forms of reparative enzymes may increase the accumulation of oxidative damage in the DNA, so that they can enhance each other. When replication of damaged DNA occurs prior to repair or when damaged DNA is repaired incorrectly, a mutation occurs (Breen and Murphy, 1995). Therefore, oxidative lesions to DNA appear to be involved not only in cell aging, but also in the pathogenesis of diseases associated with advanced age.

Mitochondrial DNA (mtDNA) suffers much more oxidative damage than nuclear (Richter et al., 1988), since it presents certain traits that make it especially susceptible to be attacked by oxidizing agents: it lacks histones that can receive the attack instead of the DNA (Johns, 1995), the repair system is less effective (Suter and Richter, 1999) and, it is very close to the mitochondrial electron transport chain, one of the main production systems of reactive oxygen species (Giulivi and Davies, 1993). Another distinctive factor of mtDNA is that it has no introns, so that modification of any base usually affects a zone of coding DNA (Ames et al., 1993) and its impact is therefore more important.

1.2.3.4 Oxidative damage to carbohydrates

Carbohydrates, such as mannose and mannitol, react easily with the hydroxyl radical for its removal. Monosaccharides and disaccharides resist the action of ROS. Glucose constitutes a sensor of the superoxide radical, by holding it and preventing its action on other molecules. Therefore, it has been observed that various polysaccharides act as cellular protective agents (Albertini et al., 1996).

Oxidative damage to carbohydrates is important when it comes to structural function polysaccharides, since polysaccharides are depolymerized by free radicals leading to degenerative processes. A special case is that of hyaluronic acid whose

structural function is to maintain the viscosity of the synovial fluid. It has been observed that superoxide dismutase is able to protect against the depolymerization of hyaluronic acid in the synovial fluid (McCord, 1974). Proteoglycans are subject to similar oxidative bursting (Greenwald and Moy, 1980).

1.2.4 Oxidative stress markers

Given the importance of the damage that oxidative stress can cause in cells and in the organism, in recent years we have tried to find indices that allow us to measure it. Among the proposed indicators, one of the most relevant is the oxidized / reduced glutathione ratio (GSSG/GSH) characteristic of oxidative stress, so that an imbalance causes an alteration of the cellular redox state (Sies, 1986).

In addition to this ratio, there are specific markers that allow measuring oxidative damage to the aforementioned biomolecules. Malondialdehyde (MDA) and hydroxynonenal (HNE) are the most commonly used to measure lipid damage, although pentane and ethane levels can also be considered. 8-oxo-dG is an indicator of oxidative DNA damage, and carbonyl groups and 2-oxohistidine are used as markers of oxidative damage in proteins (Hageman et al., 1992).

On the other hand, mitochondria play a key role in the genesis of free radicals. Mitochondrial membrane potential ($\Delta\Psi m$) is critical to maintaining the physiological function of the respiratory chain to generate ATP. Mitochondrial dysfunction supposes a collapse of the respiratory chain and therefore an increase in ROS production. Several fluorescent probes can be used to determine mitochondrial membrane potential in a variety of cell types, as well as direct determination of free radicals.

The four markers used to measure oxidative stress used in this study are described in the Methods section: Dihydrorodamine-123 (DHR123) for ROS production, the methyl ester tetramethylrodamine (TMRM) as an indicator of mitochondrial membrane potential, malondialdehyde (MDA) for lipid peroxidation and carbonyl groups for protein damage.

1.2.5 Oxidative stress and stem cells

The common cell culture is performed at 21% pO_2 (ambient oxygen tension) which is far from the physiological "normoxia" of adult stem cells, and as expected, is accompanied by an increase in ROS production and creates an oxidative stress environment. "Hyperoxia" has been described as an important destabilizing factor of cellular redox homeostasis (Fan et al., 2008).

High oxygen tension can cause oxidative damage via production of free radicals, damaging lipids, proteins and DNA (Wiseman and Halliwell, 1996). Culturing under ambient oxygen tension has a negative impact on the physiological function of stem cells: cell proliferation and migration are affected (Rodrigues et al., 2010), as well as osteogenic differentiation potential (Hung et al., 2012). p38MAPK, p21 and Nrf-2 signalling pathway has been described as the link between the oxidative stress induced by culture at 21% pO_2 and the lower proliferation rate of hDPSCs (El Alami et al., 2014).

It has also been reported that the reduction of oxygen levels leads to a decrease in intracellular levels of oxidative stress as well as oxidative damage. This is accompanied by a higher proliferation rate, suggesting that oxidative stress is a contributing factor in cellular senescence (Ho et al., 2007). On the other hand, culture at 3% pO_2 increases mRNA levels as well as protein levels of OCT4, SOX2 and c-MYC transcription factors in stem cells of dental origin (Zhou et al., 2014).

In other words, long-term culture under conditions of physiological "normoxia", without oxidative stress, has been shown to maintain the mesenchymal cell phenotype and to prevent senescence (Bigot et al., 2015).

1.3 CELLULAR SENESCENCE

1.3.1 Definition

Cellular senescence was formally described when Hayflick and colleagues showed that normal cells had a limited ability to proliferate in culture (Hayflick and Moorhead, 1961; Hayflick, 1965). These experiments showed that human fibroblasts initially underwent robust cell division in culture. However, gradually (over many cell doublings) cell proliferation declined. The non-dividing cells remained viable for many weeks, but failed to grow despite the presence of ample space, nutrients and growth factors in the medium.

Soon after this discovery, the finding that normal cells do not indefinitely proliferate spawned two important hypotheses. The first hypothesis stemmed from the fact that many cancer cells proliferate indefinitely in culture. Cellular senescence was proposed to be an anti-cancer or tumour-suppressive mechanism. In this context, the senescence response was considered beneficial because it protected organisms from cancer. The second hypothesis stemmed from the fact that tissue regeneration and repair deteriorate with age. Cellular senescence was proposed to recapitulate the ageing, or loss of regenerative capacity, of cells *in vivo* (Campisi and d'Adda di Fagagna, 2007). In fact, cancer and longevity require a durable cell proliferation potential and, therefore, those mechanisms that limit indefinite proliferation provide cancer protection but favour ageing (Serrano and Blasco, 2007).

1.3.2 Cell cycle

The cell cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. The stages are G_1-S-G_2-M (Figure 1.3). The G_1 stage stands for "GAP 1". The S stage stands for "Synthesis", where DNA replication occurs giving rise to a pair of sister chromatids linked by proteins called cohesins (Nasmyth et al., 2000). The G_2 stage stands for "GAP 2". The M stage stands for "mitosis", and it is when nuclear (chromosomes separate) and

cytoplasmic (cytokinesis) division occur. Mitosis is further divided into 4 phases, prophase, metaphase, anaphase, and telophase.

1.3.2.1 Cell cycle checkpoints

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity (Gardner and Burke, 2000). In addition, checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair. Checkpoint loss results in genomic instability and has been implicated in the evolution of normal cells into cancer cells. Recent advances have revealed signal transduction pathways that transmit checkpoint signals in response to DNA damage (Elledge, 1996).

Before initiating DNA replication, cells in the G_1 phase of the cycle may enter a resting phase known as G_0. G_0 cells are not in the state of growth or proliferation (Vermeulen et al., 2003). When cells are in G_0 phase, they are commonly named as quiescent. This cell cycle arrest is reversible and in the presence of the appropriate signals, these cells can re-enter the cycle and continue their proliferation (Blagosklonny, 2011).

1.3.2.2 Cell cycle regulation

Cyclin-dependent kinases (CDKs) are serine/threonine kinases and their catalytic activities are modulated by interactions with cyclins and CDK inhibitors (CDKIs). Close cooperation between this trio is necessary for ensuring orderly progression through the cell cycle. In addition to their well-established function in cell cycle control, it is becoming increasingly apparent that mammalian CDKs, cyclins and CDKIs play indispensable roles in processes such as transcription, epigenetic regulation, metabolism and stem cell self-renewal (Lim and Kaldis, 2013). Each stage of the cell cycle is regulated by different cyclins, CDKs and

CDKIs (Figure 1.3).

Cyclins activate CDKs by binding to them. There are multiple CDK-cyclin complexes that play specific roles at various phases in the cell cycle. These complexes include three interphase CDKs (CDK2, CDK4, and CDK6), one mitotic CDK1, and ten cyclins belonging to four different classes (A-, B-, D-, and E-type cyclins).

CDK activity is regulated by two types of inhibitors: INK4 proteins (INK4a, INK4b, INK4c, INK4d) and Cip/Kip family proteins (p21, p27, and p57) (Sherr and Roberts, 1995). Together, these cell cycle inhibitors function as a brake system that inhibit proliferation in multiple tissue types.

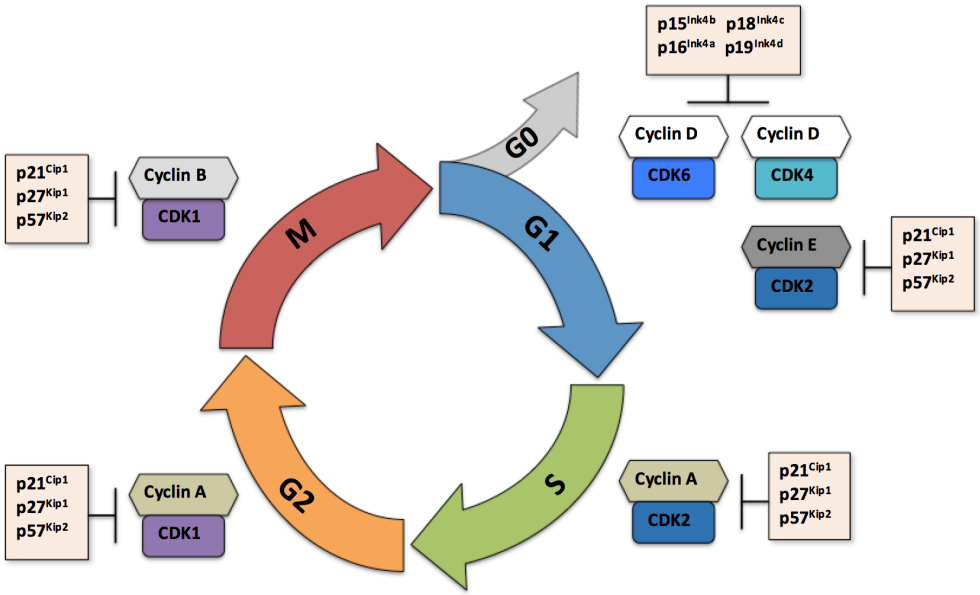

Figure 1.3 Cell cycle regulation checkpoints.
Taken from: Hochegger et al. (2008).

1.3.3 Senescence *versus* quiescence

Senescence is thought to differ from less-durable forms of cell cycle arrest (for example, quiescence) in several ways (Table 1.1). When mitogen-dependent dividing cells are deprived of extracellular growth factors or exposed to anti-proliferative cytokines or contact inhibition, they exit the cell cycle into a non-proliferating quiescent state (G_0). Although G_0 and G_1 phase cells cannot be distinguished by DNA content, quiescent cells comparatively produce reduced numbers of ribosomal RNA (rRNA) and proteins, have decreased metabolic activity, do not express G_1 CDK activities (Pardee, 1989; Sherr and Roberts, 1995).

Table 1.1 Senescence *versus* quiescence.

	SENESCENCE	QUIESCENCE
Growth arrest	Permanent	Transient
DNA content	Diploid or Tetraploid	Diploid
Metabolism	High	Low
Molecular effectors	$p16^{INK4a}$, pRb $p14^{ARF}$, p53 and $p21^{Cip1}$	$p21^{Cip1}$, $p27^{Cip2}$
Markers	SA-β-Gal, $p16^{INK4a}$, DNA damage response, SASP and SAHF	None

Taken from: Sharpless and Sherr (2015).

By contrast, adherent senescent cells emerging in culture in response to stress display an enlarged cell size and increased biomass, and they produce abundant stress granules (Kuilman et al., 2010; Rodier and Campisi, 2011; Campisi, 2013). Quiescent and senescent cells can be viably maintained in cell culture even after months of cell cycle arrest, but quiescent cells can re-enter the

cell cycle in response to mitogenic and developmental cues, whereas senescent cells cannot (Serrano et al., 1997; Beausejour et al., 2003; Blais et al., 2007; Burkhart and Sage, 2008; Indovina et al., 2013; Kareta et al., 2015).

1.3.4 Senescence biomarkers

Adherent senescent cells attached to plastic culture dishes undergo morphological alterations, such as flattening, vacuolization and accumulation of stress granules (Kuilman et al., 2010; Rodier and Campisi, 2011; Campisi, 2013). Increases in cell size relative to proliferating cells in culture may reflect a continuation of anabolic processes, such as protein and membrane synthesis, in senescent cells that have exited the cell cycle.

Senescent cells routinely express senescence-associated β-galactosidase (SA-β-Gal) and $p16^{INK4a}$, and most secrete inflammatory cytokines and other signalling molecules — including interleukin-1 (IL-1), IL-6, IL-8, vascular endothelial growth factor A (VEGFA) and matrix metalloproteinases (MMPs) — as part of a senescence-associated secretory phenotype (SASP) (Coppe et al., 2008; Rodier et al., 2009). Human cells undergoing senescence exhibit an unusual pattern of heterochromatin that is present in discrete nuclear subdomains, known as senescence-associated heterochromatic foci (SAHFs), which are associated with S-phase-promoting gene loci, such as E2F target genes (Narita et al., 2003). HP1-γ has proven to be a positive marker of SAHF in adenomas but negative in adenocarcinomas, suggesting that senescent cells exist in premalignant tumours but not in malignant ones (Collado et al., 2005).

Collado and colleagues also analysed some *de novo* markers identified by using DNA microarray analysis in order to detect oncogene-induced senescence. These *de novo* markers were $p15^{INK4b}$, Dec1 and DcR2 (Collado et al., 2005).

The next figure provides a brief description of the most commonly used markers of cellular senescence (Figure 1.4).

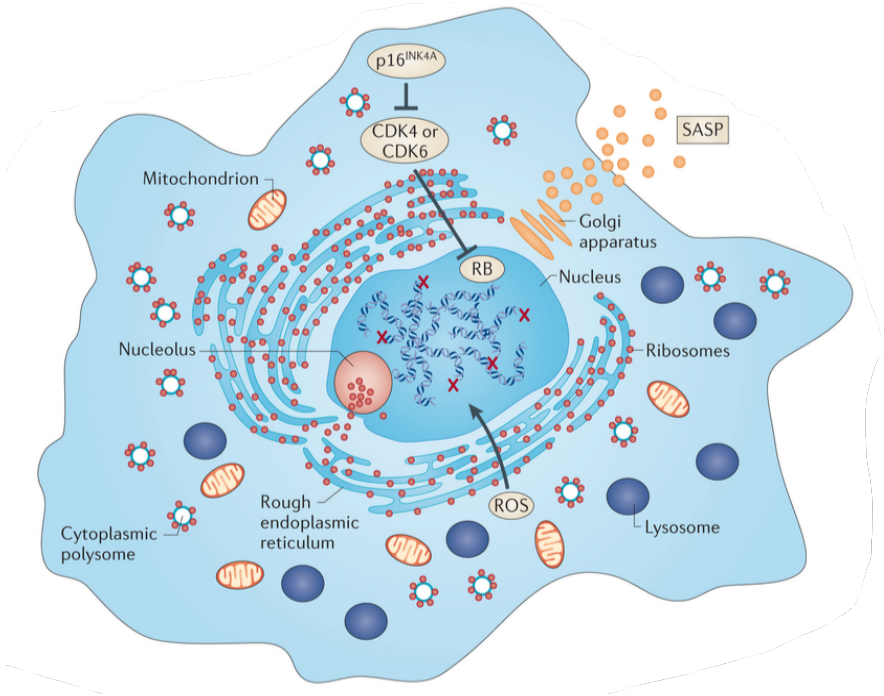

Figure 1.4 Biomarkers of senescence.
Taken from: Sharpless and Sherr (2015).

This schematic figure depicts different damaged organelles, including: the nucleus, containing damaged chromatin (SAHF); mitochondria with internal cristae producing ROS; and abundant lysosomes expressing β-galactosidase. Vesicles emanating from the Golgi apparatus contain secreted cytokines and chemokines which is known as the senescence-associated secretory phenotype (SASP), leading to paracrine signalling.

The cyclin-dependent kinase (CDK) inhibitor $p16^{INK4a}$ in the cytoplasm prevents CDK4 and CDK6 from assembling into functional holoenzymes with their allosteric regulators, the D-type cyclins. Thus, preventing the import of active kinases into the nucleus and inhibiting the phosphorylation of nuclear retinoblastoma protein (pRb) (Sharpless and Sherr, 2015).

1.3.4.1 Hypertrophic phenotype

Hayflick already stated that senescent cells show an enlarged cytoplasm that doubles the size of non-senescent cells (Hayflick, 1965). The typical senescence phenotype consist of enlarged cell with multiple or enlarged nuclei, prominent Golgi apparatus and sometimes a vacuolated cytoplasm (De Cecco et al., 2011).

1.3.4.2 Growth arrest

The hallmark of cellular senescence is an inability to progress through the cell cycle. Senescent cells arrest growth, usually with a DNA content that is typical of G_1 phase, yet they remain metabolically active. Once arrested, they fail to initiate DNA replication despite adequate growth conditions. This replication failure is primarily caused by the expression of dominant cell cycle inhibitors. In contrast to quiescence, the senescence growth arrest is essentially permanent because senescent cells cannot be stimulated to proliferate by known physiological stimuli (Campisi and d'Adda di Fagagna, 2007).

1.3.4.3 Apoptosis resistance

Apoptosis entails the controlled destruction of cellular constituents and their ultimate engulfment by other cells. Like senescence, apoptosis is an extreme response to cellular stress and is an important tumour-suppressive mechanism. But, whereas senescence prevents the growth of damaged or stressed cells, apoptosis quickly eliminates them (Roat et al., 2007).

It is not clear what determines whether cells undergo senescence or apoptosis. One determinant is cell type; i.e., damaged fibroblasts and epithelial cells tend to senesce, whereas damaged lymphocytes tend to undergo apoptosis. The nature and intensity of the damage or stress may also be important (Roux and Blenis, 2004). Most cells are capable of both responses. The senescence and apoptosis regulatory systems therefore communicate — probably through their

common regulator, the p53 tumour suppressor protein (Thakur et al., 2007).

1.3.4.4 β-Galactosidase activity

The most commonly used senescence marker, primarily because of its ease of detection in tissues, is senescence-associated β-galactosidase (SA-β-Gal) activity measured at pH 6.0 (Dimri et al., 1995). Endogenous β-galactosidase in humans is a lysosomal enzyme optimally active at pH 4.0–4.5, so its detection at suboptimal pH 6.0 connotes its very high level of expression in senescent cells (Kurz et al., 2000; Lee et al., 2006a).

This activity at pH 6.0 allowed the identification of senescent fibroblasts and keratinocytes in aged human skin biopsies, and subsequently became known as senescence-associated β-galactosidase (SA-β-Gal). Several laboratories have since then, used the SA-β-Gal assay on a variety of cells and tissues to demonstrate the onset of replicative senescence in culture (Reznikoff et al., 1996; Serrano et al., 1997; Bodnar et al., 1998; Tsukamoto et al., 1998; van der Loo et al., 1998; Matsunaga et al., 1999) and *in vivo* (Mishima et al., 1999; Sigal et al., 1999).

1.3.4.5 $p16^{INK4a}$ expression

The second most commonly used marker is the expression of $p16^{INK4a}$, a selective inhibitor of cyclin D-dependent CDK4 and CDK6 (Serrano et al., 1993). The *CDKN2A* gene encoding $p16^{INK4a}$ is closely chromosomally linked to *CDKN2B*, which encodes a second INK4 family member, $p15^{INK4b}$ (Figure 1.5). Moreover, RNAs specified by exons 2 and 3 of the *CDKN2A* gene are co-opted into distinct transcripts originating from another upstream promoter and exon, where their coding sequences are translated in an alternative reading frame (ARF) (Quelle et al., 1995); the resulting protein is $p14^{ARF}$ in human and $p19^{ARF}$ in mouse. The $p16^{INK4a}$ and $p15^{INK4b}$ proteins inhibit cyclin D-dependent CDK4 and CDK6 to prevent phosphorylation pRb. The hypophosphorylated form of pRb sequesters E2F transcription factors, preventing them from coordinately activating a suite of

genes that are required for DNA replication. The ARF protein binds to the MDM2 E3 ubiquitin ligase to prevent p53 polyubiquitylation and to facilitate p53 activation. In turn, the p53 transcription factor regulates an extensive group of genes that are commonly induced by cellular stress (Levine, 1997). These include the CDK2 inhibitor $p21^{CIP}$, which inhibits CDK2-mediated pRb phosphorylation during progression through the G_1 phase of the cell division cycle (Weinberg, 1995).

The tumour suppressor pathways, $p14^{ARF}$/MDM2/p53 and $p16^{INK4a}$/pRb, have been shown to play critical roles in the induction of cellular senescence (Sherr and DePinho, 2000; Agherbi et al., 2009). However, the relative contributions of $p16^{INK4a}$ and $p14^{ARF}$ to senescence continue to be puzzling. $p19^{ARF}$ expression is the more critical determinant of replicative senescence of cultured mouse embryonic fibroblasts (Kamijo et al., 1997), whereas $p16^{INK4a}$ is a key regulator of *in vitro* senescence in human cells (Chandler and Peters, 2013). Notably, deletion and silencing of the entire *CDKN2A–CDKN2B* locus and mutations inactivating $p16^{INK4a}$ are among the most frequent genetic events encountered in malignant human tumours, implying that, as with inactivation of *RB1* and *TP53*, loss of *CDKN2A–CDKN2B* enables cells to bypass tumour-suppressive restraints that are imposed by senescence.

However, both pathways are connected. pRb and p53 pathways are linked to each other by $p21^{CIP1}$. $p21^{CIP1}$ inhibits CDK2-mediated pRb phosphorylation during progression through the G_1 phase of the cell division cycle (Sharpless and Sherr, 2015). Unlike the roles of pRb and $p16^{INK4a}$, the role of the CDK inhibitor $p21^{CIP1}$ in senescence and tumour suppression remains controversial (el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993). Expression of $p21^{CIP1}$ in response to transient DNA damage produces a reversible cell cycle pause that provides time for DNA repair and facilitates cell survival (Deng et al., 1995; Wang et al., 1997; Martin-Caballero et al., 2001). Furthermore, inactivation of $p21^{CIP1}$ does not abrogate senescence in commonly used *in vitro* model systems (Pantoja and Serrano, 1999).

By contrast, consistent with observations that $p21^{CIP1}$ expression has been associated with prolonged proliferative arrest occurring in the setting of chronic DNA damage (Di Leonardo et al., 1994), more than 4 days of experimentally enforced $p21^{CIP1}$ expression has been reported to initiate senescence (Sang et al., 2008). Activation of high levels of $p21^{CIP1}$ by stress-induced p53 during the G_2 phase also seems to facilitate senescence induction (Spencer et al., 2013; Krenning et al., 2014). Even if $p21^{CIP1}$ is important for the initiation of senescence in some settings, its expression does not persist in senescent cells (Alcorta et al., 1996; Robles and Adami, 1998; Stein et al., 1999). Therefore, $p21^{CIP1}$ cannot be used as a reliable marker of the senescence phenotype.

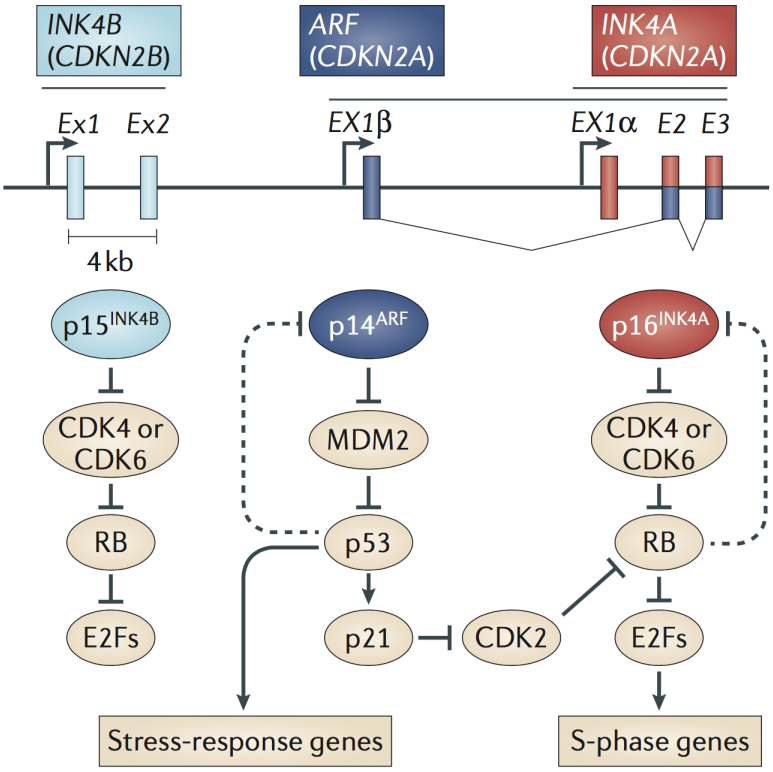

Figure 1.5 The *INK/ARF* locus (*CDKN2A-CDKN2B*).
Taken from: Sharpless and Sherr (2015).

1.3.4.6 Senescence associated secretory phenotype (SASP)

Cells undergoing senescence exhibit profound changes in their transcriptomes. A major consequence of this is the secretion of many dozens of factors, including cytokines and chemokines (Campisi, 2005). The first indication of changes in the secretome of human cells accompanying senescence was reported for fibroblasts undergoing replicative senescence. Microarray analysis revealed a strong inflammatory response, as seen in wound healing (Shelton et al., 1999). Subsequent work from various laboratories has revealed that cells undergoing either replicative or premature senescence display profound changes in their secretome, termed the senescence-associated secretory phenotype (SASP) (Coppe et al., 2008; Rodier et al., 2009).

It has been proposed that the normal function of the SASP is to restore tissue function in two ways: first, by stimulating less-damaged neighbouring cells to engage in tissue repair; and second, by attracting inflammatory cells to eliminate senescent cells and turn off SASP-mediated signals. However, this restorative process may fail when the extent, duration or frequency of the damage exceeds repair capacity. The end result is an aberrant accumulation of senescent cells that, contrary to their initial purpose, aggravate tissue dysfunction (Serrano, 2017a).

1.3.4.7 Senescence associated heterochromatin foci (SAHF)

Cellular senescence can be associated with an altered chromatin structure, at least *in vitro*. While DNA dyes display overall homogenous staining patterns in cycling or quiescent human cells, senescent cells often show strikingly different punctate staining patterns (Kuilman et al., 2010).

The organization of DNA in heterochromatin contributes to nuclear organization, chromosome structure, and gene silencing (Dillon and Festenstein, 2002; Lachner and Jenuwein, 2002). There are two types of heterochromatin: constitutive and facultative. The constitutive heterochromatin mainly comprises the pericentric regions of the chromosomes and is important for the segregation of

chromosomes and the silencing of repetitive elements. Facultative heterochromatin is controlled during development and contributes to gene regulation during differentiation. A different type of optional heterochromatin is then identified; the senescence associated heterochromatin foci (SAHF), which appears in the senescent cells (Parry and Narita, 2016).

These SAHF are specifically enriched in methylated lysine 9 of histone H3 (a modification catalyzed by the histone methyltransferase Suv39h1), while histone H3 lysine 9 acetylation and lysine 4 methylation (both euchromatin markers) are excluded from SAHF (Narita et al., 2003). Senescent cells display increased binding of heterochromatin-associated proteins in the promoters of several E2F target genes. SAHF formation is circumvented by interference with $p16^{INK4a}$/pRb pathway signalling, correlating with bypass of senescence.

Very little is known about the effector mechanism of cellular senescence, but the global chromatin reorganisation may not simply be a senescence marker, but rather play a key role in the senescence mechanism. In fact, there is a strong correlation between SAHF formation and the irreversibility of the senescence phenotype (Narita, 2007).

