Hypoxia Enhances Osteogenic Differentiation in Retinoic Acid-Treated Murine-Induced Pluripotent Stem Cells

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Hypoxic condition influences biological responses in various cell types. However, a hypoxic regulating osteogenic differentiation remains controversy. Here, an influence of short-term culture in hypoxic condition on osteogenic marker gene expression by retinoic acid-treated murine gingival fibroblast-derived induced pluripotent stem cells (RA-miPS) was investigated. Results demonstrated that hypoxic condition significantly upregulated *Vegf*, *Runx2*, *Osx*, and *Ocn* mRNA expression by RA-miPS in normal culture medium at day 3. Further, desferrioxamine significantly downregulated pluripotent marker (*Nanog* and *Oct4*) and enhanced osteogenic marker (*Runx2*, *Osx*, *Dlx5*, and *Ocn*) gene expression as well as promoted *in vitro* mineral deposition. However, the effect of cobalt chloride on osteogenic differentiation of RA-miPS was not robust. In summary, the results imply that hypoxic condition may be useful in the enhancement of osteogenic differentiation in RA-miPS.

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Key Words: Pluripotent stem cells; Hypoxia; Desferrioxamine; Cobalt chloride

INTRODUCTION

Oxygen concentration, both low and high levels, is shown to regulate induced pluripotent stem cell (iPS) differentiation [1,2]. Several publications have addressed the influence of hypoxic condition on iPS, including derivation, stemness maintaining, and differentiation ability [3,4]. Normal *in vitro* cell culture condition is performed under 21% oxygen tension [5]. However, the oxygen tension in normal tissue is in the range between 0.7–7% [5]. Hypoxia refers to the condition that the oxygen levels decrease lower than normal arterial range. However, the hypoxic condition in the *in vitro* setting refers to condition that the oxygen tension is lower than the ambient air. Hypoxia has been shown to regulate various cell responses. It participates in regulation of stemness maintenance as well as differentiation. Under hypoxic condition, the efficacy of iPS generation is significantly increased [6].

Role of hypoxia on osteogenic differentiation is widely investigated, especially in mesenchymal stem cells (MSCs). However,

an influence of hypoxic condition on osteogenic differentiation remains controversy. Hypoxic culture condition, either physical hypoxia or chemical mimic hypoxic condition, has shown to promote osteogenic differentiation in various types of stem cells [7,8]. In this regard, hypoxic condition markedly promotes mineralization and osteoblast marker gene expression by human adipose derived stem cells [7]. Similarly, low oxygen concentration enhances human periodontal ligament stem cell differentiation toward osteogenic lineage both *in vitro* and *in vivo* [8]. On the contrary, an inhibitory effect of hypoxic condition on osteoblastic differentiation is reported [9,10]. In this respect, chemical induced hypoxia attenuates osteoblast marker gene expression and *in vitro* mineralization by human periodontal ligament cells [9]. Correspondingly, hypoxic culture lead to the reduction of mineralization and osteogenic gene expression in bone marrow-derived MSCs as compared to normoxic culture [10]. The discrepancy may be due to various different factors among the experiments, including cell types, hypoxic duration, and hypoxic culturing technique.

It has been reported that mouse iPS (miPS) exhibits significant higher mineral deposition than mouse MSCs upon osteogenic differentiation *in vitro* [11], implying the potential use of iPS in bone regenerative treatment. Various approaches are utilized to promote direct osteogenic differentiation from iPS cells [12]. Though, the differentiation ability is still variable [12]. In addition, the potential mechanism(s) regulating osteogenic dif-

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ferentiation in iPS is yet ill-defined. As described above, various publications investigate the regulation of osteogenic differentiation by altering oxygen concentration in various cell types. Though, limited number of publications addresses the role of hypoxic on osteogenic differentiation of iPS cells toward osteogenic lineage. Therefore, the aim of this study was to investigate an influence of hypoxic condition on osteogenic differentiation potency of retinoic acid treated miPSs (RA-miPS).

