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Impact of low oxygen tension on stemness, proliferation and differentiation potential of human adipose-derived stem cells

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ABSTRACT

Adipose-derived stem cells (ASCs) have been found adapted to a specific niche with low oxygen tension (hypoxia) in the body. As an important component of this niche, oxygen tension has been known to play a critical role in the maintenance of stem cell characteristics. However, the effect of O_2 tension on their functional properties has not been well determined. In this study, we investigated the effects of O_2 tension on ASCs stemness, differentiation and proliferation ability. Human ASCs were cultured under normoxia (21% O_2) and hypoxia (2% O_2). We found that hypoxia increased ASC stemness marker expression and proliferation rate without altering their morphology and surface markers. Low oxygen tension further enhances the chondrogenic differentiation ability, but reduces both adipogenic and osteogenic differentiation potential. These results might be correlated with the increased expression of HIF-1α under hypoxia. Taken together, we suggest that growing ASCs under 2% O_2 tension may be important in expanding ASCs effectively while maintaining their functional properties for clinical therapy, particularly for the treatment of cartilage defects.

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1. Introduction

Nowadays, the therapeutic potential of stem cells has gained increasing scientific interest. The ability of stem cells to differentiate and self-renew makes them a potential cell source for regenerative medicine [1]. Adipose-derived stem cells (ASCs), in particular, have emerged as an attractive candidate for cell-based therapy due to their abundance and accessibility [2]. With the increased prevalence of obesity in recent years, adipose tissue can be easily obtained in large quantities by liposuction techniques [3]. Interestingly, ASCs naturally reside in a specific niche with low oxygen tension, where there is less than 4% O_2 tension present in human adipose tissue [4,5]. However, little attention has been given to the oxygen tension in the culture medium when conducting *in vitro* study. ASCs are normally cultured at normal atmospheric oxygen tension (20–21%), which does not represent their normal physiological condition [6].

In fact, the discovery of this special niche has led many studies focused on the potential role of low oxygen tension in regulating their physiological processes, particularly cellular differentiation [7–10]. This research may provide benefits for clinical applications especially for the treatment of bone and cartilage defects [11]. It has been reported that transcriptional factor hypoxia inducible factor 1-α (HIF-1α) is involved in regulating the crucial cellular processes such as stemness, proliferation and differentiation [12,13]. However, conflicting results have been reported regarding the effect of hypoxia on ASCs physiological activities, particularly stemness and differentiation. Some of the studies suggested that under hypoxia, HIF-1α enhances the stemness properties of ASCs while repressing their differentiation activities [8,13], whereas some demonstrated an increased differentiation potential in a HIF-1α dependent manner [14,15].

While there has been controversy in several research findings and literature, the effects of hypoxia on stemness, proliferation and differentiation ability of ASCs are still uncertain. Most studies have demonstrated that oxygen tension as low as 2% had the greatest impact on cell growth [16–18]. In this study, we investigated the effect of 2% O_2 on stemness and differentiation potential of human ASCs, as well as determining their proliferation rate. First, we characterized the human ASCs based on their plastic adherent property and surface marker expression and differentiation potential at 21% (normoxia) and 2% O2 (hypoxia). Subsequently, we evaluated their proliferation rate and compared the gene

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expression levels of HIF-1α, stemness markers and differentiation markers between two different culture conditions.

2. Materials and methods

2.1. Isolation and culture of human ASCs

Human adipose tissue was harvested from healthy female donors aged 25–35 who were undergoing Caesarean section. An informed consent was obtained from each donor before collecting the samples, as approved by the Medical Ethics Committee of University Malaya Medical Centre (Reference No. 996.46). Adipose tissue was washed with phosphate buffered saline (PBS) (Sigma–Aldrich, St. Louis, USA) and minced. They were digested with collagenase type I (Worthington, Freehold, USA) at 37 °C for 30 min. The digested tissue was then centrifuged and the pellet was washed and cultured in a tissue culture flask with complete growth medium containing Dulbecco's Modified Eagle's Medium (DMEM)/Ham F-12 supplemented with 10% FBS, 1% antibiotic–antimycotic solution, 1% glutamax and 1% vitamin C (Gibco, Grand Island, USA). As a normal control group, ASCs were cultured at 21% O_2 at 37 °C. In the hypoxic group, the cells were placed in an oxygen controlled incubator (Galaxy 170 R, New Brunswick Scientific, USA) with 2% O2 at 37 °C, supplied with N_2. Both oxygen levels were confirmed with a Jenway 970 portable dissolved oxygen meter (Bibby Scientific Limited, Staffordshire, UK). The cells at passage three were used for all the tests unless otherwise mentioned.

2.2. Cell phenotype and surface marker expression

Human ASCs were characterized according to the three minimal criteria proposed by Dominici et al. [19]: (i) cell adherent properties; (ii) high expression of human ASCs major surface markers, CD73, CD90 and CD105 with low expression or absent of hematopoietic markers CD14, CD19, CD34, CD45 and MHC class II HLA-DRDPDQ; (iii) adipogenic, osteogenic and chondrogenic differentiation potential.