1.3.4.8 Telomere shortening

It is unquestionable that telomere shortening is a universal mechanism that limits the proliferative potential of normal cells lacking endogenous telomerase. Most human primary cells do not express high levels of telomerase and, therefore, are subject to a progressive erosion of their telomeres with each cell generation (Blasco et al., 1999). The inability of which to add telomeric repeats to chromosome ends eventually leads to telomere deprotection and a DNA damage response (DDR) that limits cellular proliferative lifespan (Harley et al., 1990; Blasco et al., 1997).

In turn, enforced expression of telomerase can bypass replicative senescence and maintain chromosomal integrity. However, whether senescence relies

exclusively or not on telomere shortening is still an open question. In fact, telomere shortening and dysfunction can occur in non-senescent cells, and senescence can be triggered by many stresses that are independent of telomere shortening in human cells *in vitro* (Sharpless and Sherr, 2015).

Thus, even though the role of telomere shortening as a barrier to immortalization is clearly established, the full effects of "telomerization" on senescence are still to be defined (Serrano and Blasco, 2001).

1.3.5 Oxidative stress-induced senescence

According to the theory of free radical aging (Harman, 1956), ROS appear as toxic species that cause oxidative damage to biomolecules, however, there are other points of view that consider ROS as essential for cell survival. In physiological conditions, ROS have a regulatory role in a wide range of functions, since they act as second messengers. These pathways include gene regulation, cell signalling, cell differentiation, cell senescence, and apoptosis among others. Under ROS overproduction conditions, signalling regulation can be altered and could lead to the activation of dangerous signalling pathways, as well as to premature cellular senescence and its drift in the aging (Afanas'ev, 2010; Hekimi et al., 2011).

Nowadays, ROS generate a great interest because they are involved in the cellular senescence process: elevated ROS levels are associated with replicative senescence by shortening telomeres and stress-induced senescence, directly damaging DNA and inducing a DNA damage response (DDR) (Campisi and d'Adda di Fagagna, 2007; Sohal and Orr, 2012). This increase leads to damage to DNA and DDR, forming a positive feedback loop that initiates senescence (Passos and Von Zglinicki, 2006; Passos et al., 2010). Before senescence begins, cells increase the mitochondrial network, as well as the number of mtDNA molecules and the mitochondrial production of radicals. Thus, the DDR is the onset of cellular senescence irrespective of the cause of origin and is capable of causing

mitochondrial dysfunction as a late consequence (Passos et al., 2007a; Passos et al., 2007b; d'Adda di Fagagna, 2008).

Mitochondrial dysfunction leads to a high production of ROS, both being the cause and effect of a continuous DDR, causing a wide range of changes as a result of the signalling through several transcription factors, such as p53 and $p16^{INK4a}$, necessary for cellular senescence (Passos et al., 2009). ROS contribute stochastically to the long-term maintenance of DNA damage by generating a stable and self-sustaining feedback loop sufficient to maintain cell arrest in response to DNA damage both *in vivo* and *in vitro* (Passos et al., 2010).

1.4 BMI-1 AND STEM CELL BIOLOGY

1.4.1 Definition

Gene expression patterns are largely regulated by reprogrammable epigenetic regulatory mechanisms that control the local chromatin conformation (Jaenisch and Bird, 2003). The repeated specific pattern of gene expression established by epigenetic machinery during multiple rounds of cell division establishes cellular identity. Epigenetic regulation is significantly achieved by modulating nucleosome dynamics through histone tail modifications.

The major molecular machines that dictate gene expression pattern through histone tail modification and chromatin modulations are global epigenetic modifiers, such as trithorax group (TrxG) and polycomb group (PcG) proteins, which trigger transcriptional activation and repression of target gene, respectively (Mills, 2010). PcG proteins form primarily two large multi-subunit polycomb-repressive complexes (PRCs), namely, PRC1 and PRC2. In humans, PRC1 is composed by BMI-1, RING1A/B, PCGF, CBX and HPH, while PRC2 is composed by EZH, SUZ12 and EED. These complexes in association with other epigenetic markers establish target gene repression through histone tail posttranslational modifications (Muller and Verrijzer, 2009; Simon and Kingston, 2009; Margueron and Reinberg, 2011).

Generally, PRC2 initiates histone tail modification by mono-, di-, and trimethylation of histone 3 at lysine 27 (H3K27me) residue with the help of its catalytic subunit enhancer of zeste homolog 2 (EZH2) methyl transferase enzyme, and this modification promotes the recruitment of PRC1 through its chromodomain-binding proteins, which then recognizes the H3K27me marker established by PRC2 (Spivakov and Fisher, 2007). PRC1 catalyses monoubiquitination of histone 2A at lysine 119 residue (H2AK119ub) to maintain target gene repression with the help of its catalytic subunit RING1A/B, which is an E3 ligase. RING1A/B protein activity is significantly enhanced by its association with another PRC1 component BMI-1 (B-cell specific moloney murine leukaemia virus integration region 1) (van Lohuizen et al., 1991).

We are now going to summarize *BMI-1* gene and BMI-1 protein structures, the regulatory mechanisms that control BMI-1 expression, and finally the cellular pathways that are regulated by BMI-1 repressing activity.

1.4.2 *BMI-1* gene and BMI-1 protein structure

The human *BMI-1* gene, composed of 10 exons and 9 introns, is localized on the short arm of chromosome 10 and encodes 37 kDa protein composed of 326 amino acids (Alkema et al., 1993). BMI-1 protein structure is highly evolutionarily conserved and has several protein domains:

- An N-terminal RING finger domain (RF)
- A central helix-turn-helix (HTH) domain
- A carboxyl terminal PEST-like domain
- Two nuclear localization signals KRRR and KRMK

Figure 1.6 shows *BMI-1* gene and BMI-1 protein structure. The RF domain of BMI-1 is required for its association with RING1B E3 ligase, a catalytic component of PRC1, to activate PRC1 activity (Li et al., 2006). The RF and HTH domains are important for BMI-1 localization at DNA strand break and crucial for the recruitment of DNA damage repair machinery, prevention of cellular

senescence, and cancer cell survival (Itahana et al., 2003; Ginjala et al., 2011; Balasubramanian et al., 2015). The C-terminal PEST domain is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) and is critical for BMI-1 protein turnover (Yadav et al., 2010).

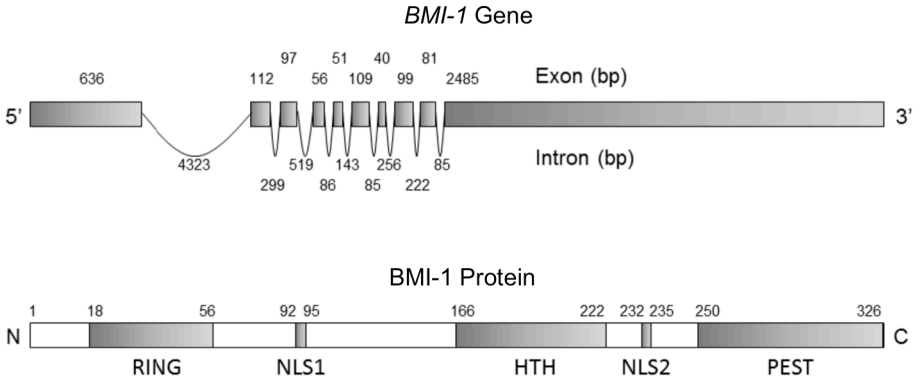

Figure 1.6 *BMI-1* gene and BMI-1 protein structure.
Taken from: Sahasrabuddhe (2016).

1.4.3 *BMI-1* gene and BMI-1 protein regulation

BMI-1 is expressed ubiquitously in almost all types of tissues. However, its expression levels are high in brain, lungs, thymus, kidney, gonads, salivary glands, placenta, blood, bone marrow, and stem cells of several lineages. It is overexpressed in several cancer subtypes and serves as a biomarker for these cancer types. BMI-1 plays a critical role in cellular physiology and hence the transcript and protein levels of BMI-1 are tightly regulated in diverse cell types. For the past several years, many investigators are trying to understand the regulation of *BMI-1* at transcriptional, posttranscriptional, and posttranslational levels. However, the comprehensive knowledge on BMI-1 regulation by different mechanisms is still scarce.

BMI-1 gene expression is transcriptionally regulated by the number of transcription factors in a context- and lineage-dependent manner. *BMI-1* gene expression is positively regulated by SP1, TWIST1, FOXM1, ZEB1, E2F1,

SALL4, n-MYC, c-MYC, and HDACs, whereas its expression is negatively regulated by MEL18, NANOG, and KLF4.

Gene expression is regulated at different levels, including regulation of mRNA activity after synthesis. This regulation may operate at the level of RNA processing for maturation, its transport to correct subcellular localization, its stability and finally translation of its coded information into protein. These regulations occur through interaction of cellular factors with mRNA sequence elements located within 5′- and 3′-untranslated regions of unprocessed mRNA, including secondary structures, internal ribosome entry sites, and poly-A tail. Post-transcriptional regulations can be achieved through the activity of target-specific microRNA expression. The miRNAs are short 21-23 nucleotide RNA sequences that control the level of expression of target gene through controlling transcription as well as mRNA stability. Several miRNAs, such as miR-452, miR-218, miR-494, miR-495, miR-215, miR-16, etc… are implicated in the regulation of *BMI-1* gene expression (Sahasrabuddhe, 2016).

Finally, post-translational regulations can be of two types: (1) reversible regulation and (2) irreversible regulations. The reversible protein modifications such as acetylation, phosphorylation, sumoylation, or ubiquitination can either alter the protein function or often direct protein to irreversible regulation through ubiquitin proteasome system (UPS)-mediated protein degradation. Post-translational regulation of BMI-1 is not significantly characterized. BMI-1 protein is reported to be regulated by UPS-mediated protein degradation. Some studies have shown that BMI-1 C-terminal PEST domain is critical for BMI-1 protein stability and within this region a consensus motif DSGSDKANS is recognized by an E3 ligase β-TrCP, which regulates BMI-1 protein turnover and consequent oncogenic activity (Sahasrabuddhe et al., 2011).

1.4.4 BMI-1 role in cellular physiology

Trough repression of target gene expression in a lineage- and context-dependent manner, BMI-1 regulates a myriad of cellular processes critical for cell growth, cell fate decision, development, senescence, aging, DNA damage repair, apoptosis, and self-renewal of stem cells (Figure 1.7) (Bhattacharya et al., 2015).

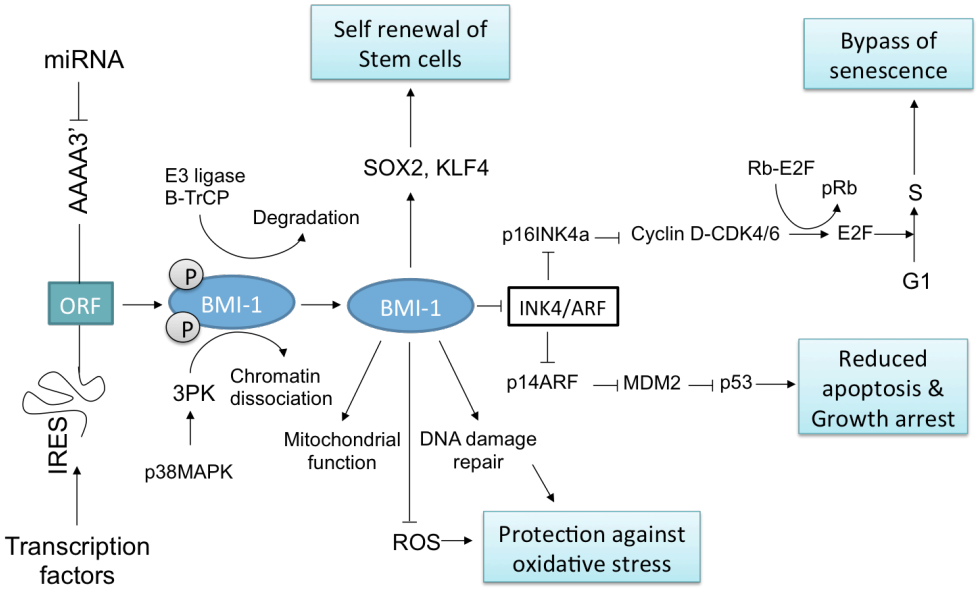

Figure 1.7 Upstream and downstream signalling pathways of BMI-1.
Taken from: Sahasrabuddhe (2016).

1.4.4.1 BMI-1, cell cycle and senescence

Since epigenetic events such as histone modification have been implicated in senescence, it follows that genes involved in chromatin remodelling and gene expression, such as members of the Polycomb and Trithorax families, might be directly involved in decisions that affect stem cell fate, including self-renewal, senescence, and possibly aging. Polycomb and Trithorax proteins form large multimeric structures, which can lead to repression or activation of gene expression, respectively, via a concerted process of chromatin modifications (Simon and Tamkun, 2002; Orlando, 2003).

As stated before, the normal cell cycle progression and regulation is tightly controlled by a variety of molecular checkpoints that supervise the various biological functions of the cell that occur within the different phases of the cell cycle (Medema and Macurek, 2012). BMI-1, being a transcriptional repressor and a PcG protein, plays an important role in cell cycle regulation (van der Lugt et al., 1994). BMI-1 controls self-renewal and cell cycle by regulating the tumour suppressor proteins $p16^{INK4a}$ y $p14^{ARF}$ (Dimri et al., 2002; Park et al., 2004). BMI-1 promotes CDK4 and CDK6 activity by repressing the *INK4A/ARF* locus (Jacobs et al., 1999). BMI-1 can also directly regulate p53 stability, further stressing its role in cellular proliferation and tumour genesis by negatively acting through the pRb/p53 pathway (Calao et al., 2013; Su et al., 2013). Thus, BMI-1 promotes cell proliferation by suppressing $p16^{INK4a}$/pRb and/or $p14^{ARF}$/MDM2/p53 tumour suppressor pathways. For example, it has been published that BMI-1 is necessary for an efficient self-renewal of hematopoietic stem cells, as well as of neural stem cells, but is less necessary for the differentiated progeny (Molofsky et al., 2003; Park et al., 2003).

1.4.4.2 BMI-1 and stem cell self-renewal

ESCs derived from the inner cell mass of a blastocyst are stem cells with unique properties of pluripotency and self-renewal. The unique identity of ESCs is governed by a network of transcriptional factors along with epigenetic features (Liang and Zhang, 2013). Compared with differentiated cells, ESCs display distinctive chromatin features related to its unique properties. The chromatin in ESCs is in an “open” state, with more accessible chromatin domains and less heterochromatin foci. In contrast, highly condensed heterochromatin foci are prevalent in lineage-committed somatic cells (Aoto et al., 2006; Meshorer and Misteli, 2006; Meshorer et al., 2006; Efroni et al., 2008). The pluripotent state of ESCs is enforced by epigenetic factors closely linked to the pluripotency transcription factor network (Orkin and Hochedlinger, 2011; Young, 2011).

Polycomb group genes (PcGs) are usually considered as being transcriptional repressors that are required for maintaining the correct spatial and temporal expression of homeotic genes during development (Bracken et al., 2006). In addition to being essential regulators of embryonic development, the PcGs have also emerged as key players in the maintenance of the adult stem cell populations (Molofsky et al., 2004; Valk-Lingbeek et al., 2004). For example, BMI-1 is required for the self-renewal of hematopoietic and neural stem cells (Lessard and Sauvageau, 2003; Molofsky et al., 2003), while overexpression of EZH2 is capable of blocking the differentiation of muscle myoblasts (Caretti et al., 2004) and preventing hematopoietic stem cell exhaustion (Kamminga et al., 2006).

Furthermore, Lee and colleagues found that PcG target genes are preferentially activated during ESCs differentiation and that the ESCs regulators OCT4, SOX2, and NANOG cooccupy a significant subset of these genes. These results indicate that PcG occupies a special set of developmental genes in ESCs that must be repressed to maintain pluripotency and that are poised for activation during ESCs differentiation (Lee et al., 2006b).

1.4.4.3 BMI-1 and oxidative stress

Genome integrity is constantly challenged by endogenous (metabolic) and exogenous (environmental) sources. To combat threats posed by DNA damage, cells have evolved mechanisms that are collectively termed as the DNA damage response (DDR). DDR involves a plethora of proteins whose sequential recruitment and function at DNA damage sites are modulated by numerous highly dynamic and reversible modifications, including phosphorylation, ubiquitination, acetylation, methylation and sumoylation.

BMI-1 is known to play a key role not only in preventing DNA damage, but also in DDR. Mitochondrial ROS has been shown to be a prominent agent for oxidative DNA damage. Some researchers have demonstrated that independent of $p16^{INK4a}$, BMI-1 has a role in maintaining mitochondrial function and redox

homeostasis (Liu et al., 2009). Further, cells lacking BMI-1 have significant mitochondrial dysfunction accompanied by a sustained increase in ROS that is sufficient to engage the DDR pathway. Thus, BMI-1 prevents oxidative DNA damage (Wang et al., 2011).

Apart from protecting against oxidative DNA damage, BMI-1 has been shown to be a key component in DDR, as it is required and sufficient to recruit the DDR machinery to DNA double-strand break sites in response to radiation (Facchino et al., 2010). Following DNA damage, BMI-1 tethers its RING finger to DNA, and associates more stably with damaged compared to undamaged chromatin (Ismail et al., 2010). Therefore, by inhibiting ROS-induced oxidative DNA damage along with facilitating DDR, BMI-1 contributes to maintaining genome integrity.

2 AIMS AND JUSTIFICATION

2.1 GENERAL AIM

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.

2.2 SPECIFIC AIMS

The specific aims of this study were:

- To analyse if ambient oxygen tension induces oxidative stress in hDPSCs during long-term culture.

- To analyse if ambient oxygen tension mediates an oxidative stress-induced premature senescence (SIPS) in hDPSCs during long-term culture.

- To analyse whether this oxidative stress-induced premature senescence (SIPS) is mediated by $p16^{INK4a}$ pathway.

- To analyse if ambient oxygen tension induces a stemness potential loss in hDPSCs during long-term culture.

- To analyse if BMI-1 controls hDPSCs fate by $p16^{INK4a}$, *SOX2* and *OCT4* regulation during long-term culture under ambient oxygen tension.

2.3 JUSTIFICATION OF THE STUDY

2.3.1 *In vitro* stem cell maintenance: 3% *versus* 21% oxygen tension

Like embryonic stem cells, mesenchymal stem cells (MSCs) also reside in low oxygen concentrations. In mammals including humans, MSCs are located in perivascular niches close to the vascular structure in almost all tissues (Shi and Gronthos, 2003; Crisan et al., 2008; Zannettino et al., 2008; Mohyeldin et al., 2010). Despite residing near the blood vessels, in different tissues where they are found, the oxygen concentrations are low (Harrison et al., 2002; Pasarica et al., 2009). In adult human tissues, oxygen concentration varies widely depending on the vascularisation and the type of microenvironment within the respective organ, and they are considerably lower than the inhaled ambient oxygen concentration (21%). The partial pressure or oxygen concentration of inspired air gradually decreases after it enters the lungs and then in the blood flowing from the alveolar capillaries that carry oxygen, towards the organs and tissues for their oxygenation. By the time oxygen reaches the organs and tissues, oxygen concentration drops to 2-9%, with a mean of 3% (Simon and Keith, 2008). Although highly vascularized, it is speculated that the oxygen concentration in the dental pulp is low. A study with rats found approximately 3% pO_2 in the pulp tissue (Yu et al., 2002).

In the last few decades there has been a great improvement in the development of culture medium and different matrix substrates as well as soluble factors that optimize cell proliferation and their maintenance (Villa-Diaz et al., 2013; Laitinen et al., 2016). However, not all the factors that compose the niche have received the same attention. Currently, the vast majority of cell cultures are performed under a hyperoxic ambient which is composed of approximately 21% oxygen tension.

The advances in cell-based therapies and regenerative medicine have aroused interest in the factors that control stem cell characteristics (Muscari et al., 2013). *In vivo*, the stem cell niche is regulated by growth factors, cytokines and low oxygen tension (Simon and Keith, 2008). Within these niches, stem cells are

present in an undifferentiated and self-renewable state (Schofield, 1983). To mimic this environment *in vitro*, simulating low oxygen tension is one of the hot topics of tissue engineering research (Bornes et al., 2015; Hutton and Grayson, 2016; Wakai et al., 2016), which aims at improving proliferation rates (Grayson et al., 2006; Grayson et al., 2007; Zhang et al., 2014; Fotia et al., 2015) and differentiation potential of stem cells (Adesida et al., 2012; Bornes et al., 2015) as well as enhancing their viability after transplantation (Yan et al., 2012; Bader et al., 2015).

The oxygen concentration *in vitro* culture under ambient oxygen tension is 4–10 times higher than in the physiological environment. Thus, there has been an increasing interest in the growth of cells under 3% oxygen tension over the last few years (Forristal et al., 2010; Basciano et al., 2011; Forristal et al., 2013).

3 MATERIAL AND METHODS

3.1 MATERIAL

3.1.1 Patient selection

This thesis summarizes a prospective, *in vitro*, and uncontrolled study. Teeth were extracted by dentists from the Department of Surgery of the Dental Clinic of the Faculty of Medicine and Odontology of the University of Valencia.

All patients were informed of the characteristics of the study, and freely agreed to collaborate, donating the extracted tooth, which was always extracted for reasons not related to this study. They were properly informed and signed an informed consent together with the patient information sheet (Annexes 1 and 2). Throughout the study, patient anonymity and data protection were maintained.

To perform this study, we used dental pulps of permanent teeth from 5 patients, men and women between 14 and 25 years old.

- **Inclusion criteria:** We included pulps from teeth that did not present any clinical and/or radiological sign or symptom of inflammation and/or infection.
- **Exclusion criteria:** We excluded all pulps from teeth that presented any clinical and/or radiological sign or symptom of the following clinical manifestations: pulpitis, apical periodontitis, periodontal disease, fractures affecting the dental pulp and those teeth in which a fracture occurred during the extraction.

3.1.2 Dissection material

For dental pulp extraction procedure, it was necessary to use the sterile dissection material detailed below:

- Extra torque 605 Kavo®
- Diamond cylindrical milling cutter
- Curettes and tweezers Hu-Friedy
- Scalpel nº10

3.1.3 Equipment

CELL CULTURE

- Cell culture hood Cultair B100
- Incubator Thermoscientific HERAcell 150i CO_2
- Incubator Binder CB150 5% pCO_2, 3% pO_2
- Water bath P Selecta Preciseterm
- Inverted Microscope Zeiss ID03
- Cell counter: Neubauer chamber
- Sterilizer Selecta Autester-G
- Precision weighing balance Sartorius Acculab (± 0.0001 g)
- Centrifuge Sorvall Heraeus Multifuge 3SR plus (for Corning tubes)
- Vacuum pump Millivac (Millipore)

FLOW CYTOMETRY

- The BD FACSVerse system includes the BD FACSVerse cytometer, the optional BD FACS Universal Loader, and BD FACSuite software running on the system workstation. The system includes customyzed steup beads.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

- The DIONEX Ultimate 3000 chromatograph (Thermo Scientific), includes a quaternary pump, an autoinyector, a UV-Vis detector, the Chromoleon software and the Hypersil GOLD column C18 5µm 175 A, 150 x 4.6 mm.

PROTEIN QUANTIFICATION

- Vortex Heidolph Reax top
- Spectrophotometer Cecil, CE3021, 3000 series
- Semi-micro cuvettes

WESTERN BLOT AND OXYBLOT™

- Weighing balance Gram Precision AHZ (± 0.01 g)
- Stuart hot plate stirrer multiposition, SB162-3
- Crison pHmeter, GLP21
- Fume hood Burdinola, OR-ST 1200
- Gel making kit:
    - Casting stand, casting frames, gaskets, releaser, glass plates and plastic combs (Bio-rad)
- Electrophoresis kit:
    - Clamping frame with electrode assembly, tank and lid from Mini Protean® Tetra Cell (Bio-rad)
- Transfer kit:
    - Gel holder cassette, filter paper, foam pads, core assembly module, tank and lid from Mini Trans-Blot® Cell (Bio-rad)
- Power supply PowerPac Basic (Bio-rad)
- Image Quant LAS 4000, GE_Healthcare Bio-Sciences

RNA ISOLATION

- Fume hood Crumair, 1100-G A
- Centrifuge Hermle Z216MK (for Eppendorf tubes)
- Centrifuge Hettich, 35 R (for Eppendorf tubes)
- Stuart, Block heater, SBH 130 D
- Spectrophotometer Thermoscientific NanoDrop 2000

REVERSE TRANSCRIPTION

- Centrifuge Sigma 1-14 (for Eppendorf tubes)
- Thermal cycler GeneAmp® PCR system 9700 (Applied Biosystems)

POLYMERASE CHAIN REACTION

- Plate centrifuge Eppendorf 5430
- Thermal cycler 7900HT Fast Real-Time PCR System (Applied Biosystems)

STORAGE

- -80ºC freezer Revco ultima II
- -80ºC freezer Platinum 500
- -20ºC freezer Liebherr Comfort Nofrost
- 2-4ºC refrigerator Lynx
- Cryogenic freezer and CoolCell LX Freezing containers

WATER PURIFICATION SYSTEM

- Millipore, Milli-Q
- Elga, purelab flex

3.1.4 Additional supplies

- Cell culture vessels (flasks, Petri dishes, multiwell plates)
- Tubes (15 mL and 50 mL Cornings)
- Eppendorfs (0.2 – 0.5 – 1.5 – 2 mL)
- Cryotubes
- Tips and pipettes
- Glass pipettes and pipettors
- Syringes and nylon membrane filters 0.2 μm
- Waste containers

3.2 METHODS

3.2.1 Cell culture

3.2.1.1 Description

Cell culture refers to the removal of cells from an animal and their subsequent growth in a favourable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation.

Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available surface (i.e., reach confluence). At this stage, the cells have to be subcultured (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth.

3.2.1.2 Reagents

The necessary reagents for hDPSCs isolation, culture, passaging and cryopreservation are:

- Krebs-Henseleit buffer + 2 mg/mL EDTA (Sigma)
- Collagenase I and Dispase II (Sigma)
- Thermolysin (Vitacyte)
- Trypsin (Invitrogen)
- Dulbecco's modified Eagle® medium (DMEM) Low Glucose (1g/L) (GIBCO)
- Inactivated Foetal Bovine Serum (FBS) (Invitrogen)
- Penicillin/Streptomycin (P/S) 10,000 U/mL (GIBCO)
- Dimetilsulfoxide (DMSO) (Sigma)
- Dulbecco's Phosphate Buffered Saline (PBS) (GIBCO)

3.2.1.3 Procedure: pulp extraction and hDPSCs isolation

Dental pulp extraction was performed by cutting the extracted tooth at the level of the amelocementary line with a torque and a milling cutter. Once removed from the pulp chamber, the pulp was extracted with tweezers (Figure 3.1).

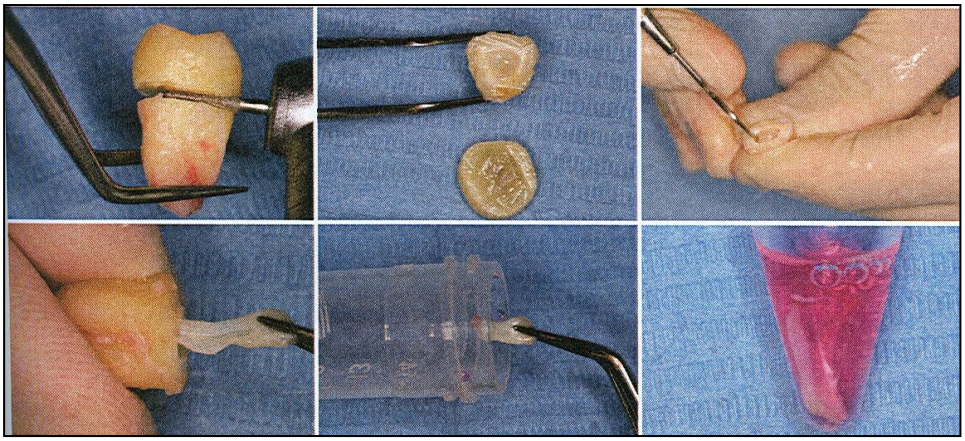

Figure 3.1 Dental pulp extraction.

