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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.



## 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 $5 \times 10^4$ 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

highly expressed CD105, CD90 and CD73 with lack expression of CD45, CD34, CD19, CD14 and HLA-DRDPDQ (Fig. 1B). Moreover, they were able to differentiate into adipogenic (Fig. 1C), osteogenic (Fig. 1D) and chondrogenic (Fig. 1E) lineages as indicated by histological staining.

### 3.2. Hypoxia increases stemness markers and HIF-1α expression

The gene expression study further showed that the expression levels of REX1, SOX2, OCT4 and NANOG under hypoxia were significantly higher over 1.23, 1.25, 1.48 and 1.39-fold, respectively as compared to normoxia, $p < 0.05$ (Fig. 2A). Furthermore, there was a significant 3.18-fold higher in the expression level of HIF-1α under hypoxia as compared to normoxia, $p < 0.05$ (Fig. 2B).

### 3.3. Hypoxia enhances the proliferation rate of ASCs

The growth curve showed that the number of cells cultured under hypoxia was significant higher as compared to normoxia starting from day 7 of the culture. Particularly, on day 10 of the culture, the number of ASCs cultured under hypoxia was 1.55-fold greater ($14.74 \times 10^4 \pm 0.04 \times 10^4$) than those of normoxia ($9.49 \times 10^4 \pm 0.031 \times 10^4$), $p < 0.05$ (Fig. 3A). In addition, we noticed that the mean PDT under hypoxia was significantly lower