Firstly, the morphology of the cells was observed, followed by the determination of their surface marker expression. The cells were trypsinized, washed and stained with specific antibodies, FITC-conjugated CD105, CD90, CD45, CD34 and HLADRDPDQ and PE-conjugated CD73, CD14 and CD19 (Becton Dickinson, San Jose, USA). FITC-conjugated mouse IgG1 & IgG2 isotypes, and PE-conjugated mouse IgG1 and IgG2 isotypes (Becton Dickinson) were used as negative controls. Flow cytometric analysis was performed using BD FACSCanto II (Becton Dickinson). Data were analyzed using FlowJo software (Treestar, OR, USA).

2.3. Differentiation assay

For adipogenic differentiation, the cells were cultured with adipogenic induction medium containing high glucose DMEM with 10% FBS (Gibco), 200 μM indomethacin (Sigma–Aldrich), 0.5 μM isobutyl-1-methyl xanthine, 1 μM dexamethasone (Sigma–Aldrich) and 10 μM insulin (Sigma–Aldrich). The cells were stained with Oil red O stain (Sigma–Aldrich) to detect the presence of lipid droplets. Osteogenic differentiation was performed using osteogenic induction medium containing high glucose DMEM with 10% FBS supplemented with 100 nM dexamethasone, 10 mM b-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate (Sigma–Aldrich). The cells were stained with alizarin red stain (pH 4.1–4.3) (Sigma–Aldrich) after 21 days to observe the formation of calcium deposits.

For chondrogenic differentiation, the cell pellet was cultured in chondrogenic induction medium composing of DMEM/F12, 1% FBS, 1% antibiotic–antimycotic, 1% vitamin C, 1% glutamax, ITS premix (Becton Dickinson), 50 μg/ml ascorbate-2-phophate, 100nM dexamethasone, 40 μg/ml L-proline (Sigma–Aldrich), 10 ng/ml TGF-β1 and 50 ng/ml IGF-1 (Peprotech, Rocky Hill, USA) for up to 21 days. Histological examination was then performed. Briefly, the pellet was fixed in 10% formalin (Sigma–Aldrich), and processed according to the standard histological procedures. Each tissue section was stained with alcian blue stain (Sigma–Aldrich) to assess the proteoglycan content. Each section was then mounted by mounting medium (DPX) (Sigma–Aldrich) and observed under the microscope (Eclipse TS100, Nikon, USA).

Apart from performing the histological staining, the expression of specific differentiation markers was determined by qPCR (stated in Section 2.5) to further compare the differentiation potential between both normoxic and hypoxic culture.

2.4. Proliferation assay

The proliferation rates of both normoxic and hypoxic cultured ASCs were assessed by seeding 5×10^4 cells per well in 24 well culture plate with complete growth media. Trypan blue exclusion assay was performed on day 1, 3, 7, 10 and 14. A growth curve showing the number of viable cells versus days was plotted for each group.

Population doubling time (PDT) was determined to further confirm the cell proliferation rate. The number of cell doublings was calculated according to the formula $n = (\log_{10} \mathrm{Nh} - \log_{10} \mathrm{Ni})/\log_{10} 2$, where Ni and Nh are the cell numbers at the beginning and at the end of the passage, respectively. PDT was calculated as a ratio of incubation period (days) divided by the number of cell doublings at each passage and a mean PDT was determined.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted using TRI reagent (Ambion, TX, USA) according to the manufacturer's instruction. cDNAs were synthesized using the high capacity RNA-to-cDNA kit (Applied Biosystems). Subsequently, real time PCR was carried out with *TaqMan* gene expression assays (Applied Biosystems) by using StepOnePlus™ Real-Time PCR system (Applied Biosystem). The genes include HIF-1α (Hs00153153_m1), adipogenic markers such as LPL (Hs00173425_m1), FABP4 (Hs01086177_m1) and PPARγ (Hs01115513_m1), osteogenic markers such as ALP (Hs01029144_m1), OSC (Hs015878914_m1), and RUNX2 (Hs00231692_m1), chondrogenic markers such as SOX9 (Hs00165814_m1), COL2A (Hs00264051_m1) and ACAN (Hs00153936_m1) as well as stemness markers such as REX1 (Hs01938187_s1), SOX2 (Hs01053049_s1), OCT4 (Hs04260367_g1) and NANOG (Hs01060663_m1). GAPDH (Hs99999905_m1) was used as a reference gene for normalization. Following the normalization, data were expressed as fold change as compared to the gene expression of normal control group.

2.6. Statistical analysis

Comparison of the data between normoxia and hypoxia was done using independent *t*-test. Paired *t*-test was used to compare the data before and after the induction of differentiation in the gene expression study. Data were presented as mean ± standard error of the mean (SEM) of six independent experiments ($n = 6$). Statistical significance was accepted at $p < 0.05$.

3. Result

3.1. Hypoxia maintains the characteristics of human ASCs

In the microscopic examination, the cells showed plastic adherent fibroblast-like morphology in the culture (Fig. 1A). They

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