Once extracted, the dental pulp was introduced into 15 mL Corning tubes with 1-2 mL of DMEM Low Glucose + 1% P/S. Between pulp extraction and its digestion, there was a time range of between 1h and 24h. During this time the pulp was stored at 4°C. hDPSCs were then isolated following this protocol:

1. Mechanical disaggregation of the dental pulp tissue with the scalpel.
2. Chemical disaggregation with Krebs-Henseleit buffer + 2 mg/mL EDTA for 10 min in a humid incubator at 37ºC, 5% pCO_2 and 3% pO_2.
3. Centrifuge at 1000 g for 2 min.
4. Enzymatic digestion during 40 min in a humid incubator at 37ºC, 5% pCO_2 and 3% pO_2. Dilute enzymes on DMEM Low Glucose + 1% P/S:
    a. Collagenase I + Dispase II at a final concentration of 4 mg/mL.
    b. Thermolysin at a final concentration of 13 ng/mL.
5. Centrifuge at 1000 g for 2 min.

6. Resuspend the pellet in new fresh complete media (DMEM Low Glucose + 10% SBF + 1% P/S).
7. Seed in T25 flask and incubate in humid incubator at 37ºC, 5% pCO_2 and 3% pO_2.

3.2.1.4 Cell culture conditions and cryopreservation

Culture conditions vary widely for each cell type, but the artificial environment in which cells are cultured invariably consists of a suitable container containing a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins and minerals), growth factors, hormones and gases (pO_2 and pCO_2), and regulates the physicochemical environment (pH, osmotic pressure and temperature).

hDPSCs, like most cells, are anchorage dependent and must be cultured in a flask that provides them with a solid base to adhere to. When the culture reaches confluence, cells need to be passaged. The purpose of the passages is to provide them with more space so that they can continue to proliferate. In this case, cells are washed with PBS and then treated with trypsin (at an average of 1 mL per 5 cm^2) to detach them. Finally, cells are transferred to a larger flask with complete medium.

For serial passaging we used T75 flasks filled with 15 mL of complete medium, which can hold 2 to 3 million hDPSCs. Every 5 passages, we collected samples. In this case, we used T25 flasks filled with 5 mL of complete medium which can hold 1 to 1.5 million hDPSCs on confluence. The exact amount of cells will depend on the morphology of the cells; as passaging number increased, the larger, elongated, flattened and irregular the cells were. A total of 25 passages were accumulated by hDPSCs cultured at 3% pO_2 while only 15 passages were reached by hDPSCs cultured at 21% pO_2. Each 5 passages, by cell counting in the Neubauer chamber, an exact number of cells was seeded in T25 flasks to perform the relevant experiments. The T25 flasks with hDPSCs cultured at 3% pO_2 were

initially seeded with 250,000 cells (50,000 cells/cm^2), while hDPSCs cultured at 3% pO_2 were initially seeded with 325,000 cells (65,000 cells/cm^2). This is 30% more cells, to compensate the loss of cells that do not adhere to the base of the flask after seeding.

As soon as a small surplus of cells becomes available from subculturing, they should be frozen as a seed stock, protected, and not be made available for general laboratory use. The best method for cryopreserving cultured cells is storing them in liquid nitrogen in complete medium in the presence of a cryoprotective agent such as dimethylsulfoxide (DMSO). Cryoprotective agents reduce the freezing point of the medium and also allow a slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death. hDPSCs surplus were cryopreserved at an average of 1.5 million cells in 1.8 mL of complete medium with 20% DMSO in each cryotube.

3.2.2 Small-interfering RNA transfection (siRNA)

3.2.2.1 Description

Gene expression can be regulated at both transcriptional and post-transcriptional levels (Hammond et al., 2001).

Transcriptional Gene Silencing (TGS) is the result of DNA modification or histone alteration. These modifications create a heterochromatin environment around a certain gene, which prevents access of the transcriptional machinery (transcription factors, RNA polymerases, etc.) by repressing the expression of that gene. Post-Transcriptional Gene Silencing (PTGS), on the other hand, is a mechanism that involves the degradation of a specific messenger RNA (mRNA). The destruction of this mRNA prevents its normal translation and consequently the corresponding protein is not synthesized.

We performed post transcriptional gene silencing. The molecular basis of this process can be divided into an initiation step and an effector and maintenance step.

Initiation step begins with the presence of a double-stranded RNA. It is recognized and digested by the Dicer enzyme, which possesses RNase type III domains (enzymes that degrade RNA molecules), to form small RNA molecules of 21-24 nucleotides length. These RNAs were called small interference RNAs (siRNA); they are short double-stranded molecules that function as specific determinants in RNA-mediated gene silencing.

During the effector step, the siRNA binds to a complex with nuclease activity (nucleic acid degrading enzymes) to form the RISC complex (RNA-induced silencing complex). RISC is a ribonucleoprotein complex with sequence homology dependent endonuclease activity that is responsible for targeted RNA degradation. The RISC helicase activity separates the two strands from the siRNA, and only one of them remains attached to the complex. Once RISC is activated, it targets the degradation of mRNAs homologous to this siRNA. The RISC nucleus is composed of proteins belonging to the Argonaute family (AGO), being the best known AGO1. These proteins are fundamental partners in the mechanisms of interference RNA in various organisms (Figure 3.2).

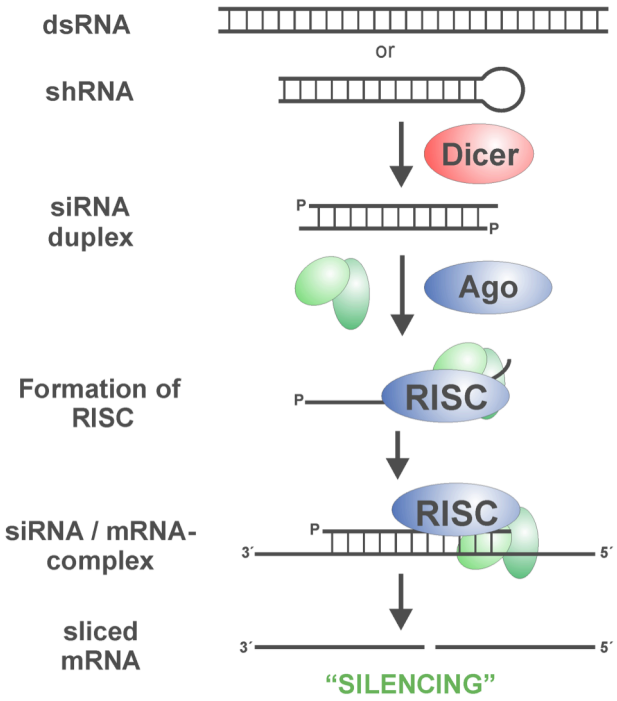

Figure 3.2 Post-transcriptional siRNA mechanism.
Taken from: Hammond et al. (2001).

3.2.2.2 Reagents

BMI-1 gene silencing was performed using the following reagents (Santa Cruz):

- BMI-1 siRNA (h)
- siRNA Control
- siRNA Transfection Medium
- siRNA Transfection Reagent

3.2.2.3 Procedure

The siRNA transfection protocol was completely performed in sterile ambient.

1. In a six well tissue culture plate, seed $2x10^5$ cells per well in 2 mL DMEM Low Glucose + 10% FBS.
2. Incubate the cells at 37°C in humid incubator at 37ºC, 5% pCO_2 and 3% pO_2 until the cells are 60-80% confluent. This will usually take 18-24 hours.
3. Prepare the following solutions:
    a. Solution A: For each transfection, dilute 3 µL of siRNA duplex into 100 µL siRNA Transfection Medium.
    b. Solution B: For each transfection, dilute 3 µL of siRNA Transfection Reagent into 100 µL siRNA Transfection Medium.
4. Add the siRNA duplex solution (Solution A) directly to the dilute Transfection Reagent (Solution B) using a pipette. Mix gently by pipetting the solution up and down and incubate the mixture 15-45 minutes at room temperature.
5. Wash the cells once with 2 mL of siRNA Transfection Medium. Aspirate the medium and proceed immediately to the next step.
6. For each transfection, add 0.8 mL siRNA Transfection Medium to each tube containing the siRNA Transfection Reagent mixture (Solution A + Solution B). Mix gently and overlay the mixture onto the washed cells.
7. Incubate the cells 5-7 hours in a humid incubator at 37ºC, 5% pCO_2 and 3% pO_2.
8. Add 1 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration (2X normal growth medium) without removing the transfection mixture.
9. Incubate the cells for an additional 18-24 hours.
10. Aspirate the medium and replace with fresh 1X normal growth medium.
11. Assay the cells using the appropriate protocol 24-72 hours after the addition of fresh medium in the step above.

3.2.3 Flow cytometry

3.2.3.1 Description

Flow cytometers are powerful analytical instruments that measure certain optical properties of particles in flow. A flow cytometer can measure these optical properties from any kind of particle as long as it (1) meets certain size requirements, and (2) the particles can be prepared as single cell suspensions. Additionally, particles must flow through the instrument to be analysed, so they must be prepared as a single-particle suspension.

Flow cytometers typically measure two distinct kinds of optical signals from a particle, light scattering and fluorescence.

Light scattering: Scattering occurs when light interacts with a cell and changes its direction as a result of this interaction. Flow cytometers measure two kinds of light scattering: forward scatter and side scatter. Forward scatter is measured at 180° relative to the illuminating light and is correlative (but not a direct measurement of) cell size. Side scatter is collected at 90° relative to the illuminating light and is correlative (but also not a direct measurement of) to cell granularity or membrane roughness.

Fluorescence: Fluorescence occurs when molecules are excited by specific colours of light and then emit specific colours of lower energy light. When we measure fluorescence with a flow cytometer, we essentially measure the intensity of specific colours of light emitted by a particle. Flow cytometers can measure many different fluorescent signals from a single particle at once. The ability to measure many fluorescent signals at once from single particles is what gives this technique its power.

The aspects of a cell that are measured using flow cytometry are called parameters. Certain parameters are intrinsic to the cell and others are extrinsic. Intrinsic parameters, such as cell size (forward scatter) or cell granularity (side scatter) do not require any special reagent to measure. Extrinsic parameters, require fluorescent molecules, or probes, to measure (Wood and Hoffman, 1998).

A flow cytometer consists of three systems that merge in the instrument to accomplish these goals (Figure 3.3):

- The fluidics system functions to facilitate the transport of particles to the analysis point. This system is based on pressure and accomplishes both the delivery of particles to the optical interrogation point as well as the focusing of particles for single cell analysis.
- The optical system consists of two sub-systems: the illumination optics and the collection optics. The illumination optics are lasers and mirrors and lenses that focus these lasers into the particles. The collection optics consists of arrays of mirrors and optical filters that direct light to the detectors.
- The electronic system transforms the optical signal into an electronic signal and then further process this electronic signal so that it can be output as data that is understandable and interpretable.

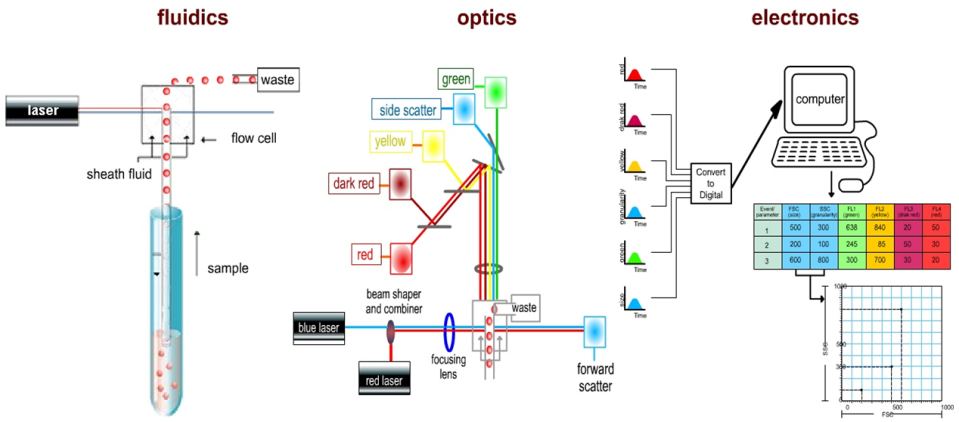

Figure 3.3 Flow cytometer components.
Taken from: Wood and Hoffman (1998).

Flow cytometry data is displayed in graphical format as either one-parameter histograms (on the right) or two-parameter histograms. The axes corresponding to the parameter are labelled with each data bin. In the plot on the right, the x-axis

lists the channel values for the forward scatter parameter. The scaling of the x-axis is linear. In other words, the intervals increase in a linear fashion. The y-axis is marked by number of cells. Therefore, we can get a graphical representation of cell count per data channel. We can see that most of the cells (the tallest peak corresponding to the biggest cell count) is at about channel 75,000. We can also see there is a distribution of cells in a wide range of channel values.

3.2.3.2 Reagents

A fluorescent compound absorbs light energy over a range of wavelengths that is characteristic for that compound. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level. The excited electron quickly decays to its ground state, emitting the excess energy as a photon of light. This transition of energy is called fluorescence.

The range over which a fluorescent compound can be excited is termed its absorption spectrum. As more energy is consumed in absorption transitions than is emitted in fluorescent transitions, emitted wavelengths will be longer than those absorbed. The range of emitted wavelengths for a particular compound is termed its emission spectrum.

When a fluorescent dye is conjugated to a monoclonal antibody, it can be used to identify a particular cell type based on the individual antigenic markers of the cell. They can also represent specific components of cells such as organelles, enzymes or surface markers.

The following stainings used for hDPSCs analysis by flow cytometry are listed below. For ROS detection ROS, we used dihydrorhodamine-123 marker; for the analysis of the mitochondrial membrane potential ($\Delta\Psi m$), the methyl ester tetramethylrodamine (TMRM) was used; and finally, for the determination of SA-β-galactosidase activity, the combination of two dyes, the fluorescein di-β-galactopyranoside and the propidium iodide (PI), were used. All were purchased from Molecular Probes (Thermo Fisher Scientific).

- Dihydrorodamine-123 (DHR123)

Dihydrorhodamine-123 is a non-charged and non-fluorescent dye for reactive oxygen species (ROS) that can passively diffuse through membranes where rhodamine 123 is oxidized, acquiring a cationic character, located in the mitochondria and showing green fluorescence.

- Tetramethylrodamine methyl ester (TMRM)

Tetramethylrodamine methyl ester (TMRM) is a red-orange fluorescent cationic dye that penetrates the cell, and where active mitochondria easily sequester.

- Fluorescein di-β-D-galactopyranoside (FDG)

Non-fluorescent FDG is sequentially hydrolyzed by β-galactosidases, first to fluorescein monogalactoside and then to highly fluorescent fluorescein. The enzyme-mediated hydrolysis of FDG is proportional to the increase in fluorescence.

- Propidium iodide (PI)

Propidium iodide (PI) is a popular chromosomal or nuclear fluorescent folk counterpart. As propidium iodide is not permeable to living cells, it is often used to detect dead cells in a population. PI binds to DNA by intercalation between bases with little or no sequence preference.

Figure 3.4 shows the excitation and emission spectra of these four molecules. Note that they are curves, which means that they absorb and emit photons in a range of wavelengths. This allows the selection of flow cytometer detectors; DHR-123 and FDG have very similar spectra and will be recognized by the fluorescein isothiocyanate (FITC) detector, which receives the light of 530/30 nm. Similarly, the TMRM and the PI will be recognized by the phycoerythrin (PE) detector, which collects 575/26 nm light.

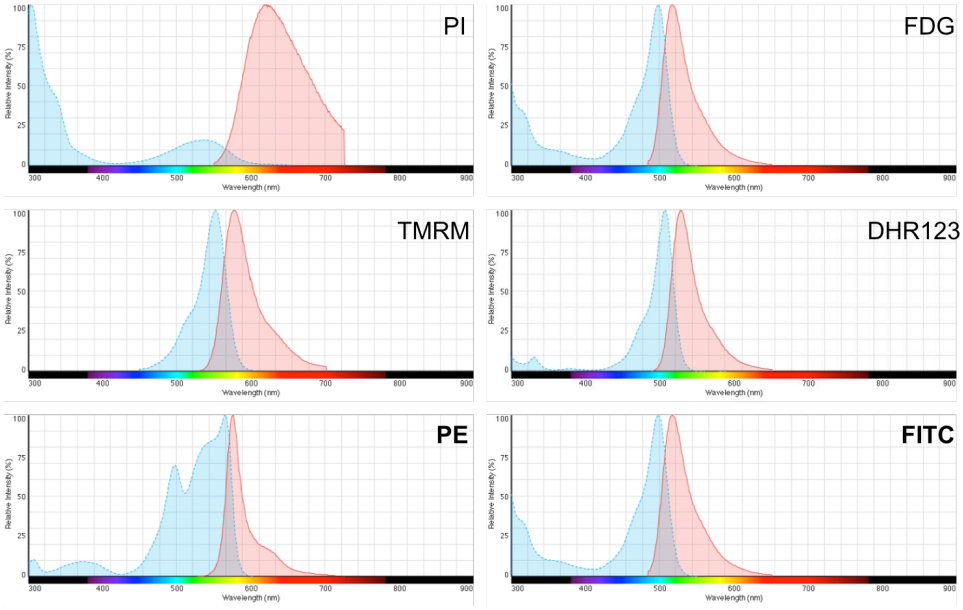

Figure 3.4 Fluorochromes excitation and emission spectra.

Table 3.1 summarizes the fluorochromes used according to their corresponding detectors and fluorescent channel.

Table 3.1 Fluorochromes and detectors correlation.

FLUORO-CHROME	FLUORESCENCE CHANNEL	DETECTOR	EXCITATION maximum	EMISSION maximum
DHR123	GREEN	FITC	494 nm	519 nm
FDG				
TMRM	YELLOW	PE	564 nm	578 nm
PI				636 nm

3.2.3.3 Procedure

Staining protocol using FDG and PI:

1. Treat cells with trypsin until they can be removed from the plate by gentle agitation. Inactivate trypsin by washing in staining medium (PBS, 4% (v/v) FBS, 10 mM HEPES, pH 7.2).
2. Centrifuge at 1000 g for 2 min.
3. Resuspend cells in staining medium to ~ 10^7 cells/mL and pipet 100 µL into an appropriate flow cytometer tube. Place cells on ice.
4. Prepare a 2 mM working solution: thaw and dilute the FDG reagent 10-fold in water. Prewarm the FDG working solution to 37ºC for 10 minutes prior to use.
5. Prepare staining medium with 1.5 µM (1 µg/mL) propidium iodide by diluting the PI reagent 100-fold in staining medium. Chill on ice.
6. Prewarm the tube containing 100 µL of cells in a 37ºC water bath for 10 minutes.
7. Start FDG loading by adding 100 µL of prewarmed (37ºC) 2 mM FDG working solution. Mix rapidly. Return to the 37ºC water bath for exactly one minute.
8. Stop the FDG loading at the end of one minute by adding 1.8 mL ice-cold staining medium containing 1.5 µM PI. Use ice-cold pipettes to aliquot the staining medium into the cells. Keep the cells on ice prior to flow cytometry analysis.

Staining protocol using DHR123 and TMRM:

1. Treat cells with trypsin until they can be removed from the plate by gentle agitation. Inactivate trypsin by washing in tissue culture growth medium.
2. Centrifuge at 1000 g for 2 min.
3. Resuspend the cells in complete medium (250,000 cells/mL).
4. Pipet 500 µL into an appropriate flow cytometer tube.

5. Add DHR123 at a final concentration of 200 µg/mL or add TMRM at a final concentration of 100 µg/mL.
6. Incubate for 30 min in a dark incubator at 37ºC prior to flow cytometry analysis.

3.2.4 High performance liquid chromatography (HPLC)

3.2.4.1 Description

The components of a basic high performance liquid chromatography (HPLC) system are shown in the simple diagram in Figure 3.5.

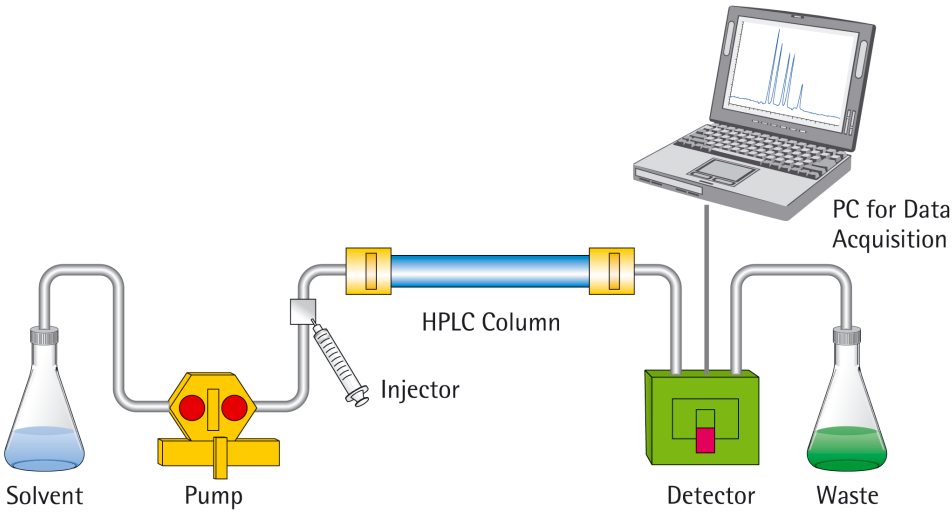

Figure 3.5 High performance liquid chromatography system.

A reservoir holds the solvent (called the mobile phase). A high-pressure pump (solvent delivery system) is used to generate and meter a specified flow rate of mobile phase, typically millilitres per minute. An injector is able to introduce the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column. The column contains the chromatographic packing material needed to effect the separation. This packing material is called the

stationary phase because it is held in place by the column hardware. A detector is needed to see the separated compound bands as they elute from the HPLC column. The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the elute containing that purified compound for further study.

We followed Wong and colleagues method (Wong et al., 1987). This method is based on the detection of the hydrolysis of the lipoperoxides and subsequent formation of an adduct between 2-thiobarbituric acid and malondialdehyde (TBA-MDA2) by HPLC. This reaction produces a pink chromogen with a maximum absorption at 532-535 nm.

3.2.4.2 Reagents

1. 50 mM Potassium phosphate-EDTA (KPi-EDTA) buffer
   - Prepare 25 mL of 50 mM K_2HPO_4 pH 6.8.
   - Prepare 25 mL of 50 mM KH_2PO_4 pH 3.5.
   - Mix them at about 4:1 until pH 7.4.
   - Add EDTA at a final concentration of 0.1 mM.
2. 2 M Sodium acetate buffer with 0.2% TBA
   - Prepare 1 L of 2 M sodium acetate pH 6.8.
   - Add 0.2% TBA and mix until complete dissolution.
3. Standards and standard curves preparation
   - Calibration MDA standards shall be prepared ahead of time for each analyte of interest. The calibration standards prepared for each analyte shall include not less than a two low end concentrations, two different mid-range concentrations, and a high end concentration for a total of five; this range of concentration values should bracket the expected range of concentrations in the sample. We used 50 – 25 – 12.5 – 6.25 – 3.125 – 1.562 – 0.781 nmol/mL concentrations.

4. Mobile phase preparation. We performed an isocratic method, thus we used two different mobile phases:
    - The elution phase (phase A) composition was 50 mM KH_2PO_4-acetonitrile (83:17) pH 6.8.
    - The washing phase (phase B) composition was a mixture of acetonitrile and purified water both at 70%.
    - All solvents were filtered through a 0.22 μm Millipore filter before use and degassed in an ultrasonic bath.

3.2.4.3 Procedure

1. Derivatization procedure
    - Cell samples should be collected with 200 μL of KPi-EDTA buffer.
    - Mix 500 μL of Sodium acetate TBA buffer with 20 μL of sample or standard.
    - Incubate 1 hour at 95ºC.
    - Centrifuge at 13,000 rpm for 5 min at 4ºC.
    - Pipet 200 μL of supernatant and proceed to HPLC analysis.
2. Chromatographic conditions
    - Flow rate: 1.0 mL/min
    - UV-visible detection at λ 532 nm
    - Reversed-phase C8 column
3. Chromatographic method
    - Each chromatogram took 30 minutes; the established sequence of mobile phases is detailed below (Table 3.2):

Table 3.2 HPLC mobile phases sequence.

PHASE A	PHASE B	PHASE A
Sample elution	Colum wash	Column restoration
12 minutes	10 minutes	8 minutes

3.2.4.4 Data analysis

HPLC method displays results as a graphical chromatogram in Chromoleon software. The area under the curve (AUC) corresponding to the TBA-MDA2 adduct is measured and compared with the standard curve. Results are presented as moles of MDA / mg of total protein.

3.2.5 Lowry method for protein quantification

3.2.5.1 Description

The most accurate method of determining protein concentration is probably acid hydrolysis followed by amino acid analysis. This procedure has no exception, but its sensitivity is moderately constant from protein to protein, and it has been so widely used that Lowry protein estimations are a completely acceptable alternative to a rigorous absolute determination in almost all circumstances in which protein mixtures or crude extracts are involved.

The method is based on both the Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu^{+}, which reacts with the Folin reagent, and the Folin Ciocalteu reaction, in which phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic amino acids. The reactions result in a strong blue colour, which depends partly on the tyrosine and tryptophan content. The method is sensitive down to about 0.01 mg of protein/mL, and is best used on solutions with concentrations in the range 0.01–10 mg/mL of protein.

3.2.5.2 Reagents

The Lowry method for protein quantification needs the following reagents:

- Bovine Serum Albumine (BSA)
- Deionized Milli-Q water
- Lowry reagent (Sigma-Aldrich)
- Folin and Ciocalteu's phenol reagent (Sigma-Aldrich)

3.2.5.3 Procedure

Prepare the solutions:

1. Lowry solution
   - Add 40 mL of deionized purified water to the powdered Lowry reagent, cover it from light, and mix for 30 minutes, according to manufacturer's instructions.
2. Folin solution
   - Add 90 mL of deionized purified water to 18 mL of the Folin and Ciocalteu's phenol reagent 2 M, according to manufacturer's instructions.
3. BSA standard protein solution
   - Weigh 50 mg of BSA and add it to a flask containing purified water.
   - Stir well to dissolve and adjust the volume to 50 mL with purified water: final concentration of the stock is (10 mg/mL).
   - Make serial dilutions in Eppendorf tubes for the standard curve: 0 – 0.01 – 0.1 – 0.5 – 1 – 2.5 – 10 mg/mL.

Then proceed to the Lowry method for protein quantification:

1. Vortex all samples and BSA dilutions.
2. Add 490 µL of purified water and 10 µL of sample or BSA dilution. Vortex briefly.

3. Add 500 µL of Lowry solution, vortex briefly and incubate for 20 minutes in the dark at room temperature.
4. Add 250 µL of Folin solution, vortex briefly and incubate for additional 30 minutes in the dark at room temperature.
5. Transfer the samples to the cuvettes and read absorbance at 660 nm starting with blank template.

3.2.5.4 Data analysis

Light absorption by a substance is a characteristic property of each substance, which can be used for its identification and quantification. All substances absorb light in some region of the electromagnetic spectrum (VIS, UV, IR, etc.). There are different methods for proteins quantification. Many of these methods are based on: the property of proteins to absorb light in the UV, the formation of chemical derivatives, or the ability of proteins to bind certain dyes.

