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Stemness and Proliferation of Murine Skin-Derived Precursor Cells under Hypoxic Environment

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Skin-derived precursors (SKPs) have potential to differentiate to various cell types including osteoblasts, adipocytes and neurons. SKPs are a candidate for cell-based therapy since they are easily accessible and have multipotency. Most mammalian cells are exposed to a low oxygen environment with 1 to 5% O_2 concentration *in vivo*, while 21% O_2 concentration is common in *in vitro* culture. The difference between *in vitro* and *in vivo* O_2 concentration may affect to the behavior of cultured cells.

In this report, we investigated the effect of hypoxic condition on stemness and proliferation of SKPs. The results indicated that SKPs exposed to hypoxic condition for 5 days showed no change in proliferation. In terms of mRNA expression, hypoxia maintained expression of stemness markers; whereas, oncogenes, such as Klf4 and c-Myc, were downregulated, and the expression of Nestin, related to cancer migration, was also downregulated. Thus, SKPs cultured in hypoxia may reduce the risk of cancer in SKP cell-based therapy.

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Introduction

Skin derived precursor cells (SKPs) are multipotent stem cells that have self-renewal potential and differentiate into various cell types such as mesenchymal lineages, neurons and glial cells. SKPs can be isolated from dorsal skin and whisker follicle and are cultured in serum-free medium containing epidermal growth factor and fibroblast growth factor [1, 2]. There are many researches related to cell therapy and these researches often use neural stem cells and mesenchymal stem cells as the cell sources [3-6]. Some researches suggest that SKPs can also be used as autologous cell sources for cell therapy because they have some advantages [7, 8]. First, SKPs can be obtained from skin. It is relatively easy to be collected by noninvasive procedure. Second, SKPs can be propagated in *in vitro* and have an ability to differentiate into several cell types [7, 9]. There are some cases that *in vivo* transplanted SKPs are functional, for example, SKP-derived schwann cells form myelin and repair in transected sciatic nerve model [10].

The control of self-renewal and cell-fate commitment is an important issue for therapeutic use of cells [4, 5]. In general, most mammalian cells are routinely cultured *in vitro* at 21% O_2 concentration (normoxia) in laboratory, whereas the cells

are exposed to much lower O_2 concentration environment (1-5%; hypoxia) in mammalian bodies. Oxygen tension affects the cellular metabolism and hypoxia has an influence on proliferation and differentiation of stem cells [11-13]. However, the effect of hypoxic culture condition on SKPs has not been investigated yet. In the present study, we focused on how hypoxic condition affect to the proliferation and stemness of SKPs.

Materials and Methods

Animals

Pregnant Imprinting Control Region (ICR) mice were purchased from Taconic Biosciences Inc. (Eumseong, Korea). E17.5 fetuses of ICR mice were used in this study. All procedures were approved by the Experimental Animal Committee of Seoul National University.

Primary cell culture and subculture

Embryonic murine SKPs were cultured as previously described [1, 14]. Briefly, dorsal back skin from mouse embryos (E17.5) was dissected and cut into 1 mm^2 pieces. Skin was digested with 0.05% trypsin-EDTA for 60 min at 37℃ and filtered through a 40 μm cell strainer (BD Biosciences, MA, USA). The filtered cells were plated in a 10 cm tissue culture dish in a 37℃, 5% CO_2 incubator. Cells were grown in DMEM/F12 (3:1; Life Technologies, CA, USA), containing 1% penicillin/streptomycin (Life Technologies), 2% B27 supplement (Life Technologies), 20 ng/ml epidermal growth factor (EGF; PeproTech, NJ, USA), and 40 ng/ml basic fibroblast growth factor (bFGF; PeproTech). Cells formed spheres and the spheres were passaged every 7 days. Spheres were collected and digested in accutase (Life Technologies) for 5min and then plated at a density of $3x10^5$ cells per ml.

Hypoxic environments

Hypoxia culture conditions were maintained by incubating SKPs in an N_2/CO_2 multi gas incubator (SMA-30D, Astec, Japan). Cells were exposed to hypoxic conditions (2% O_2 and 5% CO_2) at 37℃ for 5 days.

Cell proliferation

The proliferation was assessed by the EZ-Cytox (Daeil Lab Service, Seoul, Korea), based on the water soluble tetrazolium salt-1 (WST) method. Briefly, cultured SKPs in normoxia and hypoxia were seeded in a 96 well plate and the WST reagent solution (10 μl) was added to each well and incubated for 2 h at normoxic and hypoxic condition, respectively. The absorbance was measured at 450 nm using a microplate reader (EMax Plus, Molecular devices, CA, USA).