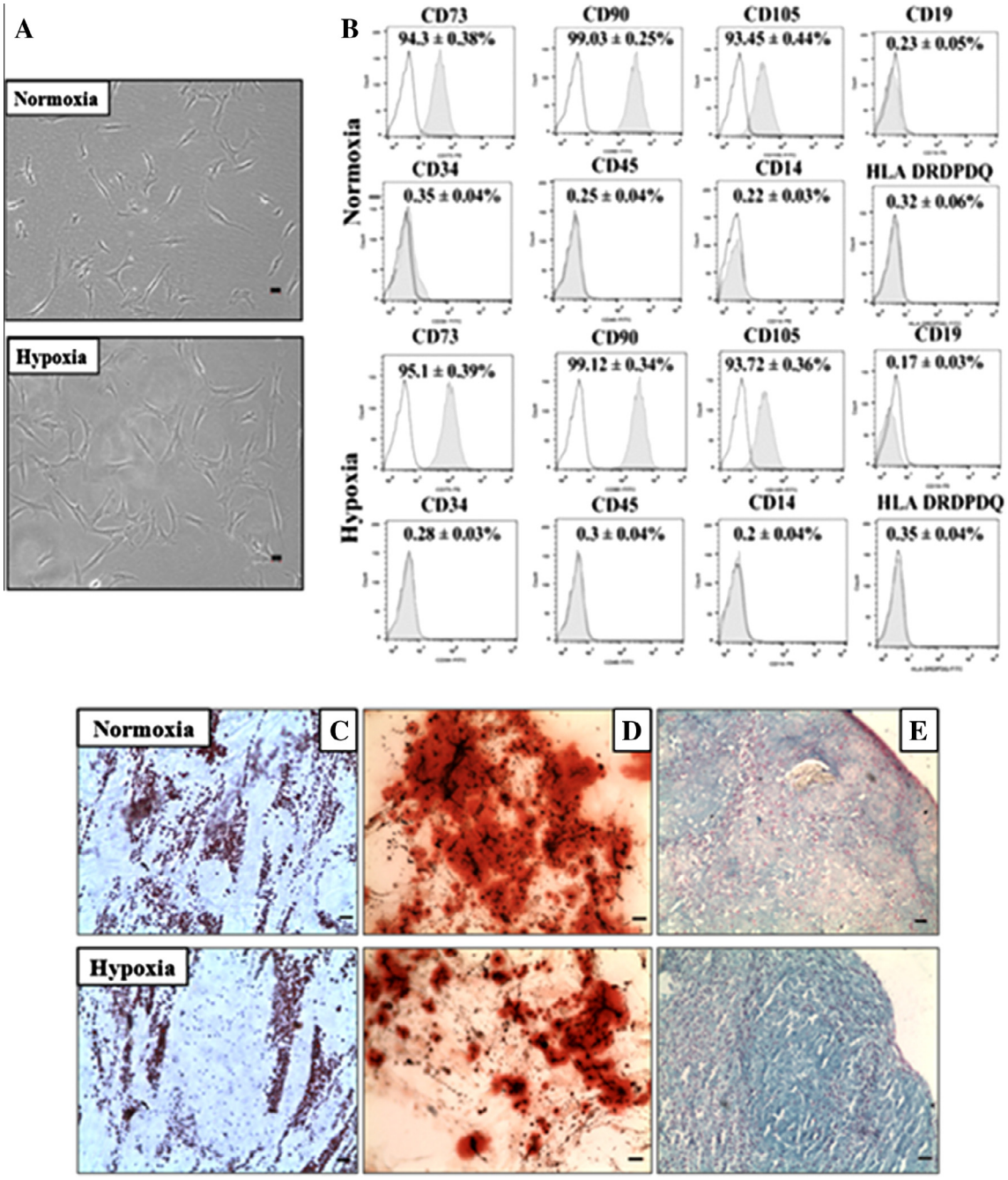


**Fig. 1.** Hypoxia maintained the characteristics of human ASCs. (A) The fibroblast-like morphology of ASCs at passage 3 under normoxia and hypoxia (magnification 100×). (B) Flow cytometric analysis showed the expression of CD73, CD90 and CD105 under both culture conditions. ASCs were positive for (C) adipogenic differentiation indicated by Oil red O staining at magnification 400×, (D) osteogenic differentiation assessed by alizarin red staining at magnification 100× and (E) chondrogenic differentiation detected by alcian blue staining at magnification 100×.

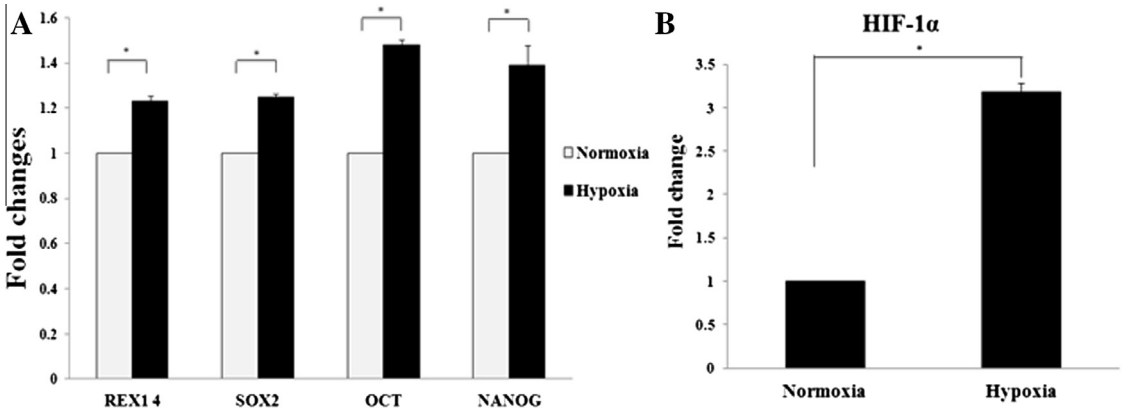


**Fig. 2.** Hypoxia increases the expression level of stemness markers and HIF-1α. (A) Hypoxia displayed a significant higher expression level of ASCs stemness markers REX1, SOX2, OCT4 and NANOG compared to normoxia. (B) A significant higher expression of HIF-1α was observed under hypoxia as compared to normoxia. (Data represents mean ± SEM, $^{*}p < 0.05$ relative to normoxia.)

(3.64 ± 0.16 days) than normoxia (7.93 ± 0.49 days), $p < 0.05$ (Fig. 3B).

### 3.4. Differentiation potential of ASCs under different culture conditions

For adipogenic differentiation, histological staining showed that the cells cultured under both culture conditions were able to exhibit intracellular lipid droplets which stained positive with Oil red O stain. However, we observed that the amount of lipid droplets under hypoxia was lower than normoxia (Fig. 1C). In addition, we found that the expression of adipogenic genes, LPL, PPARγ and FABP4 was increased under both culture conditions after the induction of adipogenic differentiation. However, it was noted that the expression of LPL, PPARγ and FABP4 was significantly lower under hypoxia as compared to normoxia. The expression level of genes PPARγ was at 1.46 ± 0.07 in hypoxia vs. 1.88 ± 0.09 in normoxia, LPL expression was at 1.49 ± 0.12 in hypoxia vs. 3.83 ± 0.12 in normoxia and FABP4 expression was at 1.64 ± 0.14 in hypoxia vs. 3.75 ± 0.27 in normoxia, $p < 0.05$ (Fig. 4A).