MATERIALS AND METHODS

Cell culture

Gingival fibroblast derived miPS culture was performed according to previous publications [11,13]. In brief, cells were maintained on mouse fibroblast STO cell line transformed with neomycin resistance and murine leukemia inhibitory factor genes (SNL) feeder cells in the embryonic stem cell (ES) medium containing Dulbecco's Modified Eagle Medium (DMEM; Nacalai Tesque, Japan), 15% fetal bovine serum (FBS; Gibco BRL, Carlsbad, CA, USA), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 50 U of penicillin, and 50 ug/mL streptomycin. For osteogenic induction (Fig. 1A), miPS were trypsinized and seeded on petri dishes to form embryoid bodies in ES medium for 2 days and subsequently maintained in ES medium supplemented with all-trans retinoic acid (RA; Sigma-Aldrich Chemical, St. Louis, MO, USA; 1 μ M) for another 2 days. Embryoid body suspension were seeded in 0.1% gelatin-coated tissue culture plated (density 250000 cell/cm²) and allowed to attach in RA supplemented ES medium for 1 day. Cells were subjected to different treatment as shown in a schematic diagram in Figure 1A. The osteogenic induction medium was DMEM (Gibco BRL, Carlsbad, CA, USA), supplemented with 100 unit/mL penicillin (Gibco BRL, Carlsbad, CA, USA), 100 ug/mL streptomycin (Gibco BRL, Carlsbad, CA, USA), 250 ng/mL amphotericin B (Gibco BRL, Carlsbad, CA, USA), 2 mM L-glutamine (1x Glutamax[®]) (Gibco BRL, Carlsbad, CA, USA), 15% FBS (Hyclone, USA), 50 ug/mL ascorbic acid (Sigma-Aldrich Chemical, St. Louis, MO, USA), 100 nM dexamethasone (Sigma-Aldrich Chemical, St. Louis, MO, USA), and 10 mM β -glycerophosphate (Sigma-Aldrich Chemical, St. Louis, MO, USA). The control condition was maintained in ES medium.

Hypoxic culture condition

Hypoxic condition was set using hypoxic chamber. The oxygen levels were monitored using oxygen detector (AsunGadget, Shenzhen, China) at less than 1 percentage of oxygen concentration. The chamber was placed in the cell culture incubator. Cells in normal tissue culture condition were used as the control

condition. For chemical induced hypoxic condition, cobalt chloride (CoCl₂, Sigma-Aldrich Chemical, St. Louis, MO, USA) or desferrioxamine (DFX, Calbiochem, EMD Biosciences, Inc., San Diego, CA, USA) was added into the culture medium at concentration of 50 or 100 μ M. These chemical agents were generally employed to mimic hypoxic condition by stabilizing HIF-1 α [14]. The equal amount of vehicle solution was added in the control condition.

Polymerase chain reaction

Isol-RNA Lysis Reagent (5 PRIME, Gaithersburg, MD, USA) was used to extract total cellular RNA. One microgram of the isolated RNA was converted to complementary DNA using reverse transcriptase enzymatic reaction (Promega, Madison, WI, USA). Real-time polymerase chain reaction was performed in a Lightcycler Nano real-time PCR system (Roche Applied Science, Indianapolis, IN, USA) using FastStart[®] Essential DNA Green Master[®] (Roche Applied Science, Indianapolis, IN, USA). The expression levels were normalized to the expression of 18S ribosomal RNA and the control condition. The primer sequences were shown in Supplementary Table 1 (in the online-only Data Supplement).

Alizarin red staining

The samples were fixed with cold methanol and rinsed with deionized water. Alizarin Red S solution (1% W/V) (Sigma-Aldrich Chemical, St. Louis, MO, USA) was added into the samples at room temperature and incubated for 2 minutes under gentle agitation. The stained calcium was solubilized by 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich Chemical, St. Louis, MO, USA). The optical density was further evaluated at 570 nm.

Statistical analyses

The data were illustrated as box and whisker plot. The line in the box indicates median value. Lower and upper borders of the box indicate 1st and 3rd quartile, respectively. Upper and lower bar represent maximum and minimum values. Mann Whitney U test was employed for independent two groups comparison and Kruskal Wallis test followed by pairwise analysis was utilized for three or more group comparison. The statistical analyses were performed using IBM SPSS Statistics for Mac, Version 22 (Armonk, NY, USA). Differences at $p < 0.05$ were considered to be statistically significant.

RESULTS

Hypoxic condition upregulated osteogenic marker gene mRNA expression

The results demonstrated that cells significantly up-regulated

Vegf messenger RNA (mRNA) expression in hypoxic culture condition in both control growth medium and osteogenic induction medium at day 1 and 3, confirming the effectiveness of hypoxic culture. Interestingly, the increase of osteogenic marker genes was noted upon culturing cells in hypoxic condition (Fig. 1B and C). *Runx2*, *Osx*, *Dlx5*, and *Ocn* mRNA levels significantly increased in hypoxia as compared to the control when cells were maintained in the control growth medium at day 1 (Fig. 1B). For day 3, the significant up-regulation of *Runx2*, *Osx*, and *Ocn* was noted in cells cultured in the control growth medium and hypoxic condition (Fig. 1C). In osteogenic culture condition, the increase of osteogenic marker under hypoxia was also noted as compared to the normoxia but there is no statistical significant difference.

Chemical inducing hypoxic condition enhanced osteogenic differentiation

Hypoxic culture condition was mimicked using chemical agents, DFX or CoCl_2 . Both agents mimic hypoxic condition by stabilizing hypoxia inducible factor-1 α (HIF-1 α), the main transcriptional factor in hypoxic condition [9,15]. At day 3, the addition of DFX or CoCl_2 in growth medium significantly up regulated *Vegf* mRNA expression compared to the untreated control (Fig. 2A and K), implying the effectiveness of these chemical agents in term of hypoxic mimicking function.