RT-PCR and Quantitative reverse transcription-polymerase chain reaction (qRT- PCR)

RNA was extracted using the PureLink™ RNA Mini Kit (Life Technologies) and the synthesis of cDNA was performed using Labopass™ M-MuLV Reverse Transcriptase (Cosmo Genetech, Seoul, Korea) according to the manufacturer's instructions. Primers are shown in Table 1. PCR was performed using T100™ Thermal cycler (Biorad, CA, USA) with the following program: 95℃ for 5 min followed by 34 cycles of 95℃ for 30 s, 62℃ for 15 s, 72℃ for 30 s and 72℃ for 5 min. PCR product were confirmed by electrophoresis on a 1% agarose gel with SYBR Safe DNA gel stain (Life Technology) and visualized by InGenius System (Syngene, Cambridge, UK). Quantitative PCR was performed using SYBR Premix Ex Taq™ Ⅱ (Takara, Shiga, Japan) and 7500 Real-time PCR System (Applied Biosystems, CA, USA). The program was set as below: 95℃ for 30 s followed by 40 cycles of 95℃ for 5 s, 60℃ for 34 s, 95℃ for 15 s, 60℃ for 1 min and 95℃ for 15 s. Comparative C_T method ddCt was used to measure the level of expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization.

Data analysis

Data were analyzed using a student's *t*-test. All data were presented as means ± standard deviation (SD). $P < 0.05$ was defined statistical significance.

Result

Morphologic differences of spheres in the normoxia and hypoxia

SKPs formed floating spheres in non-adherent conditions. Single cells from SKP were plated in culture dishes and then after 2-3 days, primary spheres were observed and

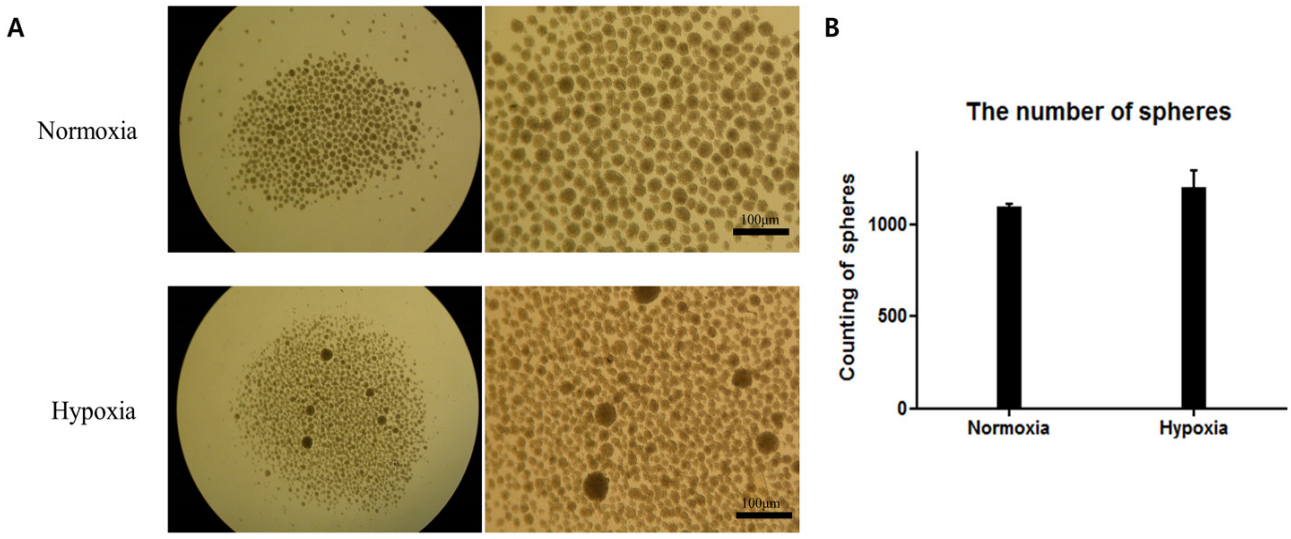

Fig 1. Effect of hypoxic condition for sphere formation of Skin-derived precursor cells (SKPs). (A) Sphere morphology of skin-derived precursor cells in normoxic and hypoxic condition. (B) The average number of spheres in normoxia group and hypoxia group. Data is expressed as mean ± standard deviation (n=4).