Lambert and Beer demonstrated that the absorbance (A) of a substance is directly proportional to the concentration (c) of the absorbing substance, the length of the light path (l) (thickness of the solution) and a constant called extinction coefficient (ε), which is characteristic for each substance at a given wavelength (λ).

$$\mathbf{A = \varepsilon \, . \, l \, . \, c}$$

The standard curve is obtained by measuring the absorbance of a series of solutions of known BSA concentrations treated with the same method and measured at the same wavelength in the same instrument. The result is expressed on a plot of absorbance (A) as a function of concentration (c). As the Lowry method follows Lambert-Beer's law (Stauffer, 1975), a straight line passing near the origin is obtained. The linear part is selected and the linear regression line that best fits is calculated and the absorption data of our samples are interpolated.

3.2.6 Specific protein determination

3.2.6.1 Description

Electrophoresis is a transport under the action of an electric field. Electrophoresis in gels with a polyacrylamide matrix, commonly referred to as polyacrylamide gel electrophoresis, is mostly used for protein separation. Specifically, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used under denaturing conditions. For the realization of this technique it is imperative that the proteins are completely denatured. To do this, we use two denaturants, sodium dodecyl sulfate (SDS) as detergent and 2-mercaptoethanol as a reducing agent, which causes disulfide bridges to rupture. In this way proteins are obtained in their primary structure.

In the presence of an SDS concentration greater than 8 mM, the proteins bind 1.4 g SDS per gram of protein, which is equivalent to the binding of one SDS molecule per 2 amino acids. The charges of the proteins are thus masked or nullified. As each SDS molecule provides a negative charge, the protein / SDS complexes are negatively charged uniformly, so that the separation depends on the charge and molecular weight of the different proteins.

Once separated, they are transferred to a membrane where detection of a particular protein is possible by incubation with specific antibodies.

3.2.6.2 Reagents

To perform western blot of specific protein, we used the reagents detailed below:

- Lysis buffer (TRIS/SDS/Glicerol), supplemented with proteases inhibitor, bromophenol blue and 2-mercaptoethanol
- Gel preparation: Acrilamide/bisacrilamide (40% w/v, 29:1), 0.5 M TRis at pH 6.8, 1.5 M Tris at pH 8.8, Milli-Q water, 10% SDS, 10% APS and TEMED

- Running buffer (25 mM Tris, 200 mM Glycin, 0.1% SDS, pH 8.3)
- Transfer buffer (25 mM Tris, 192 mM Glycin, 20% (v/v) Methanol, pH 8.3)
- Blocking buffer (5% BSA in TBS-T)
- Washing buffer TBS-T (0.1% Tween-20 in 1X TBS (20 mM Tris, 137 mM NaCl, pH 7.6)
- Nitrocellulose or PVDF Membranes
- Primary antibodies:
    - BMI-1 antibody (Santa Cruz Biotechnology)
    - Tubulin antibody (Santa Cruz Biotechnology)
- Secondary antibodies:
    - Goat Anti-Mouse IgG, Peroxidase Conjugate (Merk Millipore)
    - Anti-Rabbit IgG, HRP-linked (Cell Signaling)
- Chemiluminescent reagent: Luminata Classico Western HRP Substrate (Millipore)

3.2.6.3 Gel electrophoresis and protein transfer

We prepared gels at a 12.5% polyacrylamide final concentration. Then, we loaded prepared samples into wells in volumes corresponding to 30 μg in each well.

Electrophoresis was performed in running buffer at 120 V constant voltage until the forefront of the protein ladder almost reached the foot line of the glass plate.

Finally, separated proteins were transferred to a PVDF membrane in transfer buffer. Blotting transfer was performed at 170 mA per membrane for 1 hour in cold conditions.

3.2.6.4 Blocking, antibody incubation and detection

1. Incubate the membrane in blocking buffer for 1 hour with gentle shaking.
2. Wash the membrane with 1X PBS-T once for 15 min, then twice for 5 min each.
3. Dilute the primary antibody following manufacturer's instructions in blocking buffer. Incubate the membrane overnight at 4ºC with gentle shaking.
4. Wash the membrane as in step 2.
5. Dilute the secondary antibody following manufacturer's instructions in blocking buffer. Incubate the membrane for 1 hour at room temperature with gentle shaking.
6. Wash the membrane as in step 2.
7. Drain the excess buffer from the membrane, place it on a plastic sheet with the protein side up. Prepare the chemiluminescent reagents according to manufacturer's directions just before use. Make just enough to cover the membrane with chemiluminescent reagent.
8. Incubate for 1 min in the dark prior to exposure and analysis.

Figure 3.6 summarizes the whole western blot procedure.

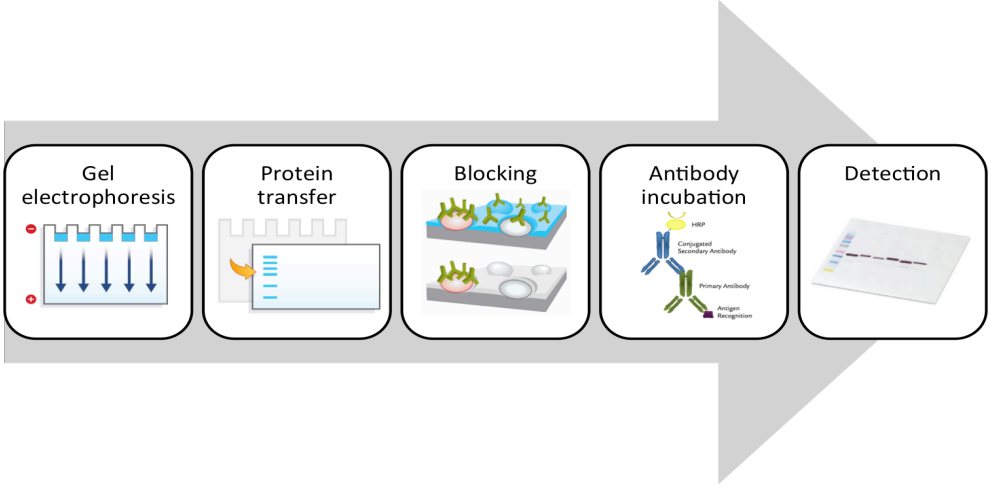

Figure 3.6 Western blot workflow.

3.2.6.5 Data analysis

Images obtained were stored in ".tif" digital format in order to perform the densitometry using "Image J" software. The values of the proteins studied were expressed in relation to the values obtained from α-tubulin in the same samples. This protein is part of the microtubules of eukaryotic cells, without significant changes in the different organisms (Little and Seehaus, 1988; Doolittle, 1992). Since it is a protein that is not altered, it is a good reference to control that the same amount of protein of each of our samples has been loaded during the experiment.

3.2.7 Carbonylated protein determination

3.2.7.1 Description

Oxidative modification of proteins by oxygen free radicals and other reactive species occurs in physiologic and pathologic processes. As a consequence of the modification, carbonyl groups are introduced into protein side chains by a site-specific mechanism. The carbonyl groups in the protein side chains are derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples are separated by polyacrylamide gel electrophoresis followed by western blot.

3.2.7.2 Reagents

To perform western blot of protein carbonylation, we used Oxyblot™ Protein Oxidation Detection Kit (Merk Millipore), which includes these reagents:

- 1X 2,4-Dinitrophenylhydrazine (DNPH) solution
- Neutralisation solution
- 1X Derivatization control solution
- 12% SDS
- Blocking buffer (1% BSA in PBS-T)
- Washing buffer PBS-T (0.05% Tween-20 in 1X PBS (pH 7.2-7.5)

- Primary antibody: Rabbit Anti-DNP
- Secondary antibody: Goat Anti-Rabbit IgG (HRP-conjugated)

3.2.7.3 Procedure

It is recommended that a reducing agent, such as either 1-2% 2-mercaptoethanol or 50 mM DTT, be added to the lysis buffer to prevent the oxidation of proteins that may occur after cell lysis.

1. Transfer 5 µL of a protein sample into Eppendorf tubes.
2. Denature each 5 µL aliquot of protein by adding 5 µL of 12% SDS for a final concentration of 6% SDS.
3. Derivatize the sample by adding 10 µL of 1X DNPH Solution.
4. Incubate tubes at room temperature for 15 minutes.
5. Add 7.5 µL of Neutralization Solution.
6. Samples are ready to load into a polyacrylamide gel.
7. Proceed as in 3.2.6.3. and 3.2.6.4 following manufacturer's instructions.

3.2.8 RNA isolation

3.2.8.1 Description

TRIzol® Reagent is a ready-to-use reagent, designed to isolate high quality total RNA (as well as DNA and proteins) from cell and tissue samples of human, animal, plant, yeast, or bacterial origin, within one hour. TRIzol® Reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components which facilitate the isolation of a variety of RNA species of large or small molecular size.

TRIzol® Reagent maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization (Chomczynski and Sacchi, 1987). After

homogenizing the sample with TRIzol® Reagent, chloroform is added, and the homogenate is allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing the DNA and proteins). RNA is precipitated from the aqueous layer with isopropanol. DNA is precipitated from the interphase/organic layer with ethanol. Protein is precipitated from the phenol-ethanol supernatant by isopropanol precipitation.

3.2.8.2 Reagents

The following additional reagents are needed:

- TRIzol® buffer
- Chloroform
- Isopropyl alcohol
- 75% ethanol (in DEPC-treated water)
- RNase free water or 0.5% SDS

3.2.8.3 Procedure

1. Homogenizing adherent cells (monolayer)
   - Remove growth media from culture dish.
   - Add 1 mL TRIzol® Reagent directly to the cells in the culture dish per 10 cm^2 of culture dish surface area.
   - Lyse the cells directly in the culture dish by pipetting the cells up and down several times.
2. Phase separation
   - Incubate the homogenized sample for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.
   - Add 0.2 mL of chloroform per 1 mL of TRIzol® Reagent used for homogenization. Cap the tube securely.
   - Shake tube vigorously by hand for 15 seconds.

- Incubate for 2-3 minutes at room temperature.
- Centrifuge the sample at 12,000 g for 15 minutes at 4°C. The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is ~50% of the total volume.
- Remove the aqueous phase of the sample by angling the tube at 45° and pipetting the solution out.
- Place the aqueous phase into a new tube.

3. RNA precipitation
   - Add 0.5 mL of 100% isopropanol to the aqueous phase, per 1 mL of TRIzol® Reagent used for homogenization.
   - Incubate at room temperature for 10 minutes.
   - Centrifuge at 12,000 g for 10 minutes at 4°C. The RNA is often invisible prior to centrifugation, and forms a gel-like pellet on the side and bottom of the tube.
4. RNA wash
   - Remove the supernatant from the tube, leaving only the RNA pellet.
   - Wash the pellet, with 1 mL of 75% ethanol per 1 mL of TRIzol® Reagent used in the initial homogenization.
   - Vortex the sample briefly, then centrifuge the tube at 7500 g for 5 minutes at 4°C. Discard the wash.
   - Air dry the RNA pellet for 5-10 minutes.
5. RNA resuspension
   - Resuspend the RNA pellet in RNase free water or 0.5% SDS solution (20-50 μL) by passing the solution up and down several times through a pipette tip.
   - Incubate in a water bath or heat block set at 55-60°C for 10-15 minutes.

3.2.8.4 RNA yield determination

Absorbance measurements were made on a NanoDrop™ 2000 Spectrophotometer, which include the absorbance of all molecules in the sample that absorb at the wavelength of interest. Since nucleotides, RNA, ssDNA, and dsDNA all absorb at 260 nm, they will contribute to the total absorbance of the sample.

- 260/280 ratio

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

- 260/230 ratio

This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm.

3.2.9 cDNA reverse transcription

3.2.9.1 Description

Reverse transcription consists of obtaining a DNA copy (cDNA) from a messenger RNA (mRNA), the reverse process of transcription. For this, particular DNA polymerases, called reverse transcriptases are necessary. The enzymes used come from retroviruses, which are viruses that present RNA as a genome, rather than DNA. In order to express their proteins, they have to pass the information to DNA. To obtain the cDNA, a reverse transcription was performed using the Applied Biosystems kit "High-Capacity cDNA Reverse Transcription".

3.2.9.2 Reagents

The High Capacity cDNA reverse transcription kit includes the reagents:

- 10X RT Buffer
- 10X RT Random Primers
- 25X dNTP Mix
- MultiScribe™ Reverse Transcriptase
- RNase inhibitor
- RNase free water

3.2.9.3 Procedure

1. To prepare the 2X Reverse Transcription Master Mix:
   - Allow the kit components to thaw on ice.
   - Referring to the Table 3.3, calculate the volume of components needed to prepare the required number of reactions. Prepare it on ice.
   - Place the 2X RT master mix on ice and mix gently.

Table 3.3 Reverse transcription components mixture.

Component	Volume / Reaction (µL)
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2.0
MultiScribe Reverse Transcriptase	1.0
RNases Inhibitor	1.0
Nuclease free water	3.2
Total volume per reaction	**10.0**

2. To prepare the cDNA RT reactions:
   - Pipet 10 µL of 2X RT master mix into each well of a 96-well plate.

- Pipet 10 µL of RNA sample into each well, pipetting up and down to mix.
- Seal the plates.
- Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
- Place the plate or tubes on ice until ready to load the thermal cycler.

3. To perform reverse transcription:
   - Program the thermal cycler conditions referring to the Table 3.4.
   - Set the reaction volume to 20 µL.
   - Load the reactions into the thermal cycler.
   - Start the reverse transcription run.

Table 3.4 Temperature conditions in reverse transcription assay.

	Step 1	Step 2	Step 3	Step 4
Temperature	25ºC	37ºC	85ºC	4ºC
Time	10 min	120 min	5 min	∞

3.2.10 Quantitative polymerase chain reaction (qPCR)

3.2.10.1 Description

The real-time polymerase chain reaction (RT-PCR) is a variant of the standard PCR, which is based on simultaneous detection and quantification of the fluorescence emitted by the PCR products that accumulate during the amplification process.

The fluorescent substance used in our experiments is SYBR Green I, which binds to DNA and emits fluorescence only when two strands of DNA are complementarily attached.

For quantitative PCR (qPCR), the measurement parameter of the expression of a given gene is not the fluorescence, but the cycle in which the amplification

begins to be exponential. This cycle is called the threshold cycle (Ct), since it is from which the amplification begins to be really appreciable (Figure 3.7). Thus, the threshold cycle values will decrease linearly as the amount of starting cDNA increases, since the more copies of starting mRNA of the gene being studied, the more cDNA will be obtained in the reverse transcription, and before the amplification will start to be exponential.

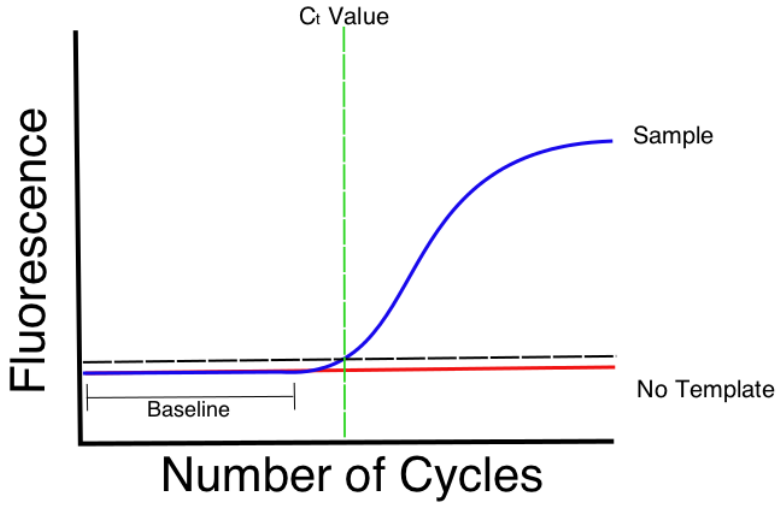

Figure 3.7 The PCR threshold cycle value.

3.2.10.2 Reagents

Maxima SYBR Green/ROX qPCR Master Mix (2X) is a ready-to-use solution optimized for quantitative real-time PCR. The master mix includes Maxima Hot Start *Taq* DNA Polymerase and dNTPs in an optimized PCR buffer. It contains SYBR Green dye and is supplemented with ROS passive reference dye. Only template and primers need to be added. Maxima Hot Start *Taq* DNA Polymerase in combination with an optimized buffer ensure PCR specificity and sensitivity. The SYBR Green intercalating dye allows for DNA detection and analysis without using sequence-specific probes.

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Prepare a reaction master mix by adding the following components as listed in Table 3.5 (except template DNA) for each reaction to a tube on ice.

Table 3.5 qPCR components mixture.

Component	Volume / Reaction (μL)
MAXIMA SYBR Green/ROX qPCR Master Mix (2X)	5.0
Primer sense	0.3
Primer antisense	0.3
DNA template	1.0
RNase free water	3.4
Total volume per reaction	**10.0**

3. Mix the master mix thoroughly and dispense 9 μL into each well of the PCR plate.
4. Add template DNA (≤ 500 ng/reaction) to each of the individual wells containing the master mix.
5. Gently mix the reactions without creating bubbles (do not vortex). Centrifuge briefly if needed.
6. Program the thermal cycler according to the recommendations below, place the samples in the machine and start the program (Table 3.6).

Table 3.6 Thermal cycling conditions in qPCR assay.

Step	Temperature (ºC)	Time	Number of cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing	60	30 sec	
Extension	72	30 sec	

The "primers" used for the determination of gene expression levels were designed using the GCG program from the gene sequences obtained in the "Genebank" of PubMed. For each of the genes to be studied, the following sequences are listed in Table 3.7.

Table 3.7 Gene primers: sense and antisense sequences.

PRIMER	Sense sequence	Antisense sequence
BMI-1	GCATCACAGTCATTGCTGCT	CAGGGCTTTTCAAAAATGA
SOX2	CTCGTCGATGAACGGCCGCT	AAAACAGCCCGGACCGCGTC
OCT4	GACTCCTGCTTCACCCTCAG	GATCCTCGGACCTGGCTAAG
c-MYC	CGTCGTCCGGGTCGCAGATG	CGCCCTCCTACGTTGCGGTC
KLF4	CAGGTCCAGGAGATCGTTGAA	CCCACATGAAGCGACTTCCC
$p14^{ARF}$	CATCATGACCTGGTCTTCTAGGAA	CCCTCGTGCTGATGCTACTG
$p16^{INK4a}$	GGTTGTGGCGGGGGCAGTT	GGGGGCACCAGAGGCAGT
GAPDH	TCCACCACCCTGTTGCTGTA	TGAACGGGAAGCTCACTGG

3.2.10.3 Procedure

The thermo cycling reaction begins once the PCR reagents are put into a thermo cycler machine, which is programmed to precisely heat and cool the reaction.

The PCR cycle begins with denaturation, which occurs for 20 to 30 seconds at 95°C, well above the melting temperature of DNA. The melting temperature is a state where half of the DNA is a double stranded helix and the other is a single stranded random coil. The denaturation temperature is well above the melting temperature, in order to ensure that all the hydrogen bonds between complementary base pairs are broken yielding only single stranded DNAs. Paired single strands are termed the sense and antisense strands. The sequence of the sense, or coding strand, is identical to the sequence of mRNA, which will ultimately code for protein.

In the second step, annealing, primers bind to the sense antisense strand. Depending on the length of primers used the annealing temperature for this step is usually 3 to 5°C below the lower melting temperature of your two primers. Annealing tends to occur between 50 and 65°C and lasts for 20 to 40 seconds.

Once the primers bind to DNA they prime the reaction by creating a 3' hydroxyl group end to which a polymerase, an enzyme that replicates DNA, will bind.

The next step called elongation or extension occurs at 72°C, which is optimal for polymerase activity. Once bound the polymerase begins to add free nucleotide triphosphates, or dNTPs, to the ends of the primer one at a time in the 5' to 3' direction to make double stranded DNA.

Once elongation completes the next cycle begins. The amount of amplicon will then increase exponentially in subsequent cycles. Figure 3.8 summarizes the qPCR procedure.

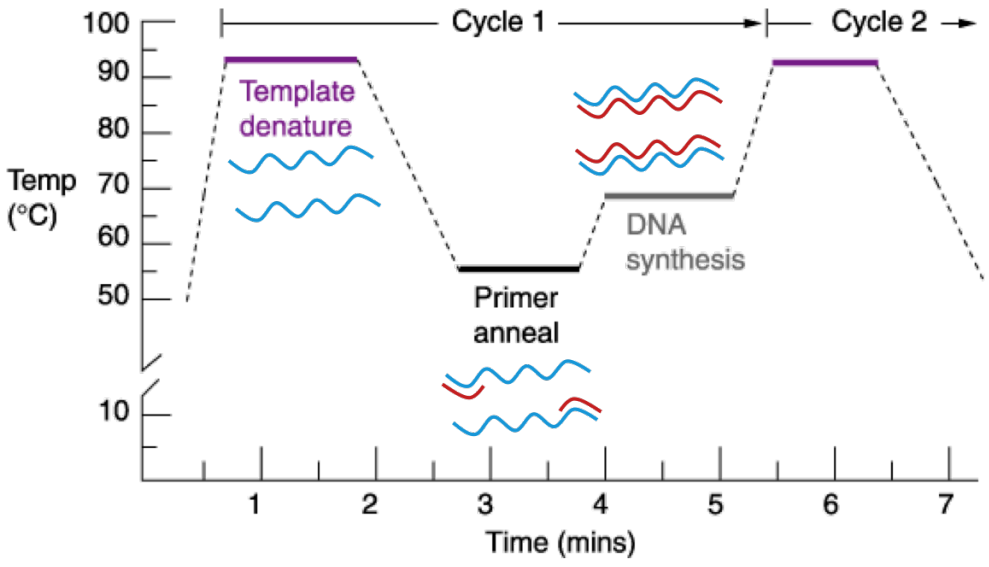

Figure 3.8 The PCR cycle steps.

3.2.10.4 Data analysis

When measuring gene expression, qPCR will tell how much of a specific mRNA there is in the samples. It amplifies a small region of this mRNA with oligos and a fluorescent probe. The qPCR machine measures the intensity of fluorescence emitted by the probe at each cycle. During the first cycles, there is not enough fluorescence to be detected, but the reaction rapidly produces more and more amplicons and the fluorescence builds up. As previously said, a qPCR curve has typically an exponential phase followed by a plateau phase. The cycle threshold (Ct) measure needs to be taken in the exponential phase, where the curve is linear.

The Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the Ct level the greater the amount of target nucleic acid in the sample).

- Cts < 29 are indicative of abundant target nucleic acid in the sample.
- Cts of 30-37 are indicative of moderate amounts of target nucleic acid.
- Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination.

The Relative Standard Curve experiment is used to determine relative target quantity in samples. In a Relative Standard Curve experiment:

1. The software measures amplification of the target of interest and of an endogenous control target in a standard dilution series, in a reference (calibrator) sample, and in test samples.
2. The endogenous control here used is GAPDH, which is a target that is expressed equally in all samples.
3. The software generates standard curves for the target of interest and the endogenous control using data from the corresponding standard dilution series.

In order to have high efficiency, the slopes must be similar, since the efficiency of the amplification reaction is given by the following equation:

Efficiency = [10 (-1 / slope)] – 1

For a slope of -3.322 we obtain an efficiency of 100%, which means that the increase of one cycle of amplification during the exponential phase of the reaction supposes exactly the duplication of the amplified material. An amplification reaction should have an efficiency close to 100% to be optimized.

4. Using the standard curves, the software interpolates the quantities of the target of interest and the endogenous control in each sample. The target quantity in each sample is then normalized to the sample's endogenous control quantity.
5. To determine the relative quantity of the target in test samples, the software divides the normalized target quantity in the sample by the normalized target quantity in the reference sample.

3.2.11 Statistical analysis

For statistical analysis, the IBM SPSS statistics program, version 19, has been used. A 95% confidence interval (p = 0.05) has been taken to accept that there is a statistically significant difference between the means of the groups.

The T-Student test was used to compare 2 means, and the analysis of variance (ANOVA) if more than 2 means were compared with a variation factor. If the n of the groups to compare is not the same in all of them, the Scheffé comparison was been used. If the n of the groups to be compared is the same in all of them, it will depend on the homogeneity test of the variances (Levene test). If it is not significant, Tukey's post-hoc comparison, if meaningful, has been used by Games-Howell.

4 RESULTS

4.1 AMBIENT OXYGEN TENSION INDUCES OXIDATIVE STRESS IN hDPSCs LONG-TERM CULTURE

It is well known that oxygen pressure (pO_2) is a critical cell culture parameter which can cause oxidative stress. As previously mentioned, oxidative stress can damage DNA, carbohydrates, proteins and lipids. However, other molecular biological markers can also be measured to provide a more immediate, quantitative risk assessment of potentially deleterious environmental exposures.

We first studied some parameters of oxidative stress in hDPSCs cultured under ambient oxygen tension compared to their physiological oxygen tension. Specifically, we assessed ROS production by dihydrorhodamine-123 (DHR123) staining, and mitochondrial membrane potential ($\Delta\Psi m$) using tetramethylrhodamine methyl ester (TMRM) staining. We next analysed protein damage by measuring protein carbonylation and finally, lipid damage by setting malondialdehyde (MDA) levels.

Antioxidant enzymes regulate the balance between production and/or accumulation of ROS and their neutralization. Under physiological conditions, this redox balance is maintained by various enzymes including superoxide dismutases (SOD), catalase, and glutathione peroxidases (GPx) (Halliwell and Gutteridge, 1995). Thus, we also analysed the expression levels of those antioxidant enzymes.

All those parameters were measured every 5 passages in hDPSCs cultured at 3% or 21% pO_2.

4.1.1 Reactive oxygen species (ROS) levels

In the presence of high oxygen pressure, the formation of ROS is favoured (Ross et al., 2001) and in the presence of ROS, such as hydrogen peroxide, the non-fluorescent dye dihydrorhodamine-123 (DHR123) passively enters into the cells, and is oxidized to rhodamine-123 (R123), a cationic green fluorescent dye that can accumulate and localize into the mitochondria (O'Connell et al., 2002).

We compared the extent of ROS production in hDPSCs grown at 3% or 21% pO_2 along passages by flow cytometry. As shown in Figure 4.1, ROS levels were higher in hDPSCs cultured at 21% pO_2 than in those cultured at 3% pO_2. As passaging number raised, the intensity of DHR123 in hDPSCs increased under either both oxygen pressure percentage. However, less ROS were accumulated when cells were cultured under physiological oxygen tension. Thus, high oxygen tension may accelerate ROS accumulation in hDPSCs during long-term culture.

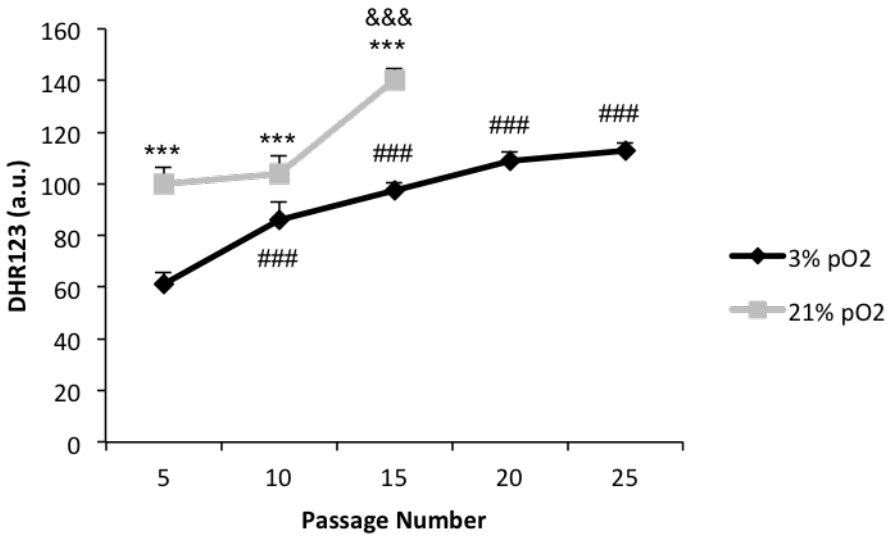

Figure 4.1 Intracellular ROS levels in hDPSCs cultured at 3% or 21% oxygen tension along passages. ROS levels measured using dihydrorhodamine-123 (DHR123) staining by flow cytometry. The data are shown as means ± SD (n=5). The statistical significance is expressed as ***$p<0.001$ for 3% pO_2 *versus* 21% pO_2, $^{###}p<0.001$ *versus* 3% pO_2 at passage 5 and $^{&&&}p<0.001$ *versus* 21% pO_2 at passage 5.