For osteogenic differentiation, the cells cultured under two different conditions were stained positively with alizarin red. However, we noted that the amounts of calcium deposits were reduced under hypoxia as compared to normoxia (Fig. 1D). In the gene expression study, both culture conditions expressed the osteogenic genes, ALPL and RUNX2. However, hypoxia showed no significant difference in expression of OSC before and after the differentiation induction. Moreover, all the osteogenic markers in hypoxic culture were expressed lower than those in normoxia. The expression levels of ALPL, RUNX2 and OSC under both hypoxia and normoxia were at 1.82 ± 0.08 vs. 2.5 ± 0.13, 1.21 ± 0.02 vs. 1.5 ± 0.05 and 1.05 ± 0.02 vs. 1.75 ± 0.04, respectively, $p < 0.05$ (Fig. 4B).

Further, the alcian blue staining showed positive results under both culture conditions. Interestingly, there were more glycosaminoglycans present in the culture under hypoxia than normoxia as demonstrated by alcian blue staining (Fig. 1E). The gene expression study revealed that ASCs expressed the chondrogenic genes COL2A, SOX9 and ACAN under two different culture conditions. Specifically, under hypoxia, there was a significant increase in the expression of COL2A (1.95 ± 0.03 vs. 1.66 ± 0.09 in normoxia), SOX9 (2.2 ± 0.08 vs. 1.88 ± 0.07 in normoxia) and ACAN (2.56 ± 0.16 vs. 1.81 ± 0.11 in normoxia), $p < 0.05$ (Fig. 4C).

## 4. Discussion

In general, given that an oxygen-limited condition represents the native physiological microenvironment of ASCs, oxygen tension is a key candidate amongst factors that regulate their properties [20]. Several studies have investigated the effects of hypoxia on their basic functional properties but yielded inconsistent results [21–23]. Apart from the oxygen level chosen, the discrepancies in previous studies particularly in terms of cell differentiation ability might be due to the variety of species involved with different gender and age, the origin of the tissue, the growth media used, the culture technique, the duration of the study and the variation of the system used to control the oxygen level [23,24].

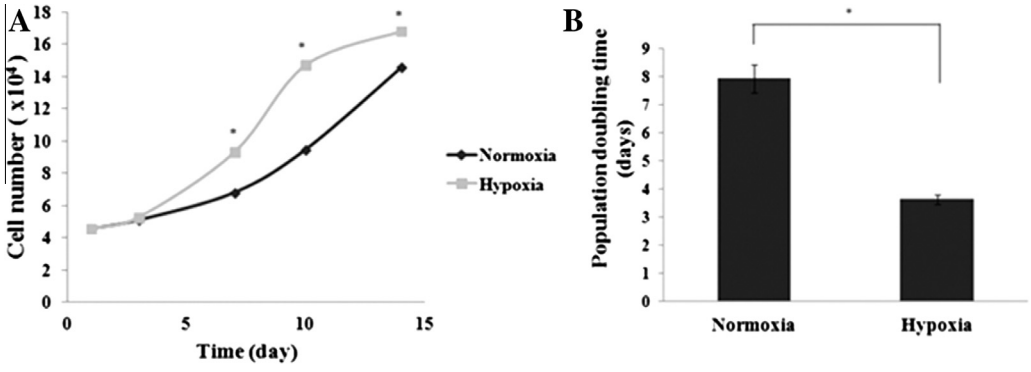


**Fig. 3.** Hypoxia enhances the proliferation rate of ASCs. (A) Growth curve showed a significant increase of ASCs growth rate under hypoxia from day 7 of the culture compared to normoxia. (B) The graph showed that the population doubling time of ASCs was significantly lower under hypoxia than normoxia. (Data represents mean ± SEM, $^{*}p < 0.05$ relative to normoxia.)