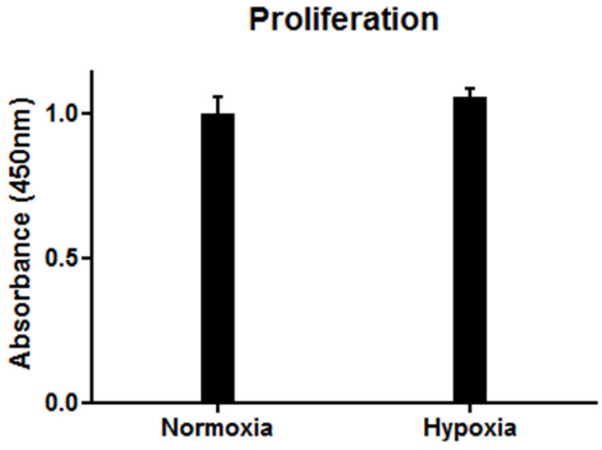

Fig. 2. Proliferation of SKPs. Proliferation was assessed by water soluble tetrazolium salt-1 assay. Data of proliferation is expressed as mean ± standard deviation (n=3).

the spheres grew in size until 5-7 days. There were no significant morphological differences between normoxic spheres and hypoxic spheres, except for their size. In hypoxia group, size of SKP spheres was smaller than the spheres in normoxia group (Fig 1A). The spheres were counted after 5 days of seeding. The number of spheres tended to increase in hypoxia condition, although the change was not statistically significant (Fig 1B).

Proliferation of SKPs in hypoxia

SKPs were cultured in the SKP medium containing EGF and bFGF. There was no effect on the proliferation of the cells between two experimental groups (Fig 2).

Gene expression changes in hypoxia

RT-PCR was performed to evaluate the change of gene expression levels in two experimental groups. As shown in Fig 3A, Hif1a expression was significantly higher in hypoxic condition. Expression level of Oct4, Sox2, Nanog, and Rex1 genes were not significantly different between normoxia and hypoxia groups. Interestingly, Klf4, c-Myc, and Nestin genes showed approximately 40% lower levels of mRNA expression in hypoxic condition when compared with in normoxic condition (Fig 3B).

Discussion

SKPs derived from dermis have ability to form floating colonies, which are called spheres in suspension cultures [1, 2, 14]. SKPs maintain their multipotency and have capacity to differentiate into multiple lineages [15, 16]. The newly isolated SKPs contain heterogenous populations that are mixed with SKPs, adipose cells, muscle cells and blood cells [1]. In this study, passage 2 SKPs were used to obtain more homogenous population of the cells.

Oxygen is important to life. It is required to produce metabolic energy and maintain cellular processes [17]. Oxygen concentration in standard cell culture is 21%