4.1.2 Mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) is critical for maintaining the physiological function of the respiratory chain to generate ATP (Joshi and Bakowska, 2011). Fluorescence probes used to assess ΔΨm are single-wavelength indicators, which increase or decrease their fluorescence intensity, proportional to a stimulus that increases or decreases the levels of ΔΨm. In our case, cells were loaded with tetramethylrhodamine methyl ester (TMRM) prior to flow cytometry analysis.

As we can observe in Figure 4.2, at 3% pO_2, hDPSCs mitochondrial membrane potential values were significantly higher in comparison to 21% pO_2. As cells were serially passaged, the ΔΨm dropped gradually. Such findings show that mitochondria depolarize during long-term culture, and high oxygen tension can precipitate such functional deterioration.

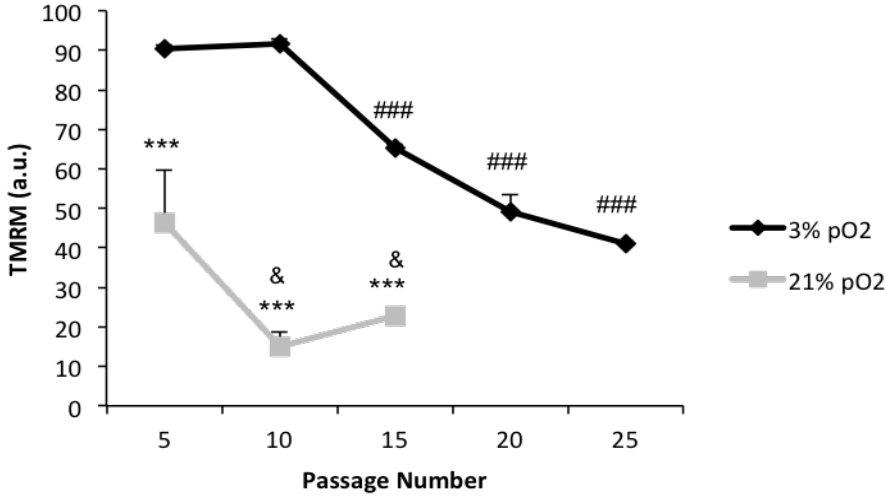

Figure 4.2 Mitochondrial membrane potential in hDPSCs cultured at 3% or 21% oxygen tension along passages.

Mitochondrial membrane potential determined using tetramethylrhodamine methyl ester (TMRM) staining by flow cytometry. The data are shown as means ± SD (n=5). The statistical significance is expressed as ***$p<0.001$ for 3% pO_2 *versus* 21% pO_2, $^{###}p<0.001$ *versus* 3% pO_2 at passage 5 and $^{&}p<0.05$ *versus* 21% pO_2 at passage 5.

4.1.3 Protein carbonylation

All amino-acid residues in the proteins are subjected to ROS attack (Stadtman, 1992). As a consequence, site-specific amino acid modification, peptide chain fragmentation, altered electric charge and increased susceptibility of proteins to proteolysis occur. Tissues injured by oxidative stress contain increased concentrations of carbonylated proteins, which is a widely used marker of protein oxidation (Moller and Kristensen, 2004). Protein carbonyl groups detection by western blot after their derivatization with 2,4-Dinitrophenylhydrazine (DNPH) is one of the most widely used measurement for protein oxidation.

As shown in Figure 4.3, protein carbonylation levels increased along passages during long-term culture. hDPSCs cultured at 21% pO_2 showed higher levels of protein carbonyls than those cultured at 3% pO_2. Thus, high oxygen tension may accelerate the damage accumulation to proteins.

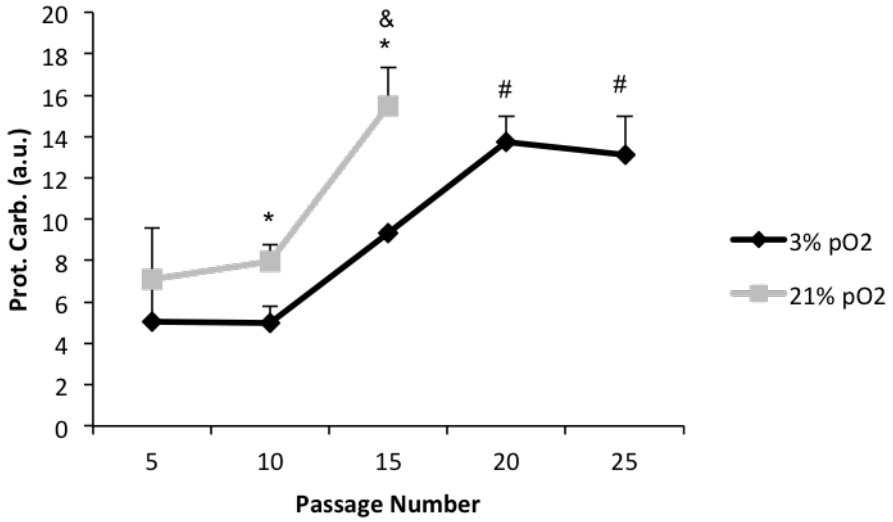

Figure 4.3 Protein carbonylation in hDPSCs cultured at 3% or 21% oxygen tension along passages. Protein carbonylation levels assessed by western blot. The data are shown as means ± SD (n=5). The statistical significance is expressed as *$p<0.05$ for 3% pO_2 *versus* 21% pO_2, $^{\#}p<0.05$ *versus* 3% pO_2 at passage 5 and $^{\&}p<0.05$ *versus* 21% pO_2 at passage 5.

4.1.4 Lipid oxidation

Lipid peroxidation plays an important role in cell aging and is most likely to be a key factor in lowering membrane fluidity (Shigenaga et al., 1994). Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids in phospholipids (Halliwell and Gutteridge, 1989). Lipid peroxidation determined as TBA-MDA2 adduct formation is an established method of accurately measuring oxidative damage to lipids (Nielsen et al., 1997).

As shown in Figure 4.4, hDPSCs cultured at 21% pO_2 displayed higher levels of MDA, which increased successively as passaging number did. In contrast, MDA levels in hDPSCs cultured at 3% pO_2 were lower and remained stable all along the 25 passages. Thus, high oxygen tension may be a cause for the increasing lipid damage in hDPSCs.

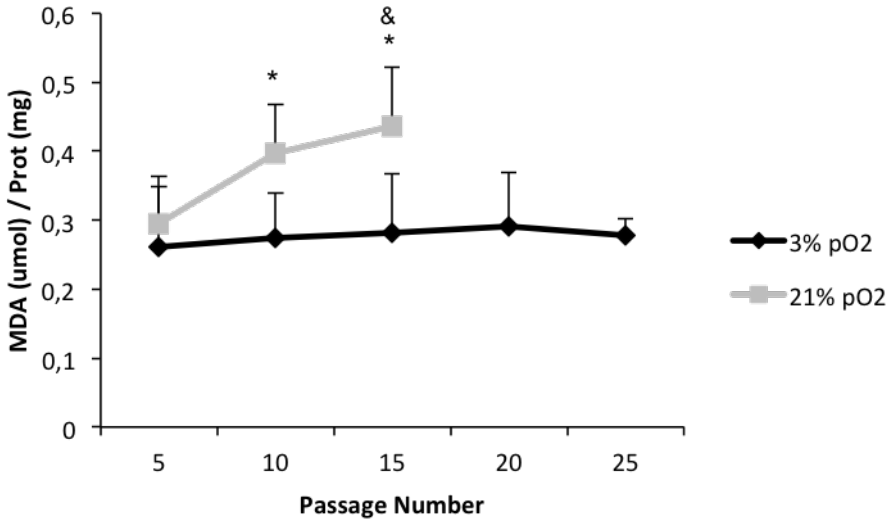

Figure 4.4 Lipid oxidation in hDPSCs cultured at 3% or 21% oxygen tension along passages. Malondialdehyde (MDA) levels determined by high performance liquid chromatography (HPLC). The data are shown as means ± SD (n=5). The statistical significance is expressed as *$p<0.05$ for 3% pO_2 *versus* 21% pO_2 and $^{\&}p<0.05$ *versus* 21% pO_2 at passage 5.

4.1.5 Antioxidant gene expression

Since ROS could potentially damage lipids, proteins and DNA, cells developed several defence mechanisms, which include antioxidant enzymes and targeted degradation pathways (Fan et al., 2008). Associated with their isoforms, manganese superoxide dismutase (MnSOD), catalase (CAT) and glutathione peroxidase (GPx), are easily induced by oxidative stress (Hermes-Lima and Zenteno-Savín, 2002). The MnSOD decomposes superoxide radicals and produce H_2O_2. H_2O_2 is subsequently removed to water by CAT in the peroxisomes, or by GPx oxidizing GSH in the cytosol (Droge, 2002; Lee and Choi, 2003).

The mRNA levels of antioxidant enzymes in hDPSCs at passage 5 were detected by qPCR and compared. Figure 4.5 shows that mRNAs levels of *MnSOD*, *CAT* and *GPx* were affected by oxygen tension. Apparently, more mRNAs of all three enzymes were observed in young hDPSCs cultured at 21% compared to 3% pO_2, suggesting that these cells might need to increase their antioxidant mechanisms to face ROS accumulation since early stages.

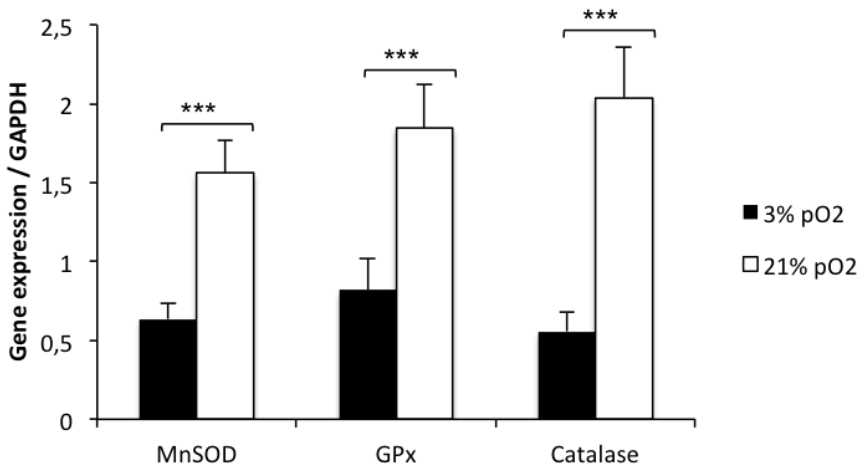

Figure 4.5 Antioxidant enzymes gene expression levels in hDPSCs cultured at 3% or 21% oxygen tension.

MnSOD, *GPx* and *CAT* mRNA levels analysed by qPCR against housekeeping gene. The data are shown as means ± SD (n=5). The statistical significance is expressed as ***$p<0.001$ for 3% pO_2 *versus* 21% pO_2.

4.2 OXIDATIVE STRESS INDUCES PREMATURE SENESCENCE UNDER AMBIENT OXYGEN TENSION DURING LONG-TERM CULTURE OF hDPSCs

Long-term *in vitro* culture undergoes the risk of senescence (Fu et al., 2015). Therefore, we measured some senescence biomarkers in hDPSCs cultured at both oxygen pressures. We first analysed hDPSCs morphology and proliferation kinetics, then cells were stained for senescence-associated expression of β-galactosidase by flow cytometry.

4.2.1 hDPSCS morphology

After a long period of *in vitro* culture, hDPSCs showed abnormalities typical of the Hayflick model of cellular aging. The cells varied in size and shape, the cytoplasm began to be granular with many cell inclusions, and debris was observed in the medium. However, as we can observe in Figure 4.6, hDPSCs cultured under ambient oxygen tension showed larger shapes at passage 15 than to those cultured at 3% pO_2 at passage 25.

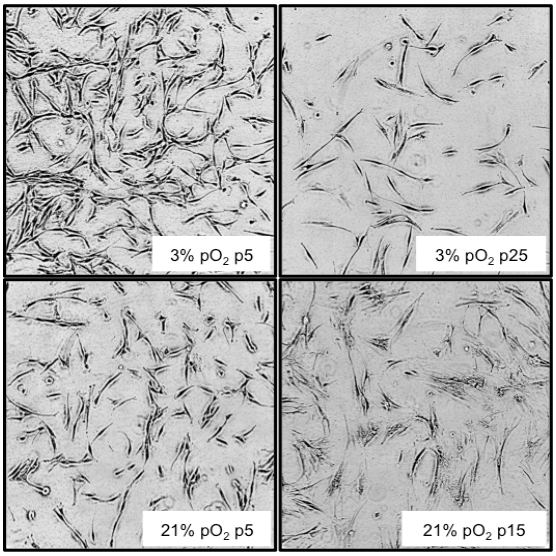

Figure 4.6 Morphological changes of hDPSCs cultured at 3% or 21% oxygen tension. Optical microscope images (20x).

4.2.2 hDPSCS proliferation

In order to examine long-term growth kinetics of hDPSCs culture, we performed serial passaging until culture exhaustion. hDPSCs from the same donor were separated and cultured at 21% or 3% pO_2; the beginning of the serial passaging was the same in both conditions. As passage number increased, the cells cultured at 21% pO_2 began to proliferate more slowly and the time between sub-culturing doubled. As a consequence, we can see in Figure 4.7 that hDPSCs cultured under ambient oxygen tension barely achieved 15 passages while hDPSCs cultured under physiological oxygen tension reached 25 passages. This is the reason why all figures show data up to 15 passages when hDPSCs were cultured at 21% pO_2, and up to 25 passages when cultured at 3% pO_2.

Taken together, these facts suggest that after 25 passages, hDPSCs cultured in physiological oxygen tension were less senescent according to their morphology and their proliferative potential than those cultured at 21% pO_2. Thus, hDPSCs cultured at standard culture condition, are prone to premature senescence upon serial passage.

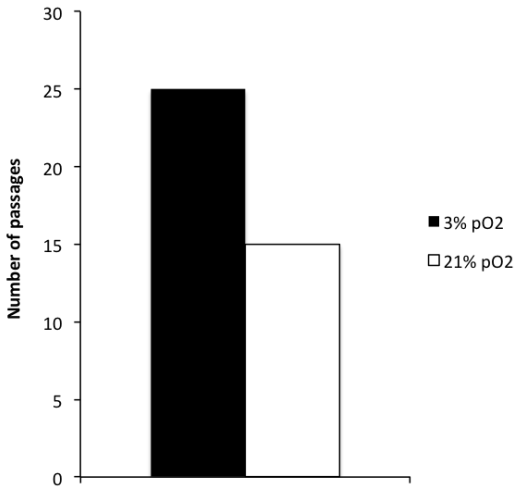

Figure 4.7 Proliferation potential of hDPSCs cultured at 3% or 21% oxygen tension.
Passaging number achieved by hDPSCs cultured under both oxygen conditions.

4.2.3 Senescence-associated β-galactosidase (SA-β-Gal) activity

SA-β-Gal is a commonly used senescence biomarker (Dimri et al. 1995; Debacq-Chainiaux et al. 2009). Accordingly, the increase in SA-β-Gal activity in senescent cells is likely due to an expansion of the lysosomal compartment, giving rise to an increase in β-galactosidase activity that can be measured also at suboptimal pH 6 (Kurz et al. 2000; Yang and Hu 2005; Lee et al. 2006).

To confirm our morphological observations of senescence, cells were stained for senescence-associated expression of β-galactosidase and analysed by flow cytometry. The proportion of cells staining positive for such expression increased as passage number did (Figure 4.8) either at 3% and 21% pO_2. However, hDPSCs cultured at 21% pO_2 showed significantly higher levels of SA-β-Gal at all stages. This result confirms our morphological studies, suggesting that senescence increases with long-term culture, and is accelerated by ambient oxygen pressure.

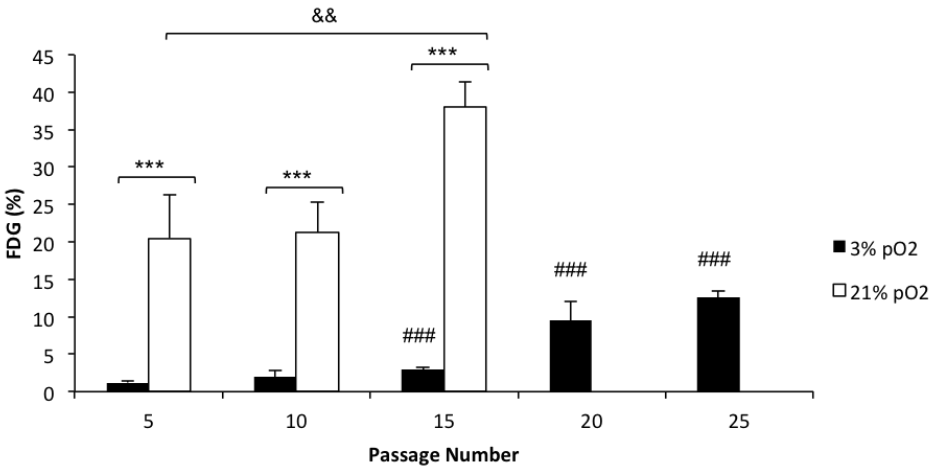

Figure 4.8 SA-β-Gal activity in hDPSCs cultured at 3% or 21% oxygen tension along passages. Senescence-associated β-galactosidase activity measured using FDG staining by flow cytometry. The data are shown as means ± SD (n=5). The statistical significance is expressed as ***$p<0.001$ for 3% pO_2 *versus* 21% pO_2, $^{###}p<0.001$ *versus* 3% pO_2 at passage 5 and $^{&&}p<0.01$ *versus* 21% pO_2 at passage 5.

4.3 OXIDATIVE STRESS-INDUCED PREMATURE SENESCENCE (SIPS) IS MEDIATED BY $p16^{INK4A}$ PATHWAY

It is now clear that many stimuli, including oxidative stress, cause cells to arrest growth with a senescent phenotype (Serrano et al., 1997; Dimri et al., 2000; Sherr and DePinho, 2000). *In vitro*, premature senescence can result from inadequate culturing conditions. When cells are explanted from an organism and placed in culture, they have to adapt to an artificial environment, characterized by abnormal concentrations of nutrients and growth factors and the presence of ambient oxygen levels, as well as the absence of surrounding cell types and extracellular matrix components. One or more of these conditions can induce a culture shock, resulting in stress-induced senescence (Kuilman et al., 2010).

Long-term exit from the cell cycle is the central and indispensable marker for the identification of all types of cellular senescence *in vitro*. Cell cycle is controlled by the *CDKN2A-CDKN2B* locus, also known as the *INK/ARF* locus. This locus encodes 3 tumour suppressor genes; the *CDKN2B* gene encodes $p15^{INK4b}$, and the *CDKN2A* gene encodes both $p16^{INK4a}$ and $p14^{ARF}$. On one hand, $p15^{INK4b}$ and $p16^{INK4a}$ proteins inhibit cyclin D-dependent CDK4 and CDK6 to prevent phosphorylation of pRb, thus inhibiting the transcription of genes involved in the transition to S phase (importantly the pRb/E2F pathway), so regulating cell cycle progression (Yaswen et al., 2015). On the other hand, $p14^{ARF}$ protein binds to MDM2 E3 ubiquitin ligase to prevent p53 polyubiquitination and to facilitate p53 activation and stabilization of p53 (Matheu et al., 2008). Finally, this leads to induction of various p53 target genes involved in cell cycle arrest and apoptosis. Both cascades commonly mediate the activation of the senescence program (Sharpless and Sherr, 2015).

We therefore sought to determine which pathway, either $p14^{ARF}$/MDM2/p53 or $p16^{INK4a}$/pRb/E2F, was mediating the oxidative stress-induced premature senescence of hDPSCs when culturing at 21% pO_2.

4.3.1 $p14^{ARF}$ gene expression pattern

$p14^{ARF}$ mRNA levels were assessed in hDPSCs cultured under both oxygen pressures and compared. As shown in the figure below, hDPSCs expressed $p14^{ARF}$, but its levels remained unchanged along passages when cells were cultured at 3% pO_2 as no statistical difference was detected (Figure 4.9). This result may suggest that $p14^{ARF}$/MDM2/p53 pathway is not mediating the oxidative stress-induced premature senescence of hDPSCs cultured at 21% pO_2.

Furthermore, as we can see in the same figure, hDPSCs cultured under ambient oxygen tension showed a downregulation of $p14^{ARF}$ expression levels as passages accumulated. At passage 15, $p14^{ARF}$ levels of hDPSCs cultured at 21% pO_2 are significantly lower than those of hDPSCs cultured at 21% pO_2.

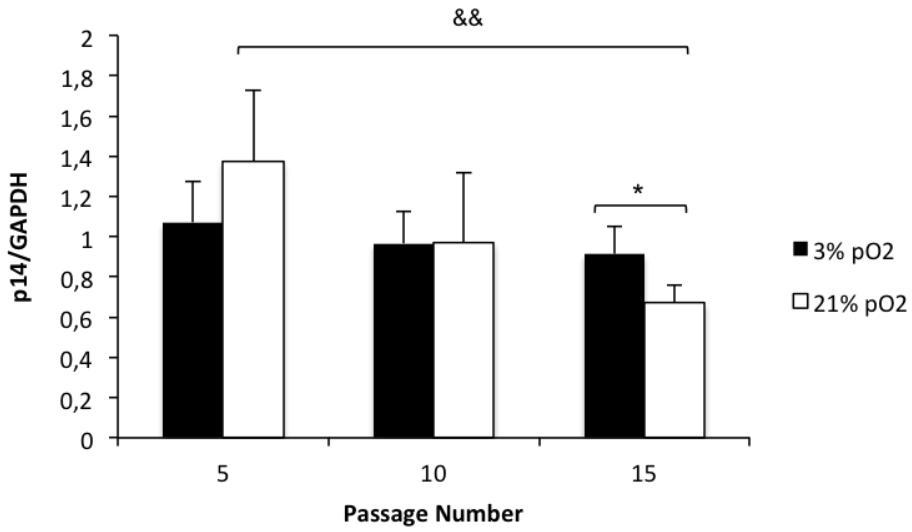

Figure 4.9 $p14^{ARF}$ gene expression levels in hDPSCs long-term culture at 3% or 21% oxygen tension. $p14^{ARF}$ mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=5). The statistical significance is expressed as *$p<0.05$ for 3% pO_2 *versus* 21% pO_2 and $^{\&\&}p<0.01$ *versus* 21% pO_2 at passage 5.

4.3.2 $p16^{INK4a}$ gene expression pattern

We next compared $p16^{INK4a}$ mRNA levels in hDPSCs cultured under both oxygen pressures. Figure 4.10 shows that hDPSCs also expressed $p16^{INK4a}$, but in this case, the levels of $p16^{INK4a}$ increased along passages in 21% pO_2 cultured-hDPSCs. Furthermore, 3% pO_2-cultured hDPSCs retained a lower level of the $p16^{INK4a}$ expression, which was constant along passages.

Taken together, these results may suggest that $p16^{INK4a}$, but not $p14^{ARF}$ pathway, is mediating oxidative stress-induced premature senescence in hDPSCs cultured under ambient oxygen tension.

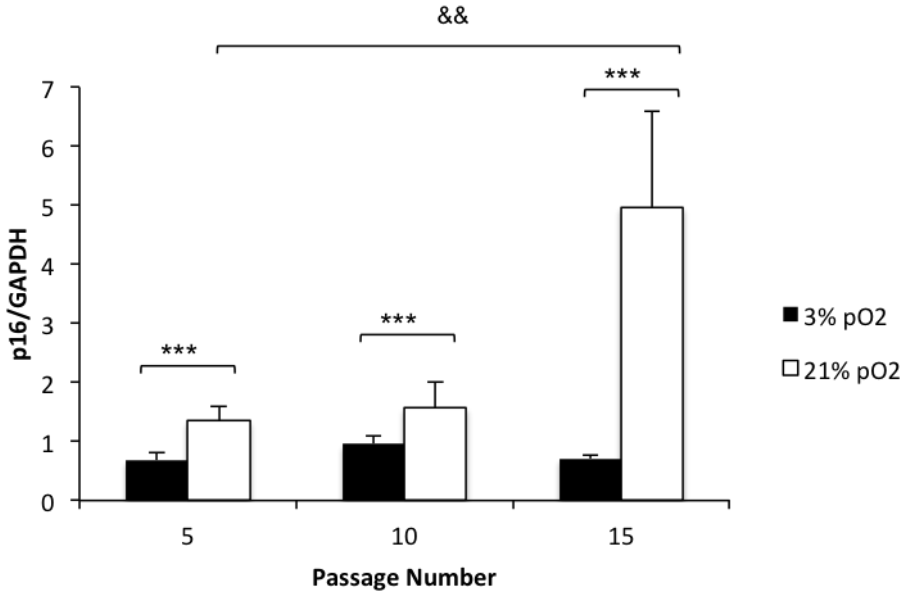

Figure 4.10 $p16^{INK4a}$ gene expression levels in hDPSCs long-term culture at 3% or 21% oxygen tension.

$p16^{INK4a}$ mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=5). The statistical significance is expressed as ***$p<0.001$ for 3% pO_2 *versus* 21% pO_2 and $^{\&\&}p<0.01$ *versus* 21% pO_2 at passage 5.

4.3.3 Trolox can reverse oxidative stress effect on $p16^{INK4a}$ expression

In order to further investigate the $p16^{INK4a}$ implication on this ROS-mediated upregulation, we wanted to ensure if its expression was effectively submitted to ROS production at 21% pO_2 culture. We therefore treated hDPSCs cultured under ambient oxygen tension with an antioxidant. We used Trolox, a water soluble derivate of vitamin E, at a final concentration of 50 μM.

hDPSCs cultured under ambient oxygen tension and treated with Trolox showed comparable levels of $p16^{INK4a}$ mRNA expression as hDPSCs cultured at 3% pO_2, which means that Trolox can restore $p16^{INK4a}$ levels under oxidative stress conditions (Figure 4.11). Taken together, this result suggests that the $p16^{INK4a}$-associated premature senescence observed in hDPSCs cultured under ambient oxygen tension, is due to an increased ROS.

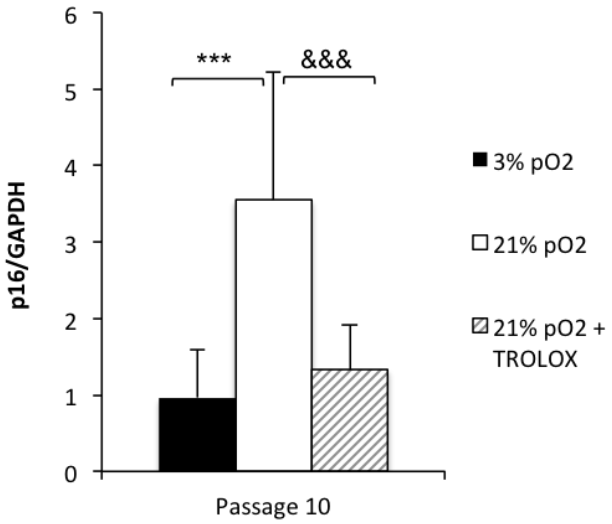

Figure 4.11 $p16^{INK4a}$ gene expression levels in hDPSCs with 50 μM Trolox treatment.
$p16^{INK4a}$ mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=5). The statistical significance is expressed as ***$p<0.001$ versus 3% pO_2 and $^{\&\&\&}p<0.001$ versus 21% O_2 + 50 μM Trolox.

4.4 LOSS OF STEMNESS UNDER AMBIENT OXYGEN TENSION DURING LONG-TERM CULTURE OF hDPSCs

Senescence-associated markers are usually not restricted to senescent passages but are continuously acquired since the beginning of the *in vitro* culture. Moreover, no marker or hallmark has been proved completely unique to the senescent state in cells, and not all senescent cells express all senescence markers. Thus, alternative markers would be useful in the assessment of cellular senescence (Wagner et al., 2008).