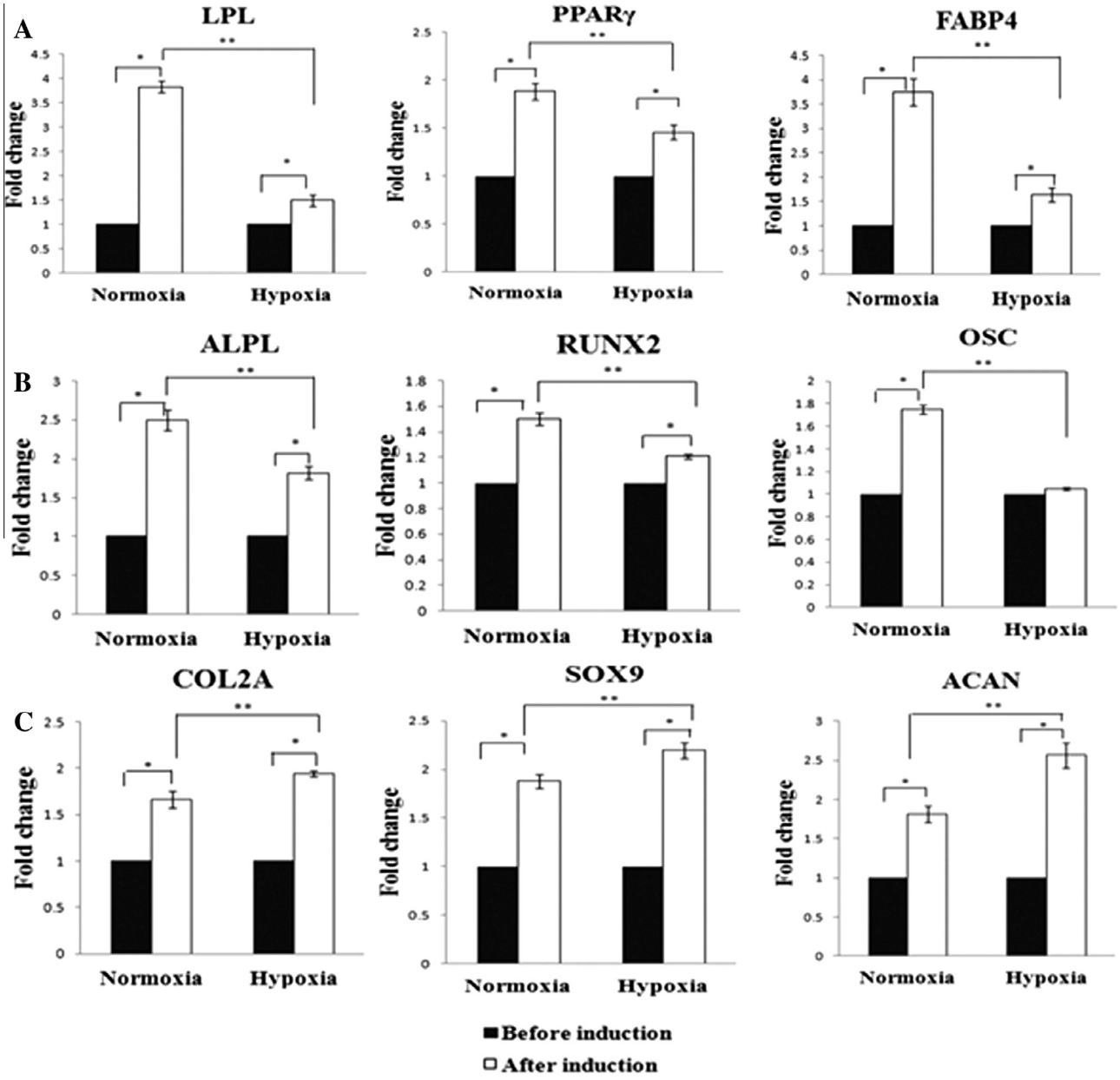


**Fig. 4.** The effect of hypoxia on tri-lineage differentiation of ASCs. (A) The gene expression profile showed reduced expression of adipogenic markers LPL, PPARγ and FABP4 under hypoxia as compared to normoxia. (B) The expression of osteogenic markers ALPL, RUNX2 and OSC was reduced under low oxygen tension. (C) ASCs cultured under hypoxia displayed a significant higher expression level of chondrogenic markers COL2A, SOX9 and ACAN in comparison to normoxia. (Data represents mean ± SEM, $^{*}p < 0.05$ relative to before induction, $^{**}p < 0.05$ relative to normoxia.)

The cells used in this study have been proven to be human MSCs as they met the important criteria of human MSCs [19]. Generally, stemness markers play a crucial role in regulating self-renewal and differentiation activity [25]. In the present study, an enhanced expression of the stemness markers OCT4, NANOG, REX1 and SOX2 under hypoxia was observed, indicating that ASCs had a greater ability to maintain their stemness properties under hypoxia as compared to normoxia. This result was consistent with other findings, suggesting that a low oxygen microenvironment provides an optimal condition for the maintenance of ASCs properties [21,26].