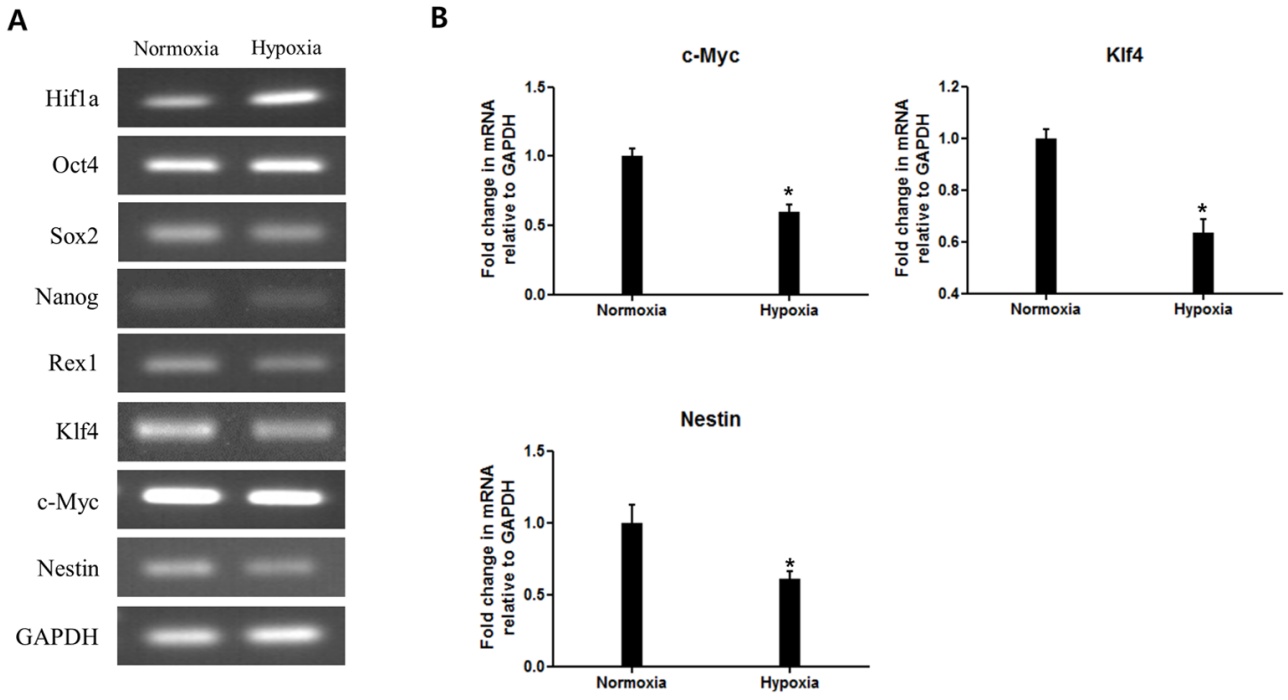

Fig. 3. Expression patterns of mRNA in skin-derived precursor cells. (A) Expression of patterns of Hif1a, Oct4, Sox2, Nanog, Rex1, Klf4, c-Myc and Nestin. (B) Relative mRNA levels of the Klf4, c-Myc and Nestin genes. GAPDH was used for normalization. Data are expressed as mean ± standard deviation (n=3). * indicates statistical significance between two groups. *$P < 0.05$.

(normoxia). However, most tissues are exposed to low O_2, 1-5% (hypoxia) in mammalian body and hypoxic condition is considered as stem cell niche [18]. In previous studies, hypoxic condition has influences on the proliferation and differentiation of stem cells including neural stem cells [19], mesenchymal stem cells [20], and induced pluripotent stem cells [21]. In the present study, the size of SKP spheres in hypoxia were smaller than normoxia group ones. Hypoxia had no effect on the proliferation of the cells although a few more spheres were observed. These results indicate that O_2 concentration is not critical for the formation of spheres and proliferation in SKPs when SKPs were cultured in hypoxia for 5 days. The proliferation of cells is not affected in some studies [22, 23]. Since the lifespan of multipotential stromal cells (MSCs) is prolonged by increasing senescence marker, $P16^{INK4A}$, under normoxic condition, the proliferation of MSCs has no changes between hypoxia and normoxia [23]. Increased level of senescence marker in normoxic condition also might be the reason for our results.

Maintenance of oxygen homeostasis is essential for cell survival in hypoxic condition [24]. Hypoxia-inducible factor (Hif) is a heterodimeric transcription factor composed of alpha (a) subunit and beta (b) subunit [25]. Hif b subunit is constitutive expressed, whereas Hif a subunits (Hif1a, Hif2a and Hif3a) are expressed in an O_2 dependent manner [24, 26]. The role of Hif3a is not yet clearly understood while Hif2a is insensitive to O_2 changes [27]. Hif1a is sensitive to hypoxia [27], and is also confirmed in this report. Hypoxic condition suppressed the expression of Klf4, c-Myc, and Nestin. Klf4 and c-Myc are well known as transcription factors that have an oncogenic function [28, 29]. These oncogenes are required for maintenance of cancer stem cells and tumor growth was also delayed and reduced when these oncogenes were downregulated [30, 31]. Nestin, an intermediate filament protein and marker of several progenitor cells, is expressed in some cancer cells such as lung [32], breast [33] prostate [34, 35] and pancreatic [34, 35] cancer cells. Since the expression of Nestin is related to malignancy and metastasis of cancers [36], downregulation of this gene inhibits the migration and growth of tumors [37]. Although hypoxia is also known as a critical factor of cancer, the effect of hypoxia in cancer has not been clearly determined yet. The tumor growth and metastasis are increased in acute hypoxia, from 30 min to 72 h [38]. On

the other hand, tumor progression was repressed when tumor cells were exposed to the chronic hypoxia, from 72 h to 2 weeks. Interestingly, this effect was maintained for 3 weeks even after the hypoxic condition was removed [39]. Although the precise effect of chronic hypoxia in stem cells and precursors has not been understood yet, acute hypoxic condition promote the proliferation and increase the expression of stemness genes in various stem and cancer cells [38, 40]. In the present study, the SKPs exposed to hypoxic condition for 5 days, which can be considered as chronic hypoxia, show repression of oncogenes similar to tumor cells under hypoxic condition whereas the genes related with stemness maintenance was not affected under same condition.

In conclusion, hypoxic culture condition may reduce the risk of cancer, when SKPs are used for cell transplantation therapy.

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Conflict of interest

The author's declare that there is no conflict of interest that would prejudice the impartiality of this work.

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