Because they exhibit an opposite biological function compared to senescence, pluripotency-related transcription factors could be pointed as a potential candidate marker of the senescent state of cells, especially for stem cells. In fact, it has been suggested that the expression of these markers is essential in maintaining the stem cell properties (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007).

Low oxygen tension in cell culture has proved to have positive effects on the *in vitro* survival and self-renewal of stem cells. However, the effects of low oxygen tension on the expression of these pluripotency markers and stemness maintenance in hDPSCs has not been investigated yet. In this study, we examined the gene expression pattern of the above mentioned pluripotency markers in hDPSCs under both ambient and physiological oxygen tension culture conditions along passages. To this end, we analysed the gene expression of four stemness markers, *OCT4*, *SOX2*, *KLF4* and *c-MYC*, which make the core transcription network responsible for the regulation of stem cell self-renewal and pluripotency, by using quantitative real-time polymerase chain reaction technique.

4.4.1 *OCT4* gene expression pattern

As we can see in Figures 4.12 to 4.15, hDPSCs expressed all four transcription factors.

Focusing just on Figure 4.12, we can observe that *OCT4* expression levels were higher at passage 5, and then, as passages accumulate, they rapidly plummeted. Furthermore, cells cultured at 3% pO_2 showed significantly higher levels of this transcription factor at passage 5 in comparison with hDPSCs cultured at 21% pO_2.

Thus, high oxygen tension seems to have a negative effect on *OCT4* expression at early passages.

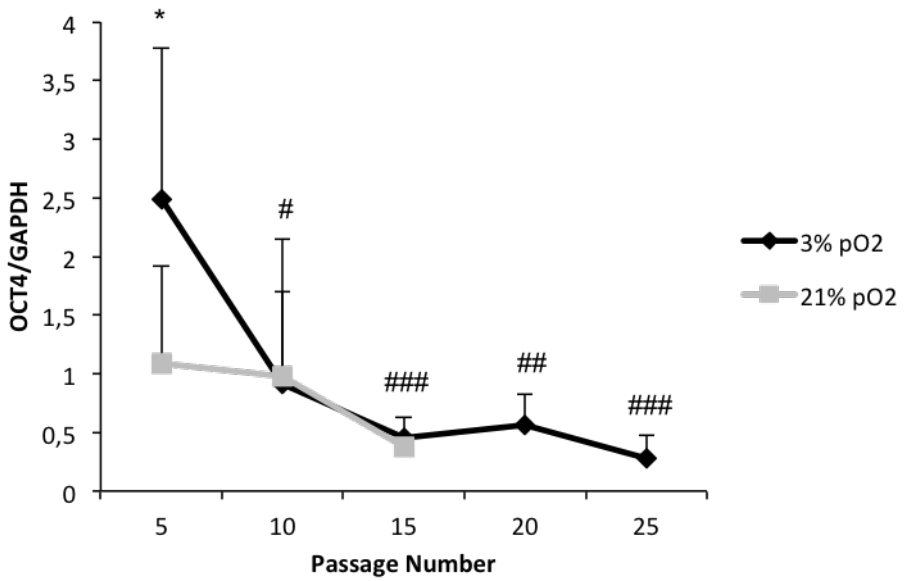

Figure 4.12 *OCT4* gene expression levels in hDPSCs long-term culture at 3% or 21% oxygen tension. *OCT4* mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=5). The statistical significance is expressed as *$p<0.05$ for 3% pO_2 *versus* 21% pO_2 and $^{\#}p<0.05$, $^{\#\#}p<0.01$ or $^{\#\#\#}p<0.001$ *versus* 3% pO_2 at passage 5.

4.4.2 *SOX2* gene expression pattern

Similarly, hDPSCs also expressed *SOX2* transcription factor. As we can see in the figure below, both *OCT4* and *SOX2* appeared to have a comparable expression pattern (Figure 4.13).

SOX2 gene expression levels were higher at early stages, and they were downregulated along passages. Moreover, ambient oxygen tension caused a reduction of *SOX2* expression levels at passage 5.

Taken together, our data suggest that ambient oxygen tension may reduce *SOX2* and *OCT4* expression levels in young hDPSCs.

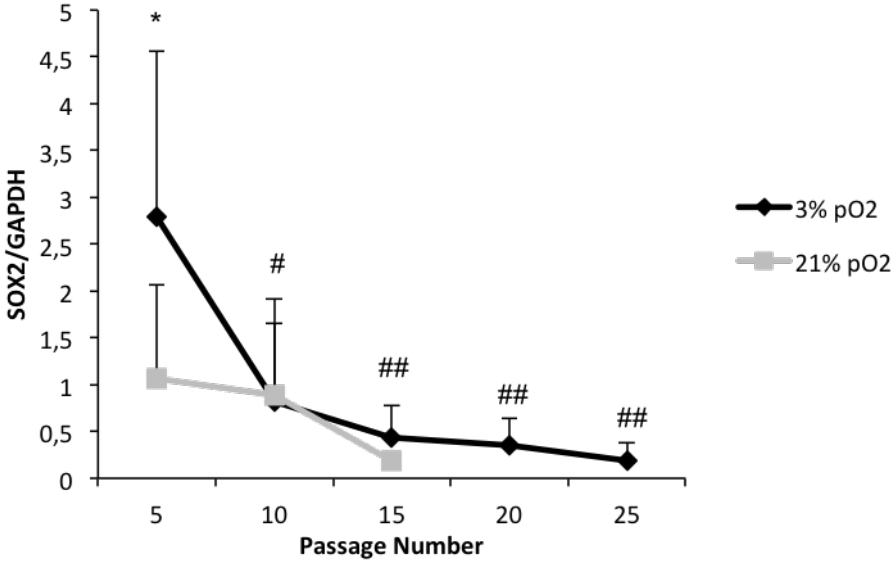

Figure 4.13 *SOX2* gene expression levels in hDPSCs long-term culture at 3% or 21% oxygen tension. *SOX2* mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=5). The statistical significance is expressed as *$p<0.05$ for 3% pO_2 *versus* 21% pO_2 and $^{\#}p<0.05$ or $^{\#\#}p<0.01$ *versus* 3% pO_2 at passage 5.

4.4.3 *KLF4* gene expression pattern

Figure 4.14 reveals that hDPSCs also expressed *KLF4* transcription factor. Contrarily to *OCT4* and *SOX2* expression pattern, *KLF4* gene expression levels increased along passages at 3% pO_2. Interestingly, *KLF4* expression was not affected by oxygen tension at passages 5 and 10, as no statistical difference were found. However, *KLF4* expression levels in hDPSCs cultured under ambient oxygen tension were significantly reduced at passage 15.

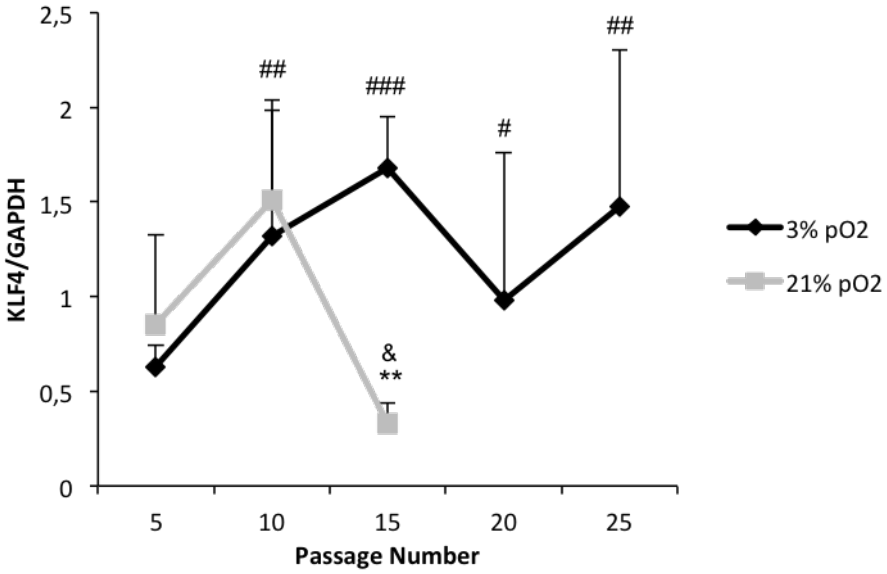

Figure 4.14 *KLF4* gene expression levels in hDPSCs long-term culture at 3% or 21% oxygen tension. *KLF4* mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=5). The statistical significance is expressed as **$p<0.01$ for 3% pO_2 *versus* 21% pO_2, $^{\#}p<0.05$, $^{\#\#}p<0.01$ or $^{\#\#\#}p<0.001$ *versus* 3% pO_2 at passage 5, and $^{\&}p<0.05$ *versus* 21% pO_2 at passage 5.

4.4.4 *c-MYC* gene expression pattern

Finally, hDPSCs also expressed *c-MYC* transcription factor. In the below figure, we can observe that *c-MYC* expression pattern was similar to the one of *KLF4* (Figure 4.15). *c-MYC* expression levels increased along passages in hDPSCs cultured at 3% pO_2. We did not found statistical differences at passages 5 and 10, but we did found at passage 15 when comparing both oxygen culture pressures.

Taken together, we can establish that high oxygen tension has a negative effect on OSKM gene expression during long-term culture of hDPSCs.

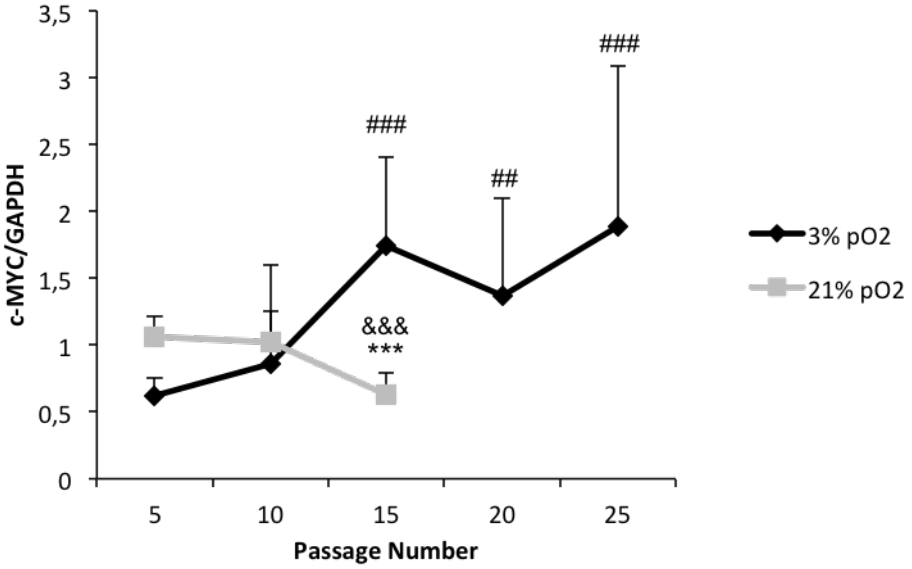

Figure 4.15 *c-MYC* gene expression levels in hDPSCs long-term culture at 3% or 21% oxygen tension.

c-MYC mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=5). The statistical significance is expressed as ***$p<0.001$ for 3% pO_2 *versus* 21% pO_2, $^{\#\#}p<0.01$ or $^{\#\#\#}p<0.001$ *versus* 3% pO_2 at passage 5 and $^{\&\&\&}p<0.001$ *versus* 21% pO_2 at passage 5.

4.5 BMI-1 CAN RESCUE *SOX2* AND *OCT4* GENE EXPRESSION UNDER AMBIENT OXYGEN TENSION

Polycomb group (PcG) genes, such as *BMI-1*, are epigenetic gene silencers that preserve transcription patterns to maintain cell identity, a function clearly compatible with a role in self-renewal and stem cell maintenance (Lund and van Lohuizen, 2004; Seo et al., 2011). Understanding the molecular events downstream of *BMI-1* should provide valuable insight into how stem cells regulate proliferation and self-renewal.

Although $p16^{INK4a}$ and $p14^{ARF}$ have been shown to be BMI-1 downstream targets in the context of stem cell self-renewal, they do not account for all BMI-1 actions (Sparmann and van Lohuizen, 2006; Fasano et al., 2007). Recently, a relationship between BMI-1, SOX2 and OCT4 has been described (Seo et al., 2011; Kaufhold et al., 2016), as well as BMI-1 and c-MYC (Sahasrabuddhe et al., 2011). Thus we wanted to investigate whether BMI-1 was responsible for the loss of the stemness properties observed in hDPSCs cultured under ambient oxygen tension.

We first aimed to describe both *BMI-1* gene and BMI-1 protein expression in order to see if oxygen tension could also have any effect on its regulation. We next investigated the role of BMI-1 by using small interference RNA silencing to reduce BMI-1 levels in hDPSCs cultured under ambient oxygen tension at early stages, and then assessing downstream OSKM gene expression changes.

4.5.1 *BMI-1* gene expression pattern

BMI-1 gene expression levels in hDPSCs cultured under both oxygen pressures were assessed by qPCR and compared. As we can observe in Figure 4.16, *BMI-1* gene expression levels in hDPSCs cultured at 3% pO_2 increased as passaging number did. However, *BMI-1* expression remained constant at 21% pO_2.

Interestingly, ambient oxygen tension upregulated *BMI-1* expression at passage 5 in hDPSCs cultured at 21% pO_2 in comparison to 3% pO_2. Thus, oxygen tension may have an influence on *BMI-1* gene expression.

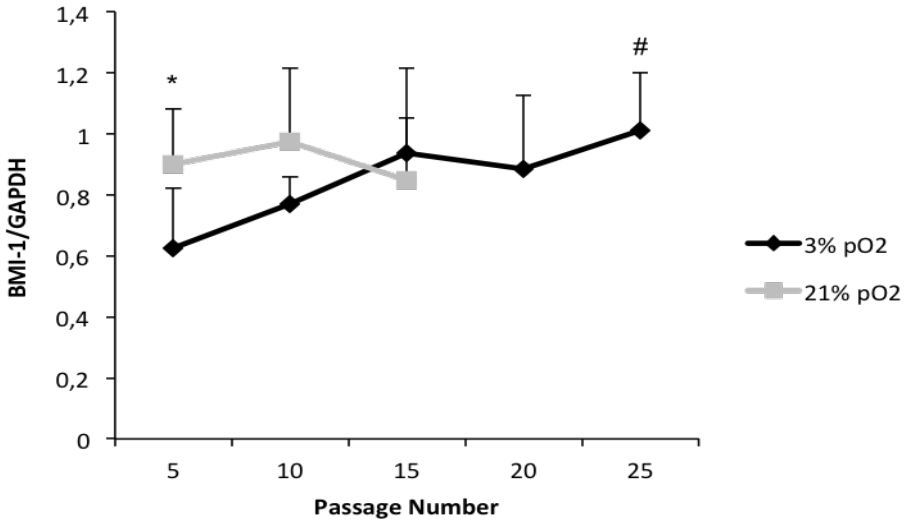

Figure 4.16 *BMI-1* gene expression levels in hDPSCs long-term culture at 3% or 21% oxygen tension.

BMI-1 mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=5). The statistical significance is expressed as *$p<0.05$ for 3% *versus* 21% pO_2 and $^{\#}p<0.05$ *versus* 3% pO_2 at passage 5.

4.5.2 BMI-1 protein expression pattern

Figure 4.17 shows BMI-1 protein levels assessed by western blot technique. We can observe that BMI-1 levels in hDPSCs cultured at 3% pO_2 remained constant along 20 passages, and then they began to decrease. By contrast, ambient oxygen tension accelerated the decrease of BMI-1 levels in hDPSCs, significantly reducing its levels from passage 5 to passage 15 when comparing to 3% pO_2.

Furthermore, 21% pO_2 cultured hDPSCs showed significantly higher levels of BMI-1 at passage 5 but significantly lover levels at passage 15 in comparison to those cultured at 3% pO_2.

Taken together, our data suggest that ambient oxygen tension may accelerate BMI-1 protein degradation machinery in hDPSCs.

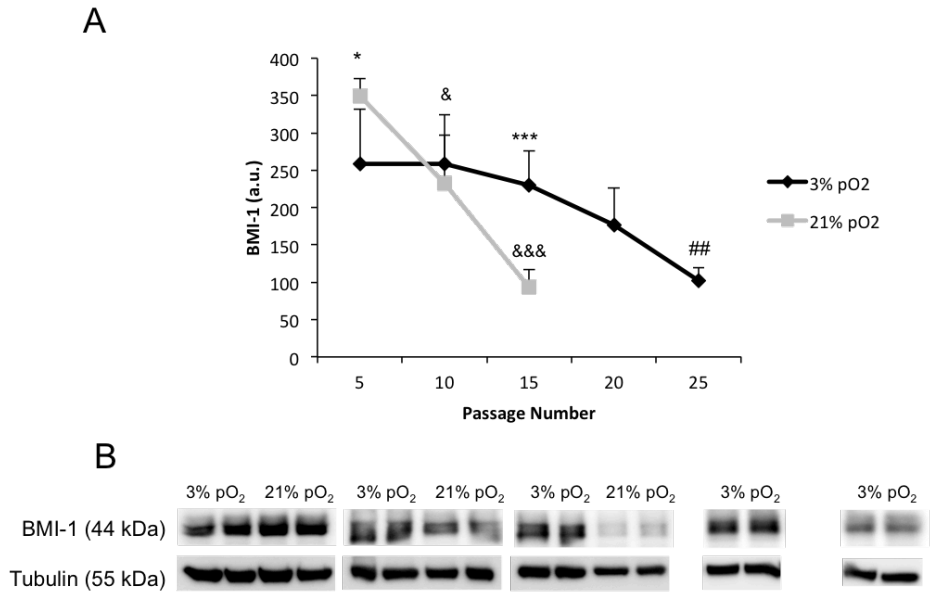

Figure 4.17 BMI-1 protein levels in hDPSCs long-term culture at 3% or 21% oxygen tension.
(A) BMI-1 protein levels assessed by western blot and (B) representative western blot image. The data are shown as means ± SD (n=5). The statistical significance is expressed as *$p<0.05$ or ***$p<0.001$ for 3% pO_2 *versus* 21% pO_2, $^{\#\#}p<0.01$ *versus* 3% pO_2 at passage 5 and, $^{\&}p<0.05$ or $^{\&\&\&}p<0.001$ *versus* 21% pO_2 at passage 5.

4.5.3 *BMI-1* gene and BMI-1 protein knockdown

Given the fact that there is a possibility that *SOX2* and *OCT4* expression are regulated by BMI-1 under oxidative stress conditions, we aimed to test whether restoring BMI-1 protein levels in hDPSCs cultured at 21% pO_2 could also recover *SOX2* and *OCT4* gene expression levels. To this end, we performed a mild knockdown of *BMI-1* gene expression using siRNA transfection protocol.

Figure 4.18, shows in (A) *BMI-1* gene expression levels (determined by quantitative real-time polymerase chain reaction) and in (B) BMI-1 protein levels (determined by western blot). After transfection, hDPSCs cultured at 21% pO_2 expressed significantly reduced levels of BMI-1 at passage 5, both at gene and protein level. Furthermore, silenced *BMI-1*gene and BMI-1 protein levels in hDPSCs cultured at 21% pO_2 were comparable to those in hDPSCs cultured under physiological oxygen pressure.

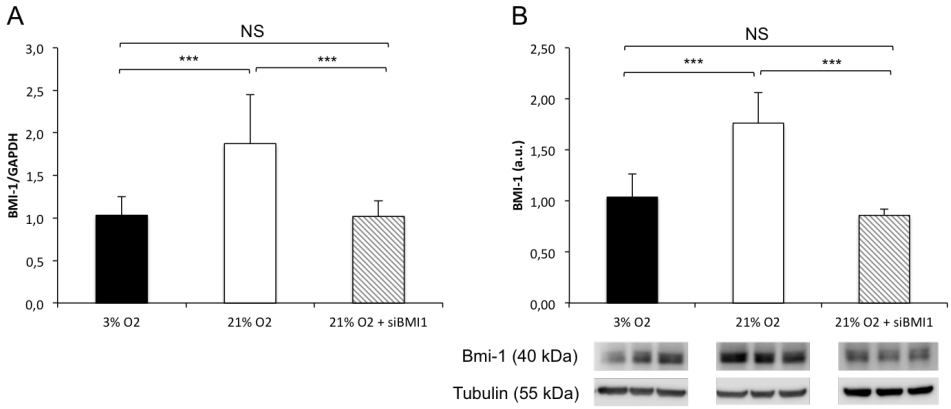

Figure 4.18 *BMI-1* gene and BMI-1 protein knockdown confirmation.
(A) *BMI-1* mRNA levels determined by qPCR against housekeeping gene and (B) BMI-1 protein levels measured by western blot. The data are shown as means ± SD (n=3). The statistical significance is expressed as ***p<0.001 *versus* 21% pO_2.

4.5.4 *SOX2* and *OCT4* rescue following BMI-1 knockdown

As previously stated, hDPSCs cultured at 21% pO_2, showed at early stages, significantly increased BMI-1 protein levels, as well as a reduced expression of *SOX2* and *OCT4* transcription factors. Thus, we focused on BMI-1 silencing effect on the expression of these two transcription factors.

To this end, we analysed *SOX2* and *OCT4* expression levels in transfected hDPSCs cultured at 21% pO_2 using quantitative real-time polymerase chain reaction. The following figures show *SOX2* (Figure 4.19) and *OCT4* (Figure 4.20) gene expression levels in non-transfected hDPSCs cultured at 3% and 21% pO_2, as well as in transfected hDPSCs cultured at 21% pO_2.

As we can observe in both figures, after transfection, *SOX2* and *OCT4* expression levels were significantly increased. Moreover, *SOX2* and *OCT4* expression levels achieved in transfected hDPSCs cultured at 21% pO_2 were comparable to those expressed by hDPSCs cultured at 3% pO_2.

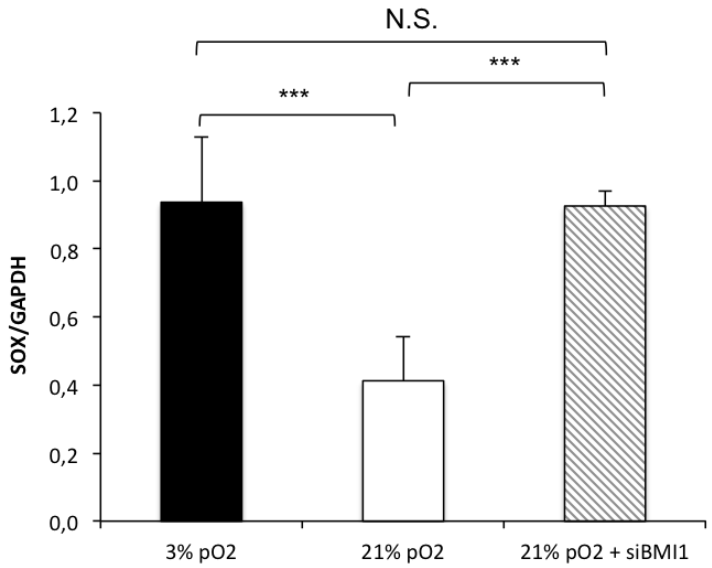

Figure 4.19 *SOX2* gene expression following BMI-1 knockdown in young hDPSCs.
SOX2 mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=3). The statistical significance is expressed as ***$p<0.001$ *versus* 21% pO_2.

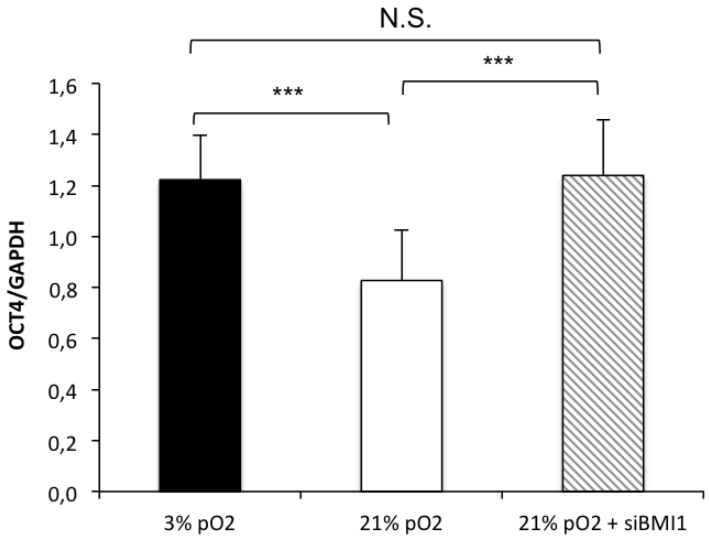

Figure 4.20 *OCT4* gene expression following BMI-1 knockdown in young hDPSCs.
OCT4 mRNA levels determined by qPCR against housekeeping gene. The data are shown as means ± SD (n=3). The statistical significance is expressed as ***$p<0.001$ *versus* 21% pO_2.

In accordance to our hypothesis, *BMI-1* knockdown restores *SOX2* and *OCT4* expression levels in young hDPSCs cultured under oxidative stress conditions.

5 DISCUSSION

5.1 AMBIENT OXYGEN TENSION INDUCES OXIDATIVE STRESS IN hDPSCs LONG-TERM CULTURE

5.1.1 Low oxygen tension alleviates oxidative damage in hDPSCs long-term culture

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 (Shi and Gronthos, 2003; Crisan et al., 2008; Zannettino et al., 2008). 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 (Yu et al., 2002; Simon and Keith, 2008). Despite this fact, it is still common to culture cells at high non-physiological 21% pO_2. However, the normal cell function requires a stable oxidation-reduction environment. The excess in the oxygen tension has been described as an important factor which could destabilize the cellular redox homeostasis (Fan et al., 2008).

In the present study, we show that reduction of the oxygen level led to decreased intracellular oxidative stress and damage during long-term culture. This was evidenced by reduced ROS production, less protein and lipid damage, as well as a better conservation of the mitochondrial membrane potential ($\Delta\Psi m$). Such effects were prominent for hDPSCs cultured at 3% pO_2.

In accordance with our results, it has been demonstrated that high concentrations of oxygen can cause oxidative stress damage via production of reactive oxygen species (ROS) and free radicals that can damage lipids, proteins and DNA (Wiseman and Halliwell, 1996). Accumulation of damaged macromolecules, including oxidative damaged proteins, is a hallmark of cellular and organismal aging. This accumulation has been viewed as the combined result of increased production of ROS, and other toxic compounds coming from cellular metabolism and external factors as well as the failure of protein maintenance (i.e. degradation and repair) with age. Protein oxidation is particularly detrimental as the resulting damages can render oxidized proteins inactive and lead to cellular

functional abnormalities (Berlett and Stadtman, 1997). Various types of oxidative damage to proteins are induced either directly by ROS or indirectly by reactions with secondary products of oxidative stress such as reactive aldehydes as 4-hydroxy-2-nonenal and MDA (Baraibar and Friguet, 2013). In accordance with this, we recently published a study that correlates high levels of MDA in hDPSCs with ambient oxygen pressure culture (El Alami et al., 2014).

There is growing evidence that mitochondria are a critical player in the vicious cycle of ROS-mediated oxidative damage and cellular replicative senescence. In fact, it has been demonstrated that mitochondrial depolarization, together with increased ROS generation, was indicative of mitochondrial dysfunction during continual passage of G6PD-deficient cells (Ho et al., 2007). Furthermore, it has also been suggested that low oxygen tension protects mitochondria from oxidative damage, helps to maintain mitochondrial function, and prevents the increase in mitochondrial mass. This is in consistence with previous reports that mitochondrial membrane potential is reduced in cells from aged animals (Hagen et al., 1997; Rottenberg and Wu, 1997).