Besides that, the greater number of cells and shorter population doubling time indicate an increased proliferation rate of ASCs under hypoxia. The results suggest that the hypoxic microenvironment is crucial in supporting the growth of ASCs, which might be due to the activation of HIF-1α [20]. HIF-1α is one of the subunits of heterodimeric transcription factor, HIF-1. Under a low oxygen level, HIF-1α is stabilized and translocates into the nucleus, where it dimerizes with HIF-1b, another subunit of HIF-1, to form the HIF-1 complex, which then binds to the hypoxia-responsive elements (HREs) in the promoters of target genes [12]. As a result, it triggers the activation of hypoxia-regulated genes which involve in a wide range of cellular processes [12,27]. Specifically, HIF-1α has been known to regulate cell proliferation under low oxygen condition [28]. This factor is involved in activating its downstream targets, VEGF which regulates the growth of ASCs [29]. In this study, the increased proliferation rate of ASCs and high expression of HIF-1α indicated that HIF-1α might play a role in regulating cell proliferation under hypoxia.

Apart from cell proliferation, some studies have provided evidence that HIF-1α is also involved in cell differentiation [8,13,14]. In the present study, the lower amount of lipid droplets

stained by Oil red O suggests that hypoxia reduced adipogenesis as compared to normoxia. This result was further supported by the reduced expression level of adipogenic markers, PPARγ, FABP4 and LPL under low oxygen tension. It has been reported that 2% and 5% $O_2$ reduced the adipogenic differentiation of ASCs [10,30]. Under low oxygen tension, the core regulator of adipogenesis, FABP4 has been found to be downregulated [7]. Similarly, Lin, et al. [13] demonstrated that 1% $O_2$ repressed the expression of essential adipogenic genes, PPARγ and FABP4. They further reported that ASCs were able to undergo adipogenic differentiation when HIF-1α was knocked down by gene-specific siRNA [13]. In agreement with their results, our findings suggested that HIF-1α plays a vital role in repressing adipogenic differentiation under hypoxia.

Upon osteogenic differentiation, there was a remarkable reduction in the amount of calcium deposition under hypoxia as compared to normoxia, indicating a reduction of osteogenesis. The reduced expression level of osteogenic genes, ALPL, RUNX2 and OSC under hypoxia further confirm that hypoxia reduced the osteogenic differentiation of ASCs. Additionally, the expression level of OSC, a late osteoblast marker [31], was not significantly increased after the induction of osteogenic differentiation under hypoxia, suggesting that most of the cells might be still in their early differentiation stage. A study by Hirao, et al. [32] demonstrated that ALPL activity and alizarin red stain were reduced under 5% $O_2$. Moreover, RUNX has been reported to be downregulated under 1% $O_2$ [33]. Xu et al. [8] noted that HIF-1α repressed osteogenic differentiation of ASCs by reducing RUNX2 transcriptional activity. The return of hypoxic cultured ASCs to normal oxygen level not only resulted in reversion of the inhibitory effect but significantly enhanced the potential of ASCs to undergo osteogenic differentiation [8]. Taken together, the increased expression of HIF-1α under hypoxia might contribute to the reduced adipogenic and osteogenic differentiation of ASCs. Additionally, adipogenesis and osteogenesis of ASCs is believed to take place in an environment with higher oxygen tension which is located closely to the blood vessels upon receiving an appropriate differentiation signal [7].

Interestingly, chondrogenic differentiation of ASCs in the present study was found to be increased under hypoxia as evidenced by the greater amount of proteoglycan formation and increased chondrogenic genes expression, ACAN, COL2 and SOX9. Consistent with our results, hypoxia has been reported to enhance chondrogenic differentiation of ASCs in most of the studies [17,34]. It has been reported that the expression level of chondrogenic markers was increased in a HIF-1α-dependent manner. The deletion of HIF-1α had resulted in decreased expression of the chondrogenic markers [14,15]. In addition, Lawyer, et al. [35] demonstrated that hypoxia stabilized the expression of HIF-1α in chondrocytes in comparison to normoxia. Given the fact that cartilage tissue is physiologically adapted to a lower oxygen tension compared to other tissues [23], we suggest that ASCs have a greater potential to undergo chondrogenic differentiation at oxygen levels as low as 2% $O_2$ than at higher oxygen tension.

In this study, we investigated the functional properties of ASCs in terms of their stemness, proliferation and differentiation potential under normoxia and hypoxia. We demonstrated that hypoxia plays a crucial role in regulating these properties which might be associated with the upregulation of HIF-1α. Further investigation is imperative to determine the response of ASCs to various $O_2$ tensions. Optimization of ASC culture conditions may reveal invaluable insights into the stem cell biology for the successful cultivation of ASCs for future clinical use. Our findings may significantly contribute to obtaining high quality ASCs for effective clinical therapy.

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