In this work, we also linked oxygen tension to altered mRNA expression of *MnSOD*, *CAT* and *GPx*. The analysis of antioxidant enzyme expression showed that *MnSOD*, *CAT* or *GPx* were overexpressed in response ambient oxygen environment. This indicates 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 $CD34^+$ cells, which implied that the increase of H_2O_2 levels could interact with more efficiently transcription of *MnSOD*, *CAT* and *GPx* (Fan et al., 2008).

Taken together, our data suggest that hDPSCs cultured under ambient oxygen tension are exposed to a hyperoxic environment which leads to oxidative stress conditions.

5.2 OXIDATIVE STRESS INDUCES PREMATURE SENESCENCE UNDER AMBIENT OXYGEN TENSION DURING LONG-TERM CULTURE OF hDPSCs

5.2.1 Low oxygen tension delays hDPSCs senescence during long-term culture

In this study, we demonstrate that hDPSCs cultured under ambient oxygen tension began to show flattened or lengthened shapes, and debris in the culture medium increased. Furthermore, hDPSCs proliferation rate was significantly reduced under ambient oxygen tension, as cells cultured at 3% pO_2 reached 25 passages while at 21% pO_2 they only reached 15 passages by the same time. These observations were accompanied with an increased activity of the senescence-associated β-galactosidase enzyme along passages, suggesting that these cells were entering a senescent state.

Hayflick and Moorhead proposed in 1961 that serial sub-cultivations of WI-38 HDFs under the usual laboratory conditions available more than 48 years ago (namely at 21% pO_2), exhaust the proliferative potential of these cells. This *in vitro* phenomenon has been historically termed as replicative senescence (Reddel, 2000). We shall review how it was shown later that cultivating human diploid fibroblasts under more physiological oxygen partial pressure (2-5% pO_2) prolongs their *in vitro* lifespan. This led to the question whether cells cultivated under usual laboratory conditions (21% pO_2) show accelerated senescence and whether the molecular mechanisms of normal senescence are really the same, even if both are characterized by critical telomere shortening (Serrano and Blasco, 2001; Toussaint et al., 2011).

The beneficial effect of low oxygen tension on mesenchymal stem cells from different sources has been demonstrated in many studies (Aranha et al., 2010; Adesida et al., 2012; Choi et al., 2014; Peng et al., 2016). The advantages of low oxygen tension in cell proliferation were reported for bone-marrow-derived mesenchymal stem cells (Grayson et al., 2006; Grayson et al., 2007; Bornes et al.,

2015). When exposed to 2-3% pO_2, the cells increased their regenerative potential (Volkmer et al., 2010) and achieved the cumulative population up to ten times compared to cells cultured in high oxygen tension (Fehrer et al., 2007). A higher regenerative potential was also observed in mesenchymal stem cells isolated from the umbilical cord at 5% pO_2 (Drela et al., 2014) and in the placental mesenchymal stem cells (Mathew et al., 2013). Adipose-derived mesenchymal stem cells submitted to low oxygen tension (2-5% pO_2) increased their regenerative potential (Kang et al., 2014).

In fact, here we show that hDPSCs can be cultured over at least 25 passages without losing their stem cell-like morphology and proliferation kinetics when cultured under physiological oxygen pressure. However, under ambient oxygen tension, hDPSCs gradually lose their proliferative potential during long-term culture and show increasing signs of senescence such as larger phenotypes and increased SA-β-Gal levels.

It has been described that depending on the senescence trigger, cells can become large, flat, and multinucleated, or rather refractile. A flat cell phenotype is commonly seen in cells undergoing H-RAS^{V12}-induced senescence (Serrano et al., 1997; Denoyelle et al., 2006), stress-induced senescence (Parrinello et al., 2003) or DNA damage-induced senescence (Chen and Ames, 1994; Chen et al., 2001). Cells senescing due to $BRAF^{E600}$ expression or the silencing of p400, however, acquire a more spindle-shaped morphology (Chan et al., 2005; Michaloglou et al., 2005). Melanocytes undergoing RAS^{V12}-induced senescence display extensive vacuolization as a result of endoplasmic reticulum stress caused by the unfolded protein response (Denoyelle et al., 2006).

SA-β-Gal is the most commonly used senescence biomarker (Dimri et al., 1995; Debacq-Chainiaux et al., 2009). This marker is detectable by histochemical staining in most senescent cells. However, it is also induced by stresses such as prolonged confluence in culture (Campisi and d'Adda di Fagagna, 2007). Still, even under normal physiological circumstances, β-galactosidase activity is

enriched in particular cell types, such as mature tissue macrophages and osteoclasts (Bursuker et al., 1982; Kopp et al., 2007), and it is detected in cells undergoing increased lysosomal activity during autophagy (Young and Narita, 2010; Ivanov et al., 2013). The SA-β-Gal probably derives from the lysosomal β-galactosidase and reflects the increased lysosomal biogenesis that commonly occurs in senescent cells (Lee et al., 2006a). Accordingly, the increase in SA-β-Gal activity in senescent cells is likely due to an expansion of the lysosomal compartment, giving rise to an increase in β-galactosidase activity that can be measured also at suboptimal pH 6 (hence, SA-β-Gal) (Kurz et al., 2000; Yang and Hu, 2005).

Taken together, our results suggest that hDPSCs cultured under ambient oxygen tension are submitted to a premature senescence, which is evidenced by enlarged phenotype, reduced proliferative potential and increased SA-β-Gal activity. This phenomenon seems to be caused by ROS accumulation, leading to the so-called stress-induced premature senescence or SIPS (Sherr and DePinho, 2000).

5.3 OXIDATIVE STRESS-INDUCED PREMATURE SENESCENCE (SIPS) IS MEDIATED BY $p16^{INK4A}$ PATHWAY

5.3.1 ROS-induced $p16^{INK4a}$ upregulation mediates SIPS in hDPSCs

Senescence is a normal feature of cells whereby they lose their replicative capacity after a finite number of divisions (Ivanchuk et al., 2001). The $p16^{INK4a}$/pRb and $p14^{ARF}$/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 (Sherr and DePinho, 2000).

Current culture conditions *in vitro* generally entails mitogenic hyper-stimulation, which in most primary cells results in upregulation of the *INK/ARF* locus (Sharpless, 2004; Li et al., 2009). In fact, reduced oxygen tension has long been known to promote the growth and extend the replicative life span of cultured human cells (Packer and Fuehr, 1977; Saito et al., 1995). ROS have important functions in cell signalling but their role in regulating cell cycle progression is poorly understood. ROS levels increase significantly as cells pass from G1 into S phase of cell cycle (Havens et al., 2006) and they are required for S phase entry, as demonstrated by the cell cycle arrest induced by quenching ROS (Menon et al., 2003; Conour et al., 2004). However, cell cycle checkpoints are also activated by increased ROS (Menon and Goswami, 2007), indicating that cellular proliferation relies on maintaining ROS levels within a functional range (Macleod, 2008).

Given the role of $p16^{INK4a}$ in cell cycle regulation and the recent implication of oxidative stress in stem cell senescence, we investigated a potential link between ROS and $p16^{INK4a}$ regulation. Our results show that hDPSCs cultured at 21% pO_2 revealed signs of senescence, which were accompanied by an increasing $p16^{INK4a}$ expression levels as passaging number did, and a reduction in $p14^{ARF}$ expression levels in relation to passaging number. This might be suggesting that these cells could have become resistant to apoptosis while they entered senescence.

In accordance with this, cultured primary human cells generally express increasing amounts of $p16^{INK4a}$ as they approach their lifespan limit *in vitro*

(Serrano et al., 1996). It was recently suggested that stressful culture conditions can induce $p16^{INK4a}$, and subsequent premature senescence, in mammary epithelial cells, keratinocytes, and some fibroblasts (Ramirez et al., 2001; Rheinwald et al., 2002). In fact, low levels of expression are detected initially in primary human fibroblasts but $p16^{INK4a}$ accumulates to high levels as these cells undergo senescence (Hara et al., 1996). Furthermore, $p16^{INK4a}$ expression has been correlated to population doubling number, that was confirmed statistically by SA-β-Gal staining (Shibata et al., 2007). Several researchers have shown that the upregulation of $p16^{INK4a}$ gene expression is important to induce cellular senescence in human MSCs (Shibata et al., 2007; Jin et al., 2010). In fact, stress signals such as ROS stimulate the activation of $p16^{INK4a}$ transcription (Jenkins et al., 2011) and play important roles in initiation, as well as maintenance, of cellular senescence (Okamoto et al., 2002; Takahashi et al., 2006; Yang et al., 2008). 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), and that treatment with antioxidant agents restored the constitutive capacity of HSCs, resulting in the prevention of bone marrow failure (Ito et al., 2004; Ito et al., 2006). 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.

If senescence and apoptosis are truly alternative cell fates, cellular changes that are pro-senescent are actively anti-apoptotic and that senescent cells are resistant to apoptosis (Childs et al., 2014). There is strong evidence for the existence of apoptosis resistance in replicative senescent fibroblasts, which they showed occurs through $p14^{ARF}$/p53 signalling (Chen et al., 2000). The outcome of apoptosis resistance can also be cell survival. Senescence induced by mild H_2O_2 promotes survival rather than apoptosis in response to apoptotic stimuli such as high-dose H_2O_2, which upregulates p53, leading to p53-dependent apoptosis (Sanders et al., 2013).

Taken together, we could say that low oxygen tension might delay senescence of hDPSCs by downregulation of $p16^{INK4a}$ expression.

5.4 LOSS OF STEMNESS UNDER AMBIENT OXYGEN TENSION DURING LONG-TERM CULTURE OF hDPSCs

5.4.1 OSKM expression pattern in hDPSCs cultured at 3% pO_2

Here we demonstrate that *OCT4* and *SOX2* expression levels were upregulated at early stages, suggesting that these two transcription factors might be involved in stemness induction. These data are in accordance with those of Wan Safwani and colleagues; they showed in adipose-derived stem cells during long-term manipulation that the expression of the stemness biomarkers *SOX2*, *NANOG* and *OCT4* was declined along passages (Wan Safwani et al., 2011).

Our results also show that *KLF4* and *c-MYC* expression levels were upregulated at advanced stages. The successive increase in the expression of those two transcription factors, could be explained as they have been shown to contribute to the long-term maintenance of the embryonic stem cell (ESC) phenotype, and the rapid proliferation of ESCs in culture (Takahashi and Yamanaka, 2006).

This indicates a close interaction between these four genes, that control the stemness status of stem cells.

5.4.2 OSKM expression pattern in hDPSCs cultured at 21% pO_2

The current results suggest that although hDPSCs were capable of being maintained under ambient conditions, pluripotency was reduced before the appearance of morphological differentiation. In fact, our data show that hDPSCs cultured at 21% pO_2 only expressed reduced levels of *SOX2* and *OCT4* compared with cells cultured at 3% pO_2 at passage 5, before these cells appeared to be morphologically differentiated.

These data are in agreement with similar *OCT4* results obtained by Ludwig (Ludwig et al., 2006). However, they are contrary to those of Forsyth and Westfall, who both observed no difference in the expression SOX2, OCT4 and NANOG between human ESCs cultured at 2% and 4% pO_2 respectively compared with atmospheric oxygen tensions (Forsyth et al., 2008; Westfall et al., 2008). However,

the latter investigators did show that OCT4-regulated genes were down-regulated under atmospheric oxygen tensions, suggesting that although mRNA expression of pluripotency markers was not reduced under 20% oxygen, their downstream targets displayed decreased expression.

5.4.3 Low oxygen tension retains hDPSCs stemness potential

hDPSCs cultured under physiological oxygen tension expressed *OCT4* and *SOX2* at early stages, and *KLF4* and *c-MYC* at advanced stages, which in turn might mean that pluripotency is conserved along passages. Ambient oxygen tension was accompanied by a reduction of all four transcription factors. According with our results, a recent study revealed that ROS accumulation in tendon stem cells was accompanied by reduced colony formation and proliferation, decreased expression of the stemness markers *NANOG*, *OCT4* and *SSEA4*, and impaired differentiation capability (Chen et al., 2016a). There is no further mention to the expression pattern of *c-MYC* or *KLF4* in relation to long-term culture or oxidative stress.

It has been suggested that culturing human embryonic stem cells by constant low oxygen tension condition may maintain pluripotency by sustaining Notch activation (Prasad et al., 2009). In a recent study, an enhanced expression of the stemness markers *OCT4*, *NANOG*, *REX1* and *SOX2* under low oxygen tension was observed, indicating that adult stem cells (ASCs) had a greater ability to maintain their stemness properties under low oxygen tension as compared to high oxygen tension (Choi et al., 2014). This result was consistent with other findings, suggesting that a low oxygen microenvironment provides an optimal condition for the maintenance of ASCs properties (D'Ippolito et al., 2006).

Recent research data also showed that using normoxic conditions (5% pO_2) increases the efficiency of generation of iPSCs from mouse embryonic fibroblasts using *OCT4*, *SOX2*, and *KLF4* retroviral transduction, as well as with non-viral vectors, such as plasmid expression vectors or piggyback transposition system

(Yoshida et al., 2009). Low oxygen tension also shows increased efficiency in the derivation of human iPSCs from dermal fibroblasts on transduction of *OCT4*, *SOX2*, *KLF4* and *c-MYC* retroviral vectors (Yoshida et al., 2009). In accordance to this, endothelial cells reprogrammed by the transduction of transcription factors in low oxygen conditions increased the number of colonies by 2.5-fold compared to ambient oxygen (Panopoulos et al., 2012). Similar results were obtained for the reprogramming of mice embryonic fibroblasts and human dermal fibroblasts, showing that low oxygen tension promotes higher reprogramming efficiencies (Yoshida et al., 2009) and accelerate the generation of iPSC colonies (Shimada et al., 2012). Thus, low oxygen tension seems to have a role not only in sustaining but also inducing pluripotency in stem cells.

Although it is true that we have not measured any parameters of stem cell differentiation potential, stem cell plasticity is also an important factor for prospective use of MSCs in regenerative medicine. Trilineage mesenchymal differentiation, known as the ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages, is a unique biological property of MSCs (Dominici et al., 2006). Several researchers reported the effect of different culture oxygen concentrations on the trilineage differentiation of MSCs. In an elegantly designed experiment, Raheja and colleagues seeded and induced MSCs for differentiation under an atmosphere of 5% carbon dioxide (CO_2) along with 1 of 4 pO_2 concentrations (1%, 2%, 5%, and 21%). According to their results, MSCs differentiated into osteoblast most rapidly at 21% pO_2, and oxygen below 5% showed reduced differentiation potential. However, no statistically significant difference in osteogenic marker was reported when oxygen tension was between 5% and 21% (Raheja et al., 2010). In addition, Basciano and colleagues have reported improved osteoblastic and adipogenic differentiation potential of early passaged MSCs at 5% pO_2 concentration (Basciano et al., 2011). Several other recent reports support that the multilineage differentiation potential of MSCs can be maintained under low oxygen tension environment (1-5% pO_2 concentration) (Grayson et al., 2007; Holzwarth et al., 2010; Nekanti et al., 2010).

5.5 BMI-1 CAN RESCUE *SOX2* AND *OCT4* EXPRESSION UNDER AMBIENT OXYGEN TENSION

5.5.1 Regulation of BMI-1 expression under oxidative stress conditions

In this study we have seen that, according to passage number, *BMI-1* gene expression levels increased while BMI-1 protein levels decreased in hDPSCs. This process was accelerated under oxidative stress conditions.

Very little is known about the post-transcriptional regulation of BMI-1. Recently, it has been reported that BMI-1 is a short-lived protein, which undergoes rapid turnover (Yadav et al., 2010; Sahasrabuddhe et al., 2011). In fact, BMI-1 is reported to be modified by several factors in context- and lineage-dependent manner. BMI-1 is phosphorylated by several kinases for different physiological consequences (Sahasrabuddhe, 2016).

Some researchers have published that BMI-1 may be a substrate of AKT and upregulation of AKT signalling coincides with upregulation of BMI-1 phosphorylation, and that phosphorylated BMI-1 is more stable. In contrast, activated oxidative stress-dependent p38 signalling causes BMI-1 to degrade, and to lose its chromatin modifying ability. Furthermore, they also show that post-transcriptional regulation of BMI-1 via proteasomal degradation causes defective proliferation in $Atm^{-/-}$ neural stem cells (NSCs) as a result of p21 upregulation (Kim and Wong, 2009).

Other researchers demonstrated that BMI-1 expression is modulated through transcriptional and posttranscriptional regulation in hematopoietic stem cells (HSCs) (Bhattacharyya et al., 2009). They further investigated whether oxidative stress could affect BMI-1 degradation. After 1 hour of treatment with H_2O_2, the BMI-1 band was significantly reduced, and after 4 hours of incubation with H_2O_2, they observed a dramatic reduction in the levels of BMI-1 (Kim et al., 2011).

Collectively, these observations strongly support the notion that down-regulation of BMI-1 is associated with oxidative stress-dependent pathways in stem cells.

5.5.2 BMI-1 levels and stemness potential in hDPSCs

OCT4 and SOX2 are components of a complex transcriptional network of positive and negative feedback loops that maintain ESC identity while repressing the expression of genes that promote differentiation (Boyer et al., 2005; Lapthanasupkul et al., 2012). Previous studies have established the importance of epigenetic modulation of gene expression in ESCs, including the chromatin-modifying polycomb group (PcG) and trithorax group (TrxG) proteins, which predominantly mediate gene repression and activation, respectively (Bernstein et al., 2006; Boyer et al., 2006). PcGs exist as two multimeric protein complexes; polycomb repressive complex 1 (PRC1) and 2 (PRC2), both of which function as gene silencers. BMI-1 is a PRC1 component that acts sequentially to repress transcription by H3K27 trimethylation (Yang et al., 2016).

In the present study, we have previously confirmed a loss in all Yamanaka transcription factors (*OCT4*, *SOX2*, *KLF4* and *c-MYC*) expression during hDPSCs *in vitro* long-term culture at 21% pO_2. Moreover, at passage 5, high oxygen tension caused a significant downregulation of *SOX2* and *OCT4* expression, which was accompanied by increased BMI-1 protein levels. Silencing of *BMI-1* gene at passage 5 resulted in a restoration of *SOX2* and *OCT4* expression levels in hDPSCs cultured at 21% pO_2. In fact, most of the transcriptionally silent developmental regulators targeted by OCT4, SOX2 and NANOG are also occupied by the Polycomb group (PcG) proteins (Boyer et al., 2006; Lee et al., 2006b).

Furthermore, with respect to transcriptional regulation, it has been shown that *BMI-1* gene expression is positively regulated by *c-MYC*, and the E2F family of transcription factors (Guney et al., 2006; Nowak et al., 2006; Guo et al., 2007; Liu et al., 2014). This is in accordance with our results where hDPSCs cultured at 3% pO_2 overexpressed both *BMI-1* and *c-MYC* genes as passage number increased.

5.5.3 BMI-1 levels and hDPSCs senescence

It has been suggested that BMI-1 controls stem cells via the *INK4/ARF* locus, because BMI-1 function in young adult hematopoietic stem cells is mediated, in large part, through its transcriptional repression of $p16^{INK4a}$ (Pollina and Brunet, 2011). In accordance to this, we can see in the figure below that BMI-1 and $p16^{INK4a}$ expression patterns are related to each other in hDPSCs cultured under both oxygen tension conditions (Figure 5.1). At 3% pO_2, BMI-1 and $p16^{INK4a}$ levels remain constant and parallel along passages, however, at 21% pO_2, $p16^{INK4a}$ expression rises as BMI-1 levels plummet.

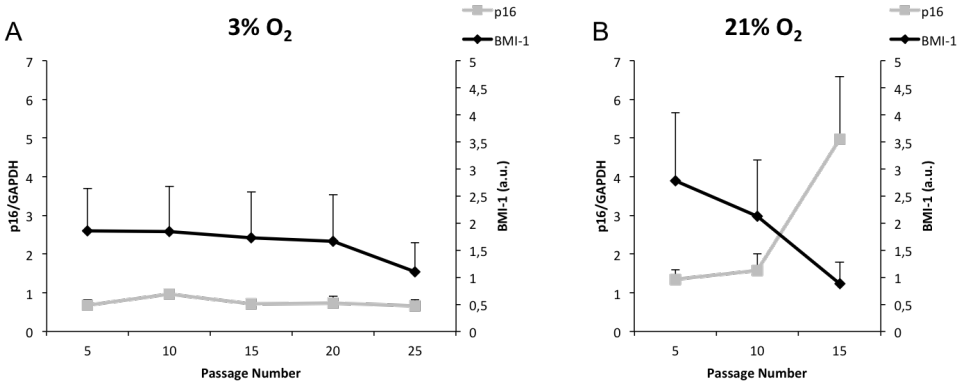

Figure 5.1 Relation between ROS, $p16^{INK4a}$ and BMI-1 expression in hDPSCs long-term culture. $p16^{INK4a}$ mRNA levels determined by qPCR against housekeeping gene, and BMI-1 protein levels measured by western blot in hDPSCs long-term culture at (A) 3% pO_2 and (B) 21% pO_2. The data are shown as means ± SD (n=5).

The role of PcG proteins is the maintenance of established gene expression states to achieve an epigenetic memory of cell identity. Dividing cells must preserve epigenetic memory in order to face disruptions such as DNA replication or mitosis, where regulatory factors may be disassembled from promoters. PcG is thus involved in the competence for switching, with every cell cycle transition providing an opportunity to either maintain the repressed state or to switch to a

derepressed state. Here we observe that decreased expression of BMI-1 along passages in hDPSCs cultured at 21% pO_2, increases the probability of a cell switching from a p16-off to a p16-on state, reducing its regenerative potential.

The decline in regenerative capacity and the subsequent loss of tissue homeostasis is one of the most striking hallmarks of aging. The results described by Ocampo and colleagues and commented by Serrano, suggested that *in vivo* OSKM induction may slow the aging process by preventing molecular changes associated with aging, including epigenetic alterations, activation of cellular senescence pathways (such as $p16^{INK4a}$/pRb), and the exhaustion of adult stem cell populations (Ocampo et al., 2016; Serrano, 2017b).

Taken together, BMI-1 maintains adult stem cell pools while controlling lifespan extension by suppression of the $p16^{INK4a}$-dependent senescence pathway. Thus, Pollina and colleagues proposed that *BMI-1* expression should be kept in a balance between stem cell self-renewal and differentiation during aging (Figure 5.2). This mechanistic balance allows stem cell normal proliferation, while preventing stem cell senescence when downregulated. Thus, maintaining the proper stem cell homeostasis (Pollina and Brunet, 2011).

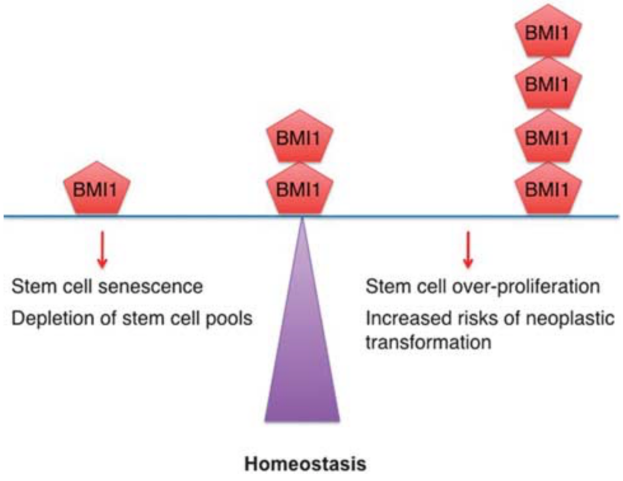

Figure 5.2 BMI-1 balance and stem cells.
Taken from: Pollina and Brunet (2011).

5.6 OUTLOOK: LOW OXYGEN TENSION AS A STRATEGY TO ENHANCE STEM CELL THERAPIES

There is growing interest in transplantation of *ex vivo* amplified cell preparations for various therapeutic applications. This has been fuelled by novel insights from stem cell biology, new molecular tools and promising preclinical model systems. To date, mesenchymal stem cells (MSCs) are tested for a wide spectrum of diseases, hence, cellular therapeutics needs standardized isolation and reliable quality control of cell preparations. This, however, is greatly hampered by the multitude of different methods to prepare MSCs (Wagner and Ho, 2007).

MSCs, which have the ability to divide and remain in an undifferentiated state, are present in perivascular niches in close association with blood vessels in virtually all tissues (Miura et al., 2003; Shi and Gronthos, 2003; Crisan et al., 2008; Zannettino et al., 2008). Even though MSCs are located close to vascular structures, the different tissues where these stem cells are found exhibit low oxygen tensions (Harrison et al., 2002; Pasarica et al., 2009). Therefore, it is possible that maintaining MSCs in an undifferentiated state may require a low oxygen environment, in addition to other factors. Furthermore, stem cell therapies require a large amount of cells which can only be achieved after long-term *in vitro* culture. There is a growing perception that even under highly standardized culture conditions, continuous effects during long-term culture entails senescence, which needs to be taken into account (Wagner et al., 2010).

An unbiased and robust assessment of MSCs senescence – valid for all *in vitro* samples, regardless of donor age and inter-donor variation, would be ideal for estimating sufficient cell expansion potential and quality for therapy (Bertolo et al., 2016). In this study we provide a simple strategy, which is to mimic the organism physiological oxygen tension when culturing in Petri dishes. This allows investigations on the proliferation, differentiation, senescence, metabolic balance, transcriptome, and other physiological aspects of MSCs, which have potentially important clinical applications.

5.6.1 Timing is everything

Timing is critical in MSC therapy, from the initial MSC isolation from adult-donors, to the best time points for patient treatment (Schepers and Fibbe, 2016).

First of all, the time point of cell infusion in relation to the progress of the treated indication in patients may affect their therapeutic efficacy. Moreover, younger patients may respond better than older patients (Ball et al., 2013). Patient clinical responses to MSC batches may not be robust and they may be transient in nature, both complicating patient response readout and MSC potency assessment.

Second, a number of time-dependent variables complicate MSCs production. Particularly intrinsic (cell donor age) and extrinsic cell ageing (*ex vivo* expansion process) may affect MSCs therapeutic properties, as reviewed elsewhere (Dimmeler and Leri, 2008; Geissler et al., 2013; Dimmeler et al., 2014; Efimenko et al., 2015; Kundrotas et al., 2016). It is now well established that bone marrow stem cell content decreases with donor age (Caplan, 1994), and that both, donor age and comorbidities, may affect cell efficacy (Stolzing et al., 2008). If allogeneic cells are applicable, MSCs from younger donors may thus optionally be preferred for clinical use.

As stated before, another evident factor affecting MSCs potency appears to be the time in culture. Stemness potential is reduced after *in vitro* expansion and repeated passaging (Banfi et al., 2000; Horwitz et al., 2002; Javazon et al., 2004; Crisostomo et al., 2006; von Bahr et al., 2012; Binato et al., 2013; Wagner and Henschler, 2013; Bertolo et al., 2016), which results in a gradual loss of progenitor properties and tissue forming capacity, reduced long-term engraftment, lower clinical response and survival benefit, thus compromising engraftment and function (Wagner et al., 2010).

5.6.2 Low oxygen tension *in vitro* preconditioning to prevent senescence

Aging or replicative senescence negatively affects proliferation of MSCs as the cells lose their stemness potential and undergo cell cycle arrest (Yu and Kang, 2013; Boyette and Tuan, 2014). The delay in developing the senescent phenotype in MSCs cultured under low oxygen tension may be because they tend to maintain a higher rate of cell proliferation than MSCs cultured under ambient oxygen tension (Kim et al., 2016). This opens the prospect of obtaining large amounts of cells with the desired biological characteristics during long-term culture. Our findings should help to formulate guidelines for the collection of optimal MSCs for cell therapy.

Several researchers are demonstrating in *in vivo* experiments how low oxygen preconditioning of MSCs (such as bone marrow mesenchymal, adipose-derived and neural stem cells) improves their regenerative potential in common diseases such as bone regeneration, diabetes, spinal cord injuries, neurological disorders and liver regeneration (Chen et al., 2016b; Lee et al., 2016; Wakai et al., 2016; Wang et al., 2016; Waseem et al., 2016; Wu et al., 2016). The consensus is that inhibition or reversal of senescence onset in adult stem cells would be of utmost benefit.

With potential for bone regeneration, hDPSCs might be an effective biological therapy for bone destruction. Wu and collaborators results show that low oxygen preconditioning significantly enhanced hDPSCs survival rate, osteogenic differentiation and its migration response *in vivo*. In mouse apical periodontitis bone destruction model, after transplantation of low oxygen preconditioned hDPSCs via intravenous injection, there is an upregulation of hDPSCs recruitment and recovery of alveolar bone mass in infected periapical tissue, and osteogenesis and bone mineralization is enhanced (Wu et al., 2016).

5.7 LIMITATIONS OF THE STUDY

There is always another experiment that could have been performed or some idea that could have been tested. Thus, this study has some limitations which I would like to briefly summarize.

It would have been of interest to set a deeper characterization of hDPSCs senescence in order to establish how low oxygen improves their regenerative potential for stem cell therapies.

Therefore, analysing the senescence associated secretory phenotype (SASP) and the immune-modulatory profile would have been very useful as novel mechanisms underlying the therapeutic effects of MSCs were shown to include the paracrine actions by cytokines, chemokines, growth factors, and their receptors (Caplan and Dennis, 2006; Hung et al., 2007; Zhang et al., 2007; Gnecchi et al., 2008; Figueroa et al., 2012; Liu et al., 2012).

Another approach could have been to set the senescence associated heterochromatin foci (SAHF) and senescence associated DNA-damage foci (SDF), along with a chromosome profile and telomere length study. SAHFs are detected by the preferential binding of DNA dyes, such as 4,6-diamidino-2-phenylindole (DAPI), and the presence of certain heterochromatin-associated histone modifications (for example, H3K9 methylation) (Campisi and d'Adda di Fagagna, 2007). Therefore, it is possible to assume a relation between SAHF formation, *BMI-1* and $p16^{INK4a}$ expression in hDPSCs. Furthermore, DNA-damage foci arise at dysfunctional telomeres that accompany replicative senescence and can be triggered by either intrinsic or environmental insults (d'Adda di Fagagna et al., 2003; Takai et al., 2003; Herbig et al., 2004; Sharpless and Sherr, 2015). Thus, we could hypothesise a connection between ROS accumulation under ambient oxygen tension and telomere shortening.

Another limitation of this study is that we could not determine the differentiation potential of hDPSCs along passages under both oxygen tension conditions. Finally, measuring the multilineage differentiation potential of hDPSCs

with biomaterial conjugation could have offer another approach of how *in vitro* culturing may affect survival after *in vivo* transplantation.

Although we are aware of the limitations of our study, it enabled us to draw interesting conclusions regarding new approaches for stem cell therapy.

6 CONCLUSIONS

6.1 GENERAL CONCLUSION

Long-term *in vitro* culture of hDPSCs at ambient oxygen tension, in comparison to those cultured at physiological oxygen tension, caused an oxidative stress-related premature senescence and a downregulation of *OCT4* and *SOX2* expression. The proteins $p16^{INK4a}$ and BMI-1 are involved in this process.

6.2 SPECIFIC CONCLUSIONS

The following conclusions can be drawn from the results obtained in this study:

1. Ambient oxygen tension induced oxidative stress in hDPSCs long-term culture as was evidenced by ROS accumulation, lipid and protein damage, mitochondrial membrane potential loss, and antioxidant gene upregulation.

2. Oxidative stress induced a premature senescence (SIPS) under ambient oxygen tension during long-term culture of hDPSCs as evidenced by a senescent phenotype, increased $p16^{INK4a}$ expression levels and SA-β-Gal activity.

3. Oxidative stress-induced premature senescence (SIPS) under ambient oxygen tension during long-term culture of hDPSCs appeared to be mediated by $p16^{INK4a}$ pathway.

4. Oxidative stress-induced premature senescence (SIPS) under ambient oxygen tension during long-term culture of hDPSCs was accompanied by a stemness potential loss as evidenced by *SOX2*, *OCT4*, *KLF4* and *c-MYC* downregulation.

5. BMI-1 proved to control hDPSCs proliferative potential by regulating 16^{INK4a} expression during long-term culture under ambient oxygen tension. Furthermore, *BMI-1* knockdown could rescue *SOX2* and *OCT4* expression in hDPSCs culture under ambient oxygen tension.

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8 ANNEXES

8.1 ANNEX 1: INFORMED CONSENT

Clínica Odontológica, Unidad de Cirugía
Facultad de Medicina y Odontología
Universidad de Valencia

CONSENTIMIENTO INFORMADO

Nombre y apellidos ……...
Edad……
Sexo……

He sido informado/a y acepto libremente entregar mi diente, extraído por motivos ajenos a este estudio, para el cultivo y análisis de las células de la pulpa dental.

Fecha:

Firma:

8.2 ANNEX 2: PATIENT INFORMATION SHEET

Clínica Odontológica, Unidad de Cirugía
Facultad de Medicina y Odontología
Universidad de Valencia

HOJA DE INFORMACIÓN AL PACIENTE

Le invitamos a participar en el presente estudio sobre la obtención de células madre de pulpa dental que se está llevando a cabo en la Universidad de Valencia.

Podrá hablar con el investigador para aclarar sus dudas y si decide no participar en el estudio, esto no afectará de ninguna manera a la calidad de su tratamiento odontológico.

Objetivos del estudio:

- Obtener células madre de pulpa dental.
- Mejorar el método de obtención de estas células.

Tratamiento del estudio: Durante el estudio, se registraran datos referentes a su historia médica (edad, sexo), así como a las características clínicas del diente a extraer.

Posibles riesgos: No existen riesgos para el paciente asociados a este estudio.

Participación voluntaria: Puede retirarse del estudio en cualquier momento sin tener que ofrecer explicación alguna sobre sus razones. El abandono del estudio no condicionará en absoluto los tratamientos odontológicos en el futuro.

Confidencialidad: Todos los datos referentes a su participación en el estudio se almacenarán y analizarán en una base de datos electrónica, sin mención expresa de su nombre.

8.3 ANNEX 3: ETHICAL COMITEE

VNIVERSITAT
DE VALÈNCIA
Vicerectorat d'Investigació i Política Científica

D. Fernando A. Verdú Pascual, Profesor Titular de Medicina Legal y Forense, y Secretario del Comité Ético de Investigación en Humanos de la Comisión de Ética en Investigación Experimental de la Universitat de València,

CERTIFICA:

Que el Comité Ético de Investigación en Humanos, en la reunión celebrada el día 18 de octubre de 2010, una vez estudiado el proyecto de investigación titulado:
"Utilización de células madre de origen dental para enriquecimiento del biomaterial fosfato tricálcico como matriz para regeneración ósea en cirugía previa a la colocación de implantes dentales",
cuyo investigador principal es D. José Viña Ribes,
ha acordado informar favorablemente el mismo dado que se respetan los principios fundamentales establecidos en la Declaración de Helsinki, en el Convenio del Consejo de Europa relativo a los derechos humanos y cumple los requisitos establecidos en la legislación española en el ámbito de la investigación biomédica, la protección de datos de carácter personal y la bioética.

Y para que conste, se firma el presente certificado en Valencia, a veinte de octubre de dos mil diez .

Firmado digitalmente por FERNANDO ALEJO|VERDU|PASCUAL
Motivo: Certifico la precisión e integridad de este documento
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8.4 ANNEX 4: ABBREVIATIONS

Abbreviation	Name	Abbreviation	Name
8-oxodG	8-hydroxy-2-deoxyguanosine	**MnSOD**	Manganese superoxide dismutase
a.u.	Arbitrary units	**mRNA**	Messenger Ribonucleic acid
AGO	Argonaute family	**MSC**	Mesenchymal stem cell
ANOVA	Analysis of variance	**mtDNA**	Mitochondrial Deoxyribonucleic acid
APS	Ammonium persulfate	**dNTP**	Deoxyribose nucleotide triphosphates
ARF	Alternative reading frame	**n**	Sample number
ASC	Adult stem cell	**NaCl**	Sodium Chloride
ATP	Adenosin-5'-triphosphate	**NADH**	Reduced nicotin adenine dinucleotide
AUC	Area under curve	**NADPH**	Reduced nicotin adenine dinucleotide phosphate
BMI-1	B-cell specific moloney murine leukaemia virus integration region 1	**NFkB**	Nuclear factor kappa B
BSA	Bovine serum albumin	**nm**	Nanometre
CDK	Cyclin dependent kinase	**Ct**	Cycle threshold
CDKI	Cyclin dependent kinase inhibitor	**nmol**	Nanomoles
CDKN2A	Cyclin dependent kinase inhibitor 2A	**º**	Angle degree
cDNA	Complementary Deoxyribonucleic acid	**ºC**	Centigrade degree
c-MYC	avian myelocytomatosis viral oncogene homolog	***OCT4***	Octamer-binding transcription factor 4 gene
cm^2	Square centimetre	**OH^-**	Hydroxyl ion
Cu^+	Cupper ion	**OSKM**	OCT4, SOX2, KLF4 and c-MYC

DDR	DNA damage response	**P/S**	Penicillin / Streptomycin
DcR2	Decoy death receptor-2	**PAGE**	Polyacrylamide gel electrophoresis
Dec1	Differentiated embryo-chondrocyte expressed-1	**PBS**	Phosphate buffered saline
DEPC	Diethyl pyro carbonate	**PBS-T**	Phosphate buffered saline - Tween
DHR123	Dihydrorhodamine-123	**PcG**	Polycomb group proteins
DMEM	Dulbecco's Eagle Modified Medium	**pCO_2**	Carbon dioxyde pressure
DMSO	Dimethyl sulfoxide	**PE**	Phycoerythrin
DNA	Deoxyribonucleic acid	**PEST**	Proline (P), glutamic acid (E), serine (S), and threonine (T) domain
DNPH	2,4-Dinitrophenylhydrazine	**pH**	Potential of hydrogen
DTT	Dithiothreitol	**PI**	Propidium iodide
EDTA	Ethylene-diamine-tetra-acetic acid	**pO_2**	Oxygen pressure
ESC	Embryonic stem cell	**pRb**	Retinoblastoma protein
EZH2	Enhancer zeste homolog 2	**PRC**	Polycomb-repressive complex
FACS	Fluorescence activated cell sorting	**PTGS**	Post-Transcriptional Gene Silencing
FBS	Foetal bovine serum	**PVDF**	Polyvinylidene fluoride
FDG	Fluorescein di-β-$_D$-galactopyranoside	**qPCR**	Quantitative polymerase chain reaction
FITC	Fluorescein isothiocyanate	**RF**	RING finger domain
g	Grams / Gravities	**RISC**	RNA-induced silencing complex
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	**RNA**	Ribonucleic acid
GPx	Glutathione peroxidase	**RNS**	Reactive nitrogen species
GSC	Germ line stem cell	**ROS**	Reactive oxygen species
GSH	Reduced glutathione	**rpm**	Revolutions per minute

GSSG	Oxidized glutathione	**rRNA**	Ribosomal Ribonucleic acid
h	Hours	**RT**	Reverse transcriptase
H_2O_2	Hydrogen peroxide	**SA-β-Gal**	Senescence associated β-galactosidase
hDPSCs	Human dental pulp stem cells	**SAHF**	Senescence associated heterochromatin foci
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulfonic	**SASP**	Senescence associated secretory phenotype
Hg	Mercury	**SD**	Standard deviation
HNE	4-Hydroxi-2,3-nonenal	**SDS**	Sodium dodecyl sulfate
HPLC	High performance liquid chromatography	**SIPS**	Stress-induced premature senescence
HRP	Horse radish peroxidase	**siRNA**	Small interference Ribonucleic acid
HTH	Helix-turn-helix domain	**SOD**	Superoxide dismutase
IgG	Immunoglobulin G	***SOX2***	Sex determining region Y-box 2 gene
IL	Interleukin	**ssDNA**	Single strand Deoxyribonucleic acid
INK4	Inhibitor of CDK4	**dsDNA**	Double strand Deoxyribonucleic acid
iPSC	Induced pluripotent stem cell	**TBA**	Thio-barbituric acid
IVF	*In vitro* fertilization	**TBST-T**	Tris buffered saline – Tween
K_2HPO_4	Potassium hydrogen phosphate	**TEMED**	Tetramethylethylenediamine
KH_2PO_4	Potassium dihydrogen phosphate	**TGS**	Transcriptional Gene Silencing
KLF4	Kruppel like factor 4 gene	**TMRE**	Tetramethylrodamine ethyl ester
KPi	Potassium phosphate	**TMRM**	Tetramethylrodamine methyl ester
L	Litre	**TRIS**	2-Amino-2-(hydroxymethyl)-1,3-propanediol
M	Molar	**TrxG**	Trithorax group proteins

mA	Milliampere	**UPS**	Ubiquitin proteasome system
MAPK	Mitogen-activated protein kinases	**UV**	Ultraviolet
MDA	Malondialdehyde	**V**	Volts
MDM2	Murine double minute 2	**v/v**	Volume/volume
MEF	Mouse embryonic fibroblast	**VEGFA**	Vascular endothelial growth factor A
mg	Milligram	**w/v**	Weight/volume
min	Minutes	**ΔΨm**	Mitochondrial membrane potential
miRNA	Micro Ribonucleic acid	**λ**	Wavelength
mL	Millilitre	**µg**	Microgram
mm	Millimetre	**µL**	Microliter
mM	Millimolar	**µM**	Micromolar
MMP	Matrix metalloproteinase	**µm**	Micrometer

8.5 ANNEX 5: ARTICLE RELATED TO THIS DOCTORAL THESIS

Redox Biology 12 (2017) 690–698

Contents lists available at ScienceDirect

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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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ARTICLE INFO

Keywords:
Oxygen tension
Regenerative medicine
Aging

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.

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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

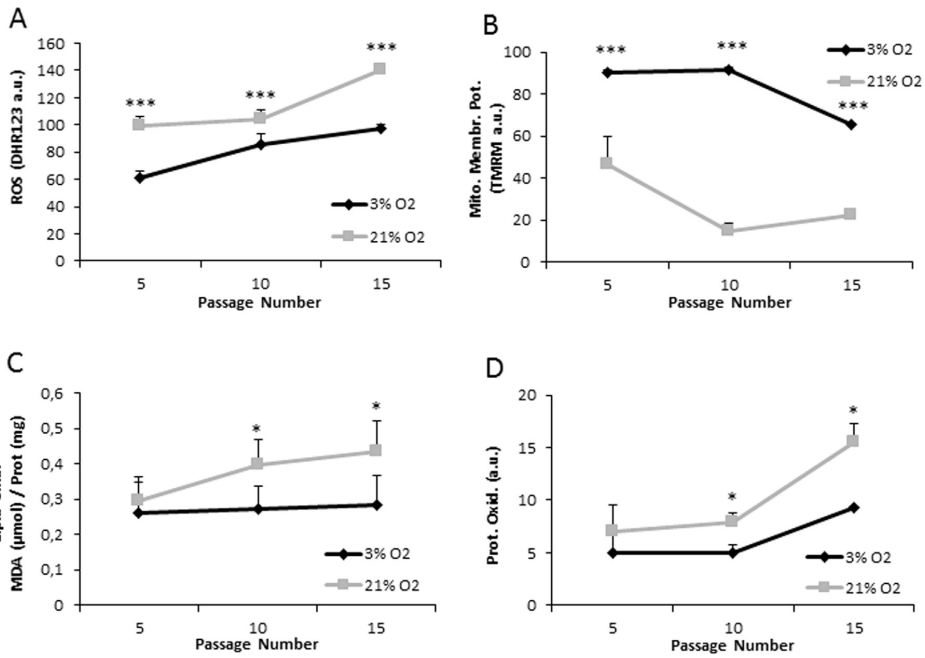

Fig. 1. Oxidative stress related parameters in hDPSCs cultured at 21% or 3% oxygen tension along passages. (A) ROS levels measured by dihydrorhodamine-123 (DHR123), (B) Mitochondrial membrane potential levels measured by tetramethylrhodamine methyl ester (TMRM), (C) Lipid oxidation levels measured by 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 $2-\Delta\Delta Ct$ 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.

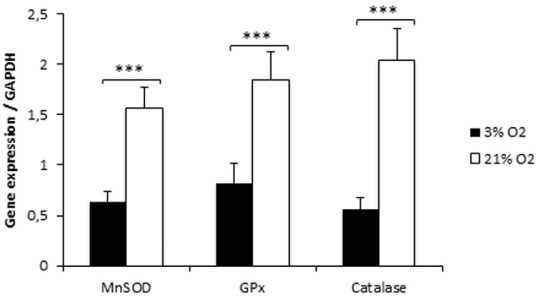

Fig. 2. Antioxidant genes expression in hDPSCs cultured at 21% or 3% oxygen tension. Manganese superoxide dismutase (MnSOD) levels, glutathione peroxidase (GPx) levels and catalase levels. The data are shown as means ± SD (n = 5). The statistical significance is expressed as ***$p < 0.001$ versus 3%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

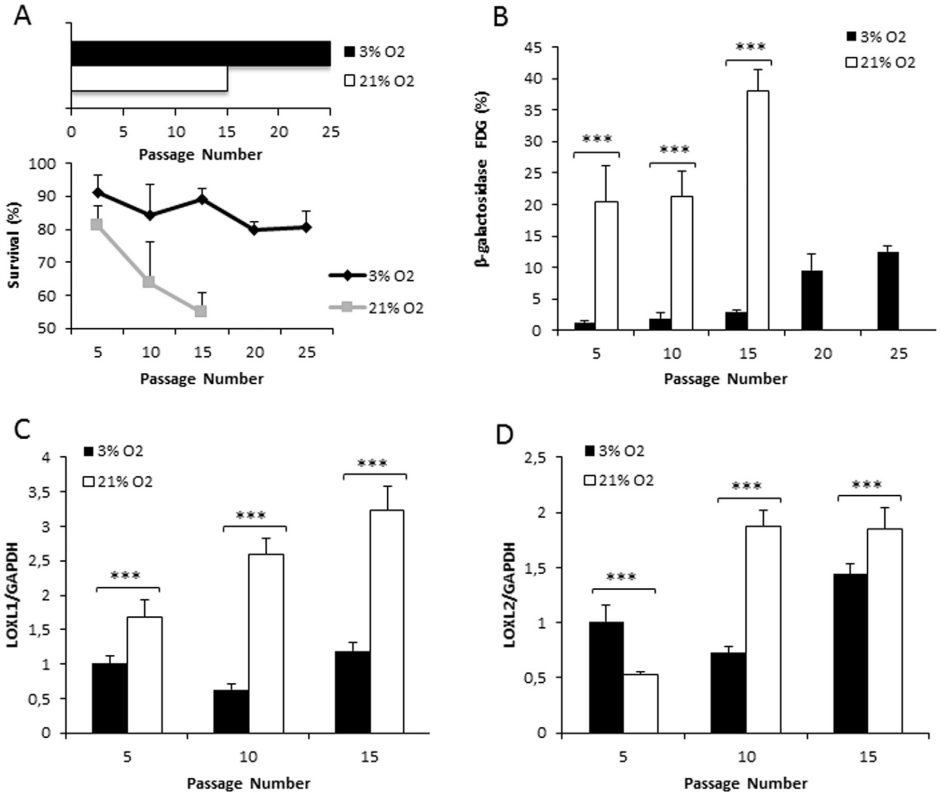

Fig. 3. Oxidative stress induces premature senescence in hDPSCs during long term culture at 21% oxygen tension. (A) Number of passages reached (upper pannel) and survival curve (lower pannel), (B) β-galactosidase activity measured by fluorescein di-β-$_D$-galactopyranoside (FDG) load, (C) *LOXL1* and (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.

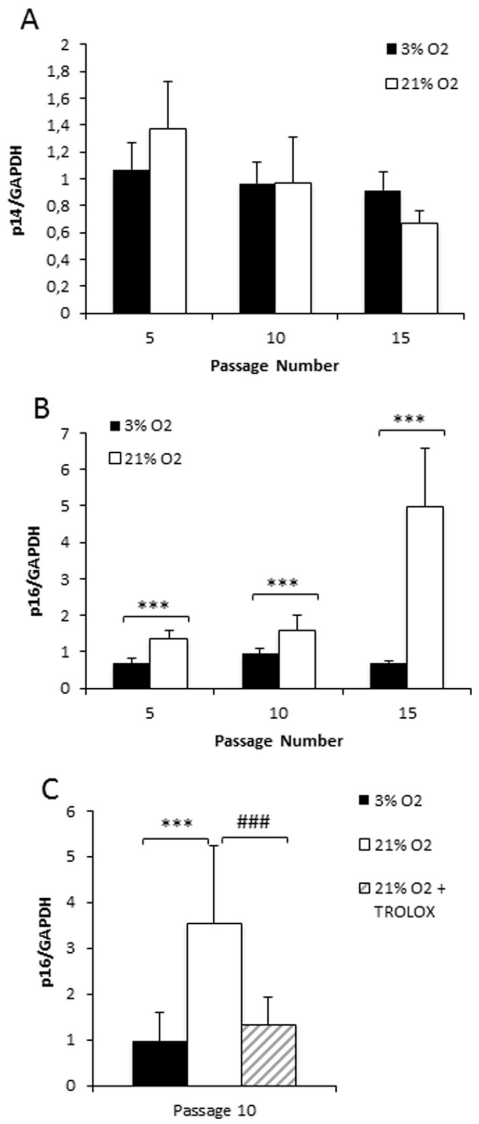

Fig. 4. Oxidative stress-induced premature senescence. Correlation with $p16^{INK4a}$ and $p14^{ARF}$. (A) $p14^{ARF}$ mRNA levels, (B) $p16^{INK4a}$ mRNA levels and (C) $p16^{INK4a}$ mRNA levels when treatment with 50 μM Trolox. The data are shown as means ± SD (n = 5). The statistical significance 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

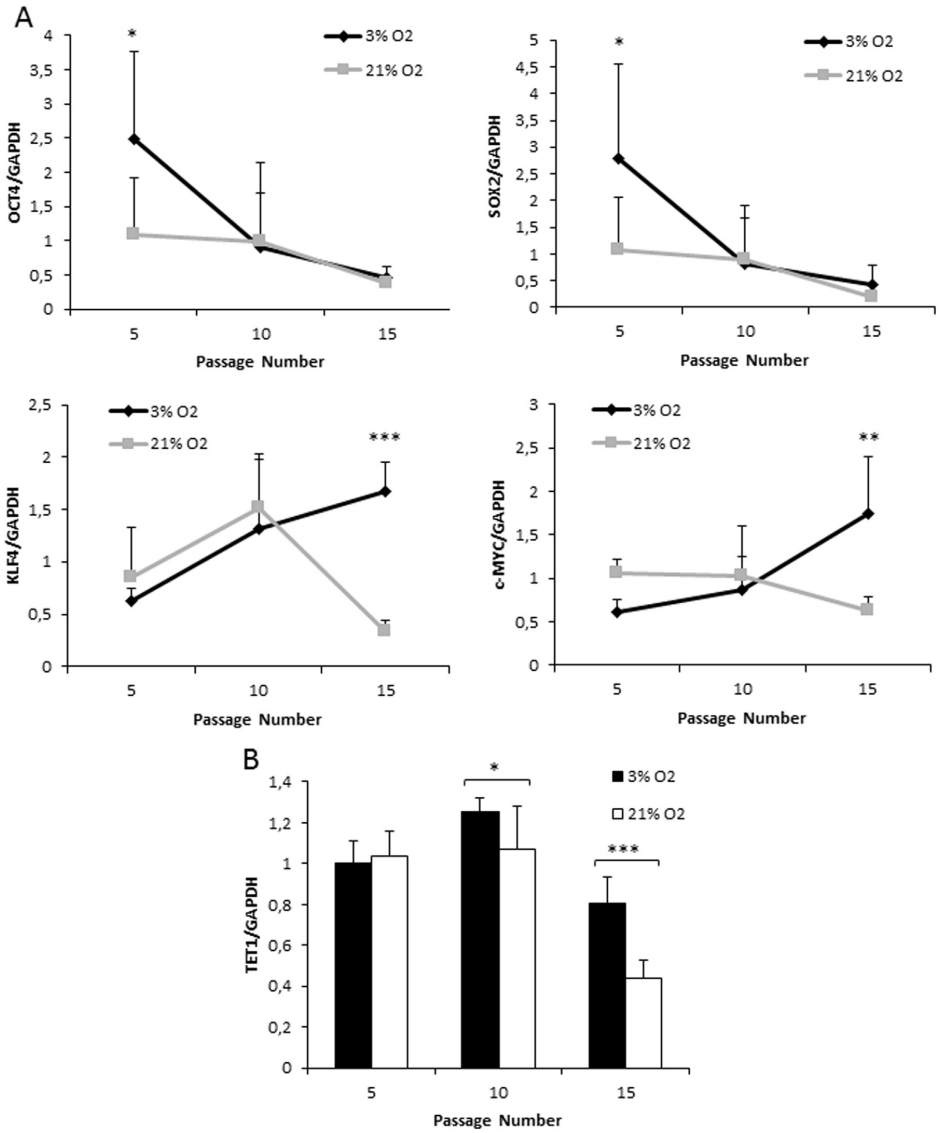

Fig. 5. Pluripotency markers in hDPSCs cultured at 21% vs 3% oxygen tension along passages. (A) *OCT4*, *SOX2*, *KLF4* and *C-MYC* mRNA levels and (B) *TET1* mRNA levels relative expressions. The data are shown as means ± SD (n = 5). The statistical significance is expressed as *p < 0.05; **p < 0.01;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

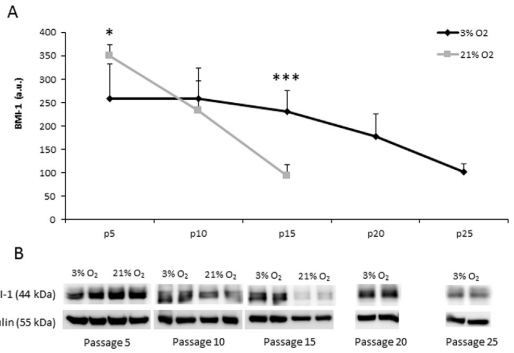

Fig. 6. *BMI-1* expression level in hDPSCs cultured at 21% vs 3% oxygen tension along passages. (A) *BMI-1* protein levels, and (B) representative western-blot images of *BMI-1* protein levels. The data are shown as means ± SD (n = 5). The statistical significance is expressed as *p < 0.05; ***p < 0.001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

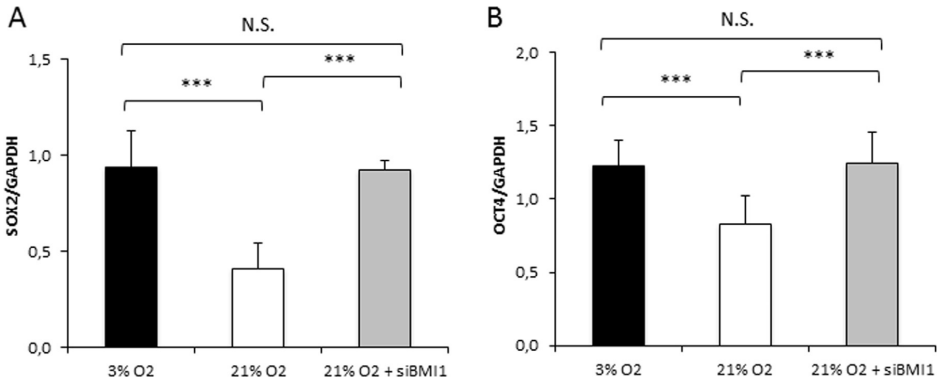

Fig. 7. *BMI-1* knockdown effect on *SOX2* and *OCT4* expression in hDPSCs cultured at 21% vs 3% oxygen tension. (A) *SOX2* mRNA expression levels and (B) *OCT4* mRNA expression levels. The data are shown as means ± SD (n = 3). *p < 0.05; **p < 0,01; ***p < 0.001.

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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