The supplementation of DFX (100 μM) for 3 days promoted the *Runx2*, *Osx*, *Dlx5*, and *Ocn* mRNA expression as compared to the untreated control (Fig. 2B-E). Moreover, the downregulation of pluripotent markers (*Nanog* and *Oct4*) was significantly

Figure 1. Hypoxic condition enhanced osteogenic marker gene expression. (A) Experimental plan illustrated the culture condition. Cells were cultured in hypoxic chamber for 1 day (B) or 3 days (C) after the adhering culture in the normal growth medium (Ctrl) or osteogenic induction medium (OM). The mRNA expression was determined using real-time PCR. Bars indicated the statistical significant difference. NC: normoxic culture condition, HC: hypoxic culture condition.

noted in DFX supplemented condition (Fig. 2F-H). Mineral deposition significantly increased in those cells treated with DFX for 3 and 7 day (Fig. 2I and J). No statistical significant difference was noted on mRNA levels of osteogenic and pluripotent marker genes upon CoCl_2 supplementation (100 μM), except *Runx2* and *Dlx5* (Fig. 2L-R). However, significant increase of mineral deposition was noted at day 7 upon treated cells with CoCl_2 (Fig. 2S and T).

Short-term exposure to hypoxia promoted mineral deposition

Short-term effect of hypoxia on osteogenic differentiation by miPS cells was evaluated. Cells were cultured in osteogenic medium under hypoxic condition, CoCl_2 , or DFX supplementation for 3 days (Fig. 3A). Subsequently, cells were maintained in osteogenic medium in normoxic condition for another 4 days. The results showed that mineral deposition was significantly enhanced in all treatment conditions compared to the control (Fig.

Figure 2. Effect of desferrioxamine (DFX) or cobalt chloride (CoCl_2) on osteogenic marker gene expression. Cells were cultured in medium supplemented with DFX or CoCl_2 for 3 days. The mRNA levels were determined using real-time PCR (A-H and K-R). Mineral deposition was examined using alizarin red s staining (I-J and S-T). Bars indicated the statistical significant difference.

Figure 3. Short-term hypoxic treatments enhanced mineral deposition. (A) Experimental plan illustrated the culture condition. (B) Mineral deposition was examined using alizarin red s staining. Bars indicated the statistical significant difference. HC: hypoxic culture condition.

3B). Though, short-term exposure to physical hypoxia resulted in the highest mineral deposition.

DISCUSSION

Under low oxygen concentration, HIFs is stabilized and further translocated into the nucleus, resulting in the activation of gene transcription. One of the main target genes of HIFs is *Vegf* [16]. Under hypoxic condition, the significantly upregulation of *Vegf* was observed [9,17]. In this present study, the significant upregulation of *Vegf* mRNA levels was noted when RA-miPS cells were exposed to physical hypoxia, DFX, or CoCl_2 . These results may further confirm the effectiveness of hypoxic condition in the experimental setting in the present study.

Effect of physical hypoxia on osteogenic differentiation was extensively investigated in MSCs. Although, miPS cells and

MSCs could be differentiation into osteoblast cells, the regulation mechanisms might different [11]. Thus, the regulation of osteogenic differentiation under hypoxic condition may dissimilar. The present study described the positive induction effect of hypoxic condition on osteogenic marker gene expression by RA-miPS cells. Correspondingly, previous study showed that exposing rabbit ES cells to hypoxia resulted in the enhancement of differentiation toward MSCs [18].

On the contrary, previous study illustrated that osteogenic induction of miPS cells under hypoxic condition (5% O_2) attenuated osteogenic differentiation as determined by the reduction of *Runx2* and *Ocn* mRNA expression as well as mineral deposition [4]. These contradict results could be explained by several aspects. First, RA-treated embryoid body formation was performed in the present study to obtain MSC-like cells prior to osteogenic induction [19]. On the contrary, previous study em-

ployed the direct osteogenic induction in co-culture of miPS cells and feeder cells. In this regard, different osteogenic induction protocols for mouse ES cells were compared and the results demonstrated that RA-treating embryoid bodies followed by adhering culture supplemented with β -glycerophosphate, ascorbic acid, and dexamethasone demonstrated the higher osteogenic marker gene expression and mineralization *in vitro* [20]. In addition, osteogenic induction of miPS in co-culture with feeder cells may result in the different effect since feeder cells could secrete unknown factors influencing miPS behaviours i.e. cell growth and stemness maintenance. Embryoid body formation technique could be utilised to isolate miPS cell population from feeder cells, thus the effects of feeder cells on osteogenic induction could be eliminated. Second, the discrepancy between the present study and previous work could be due to various different factors among the experiments, including oxygen concentration, hypoxic duration, and hypoxic culturing technique.

Physical hypoxia requires specific facilities and is technically sensitive. Several limitations are noted when using physical hypoxic chamber. For example, re-oxygenation may be occurred during medium changing. Oscillated oxygen concentration could lead to the change in biological responses [21]. Thus, an alternative strategy is to use a chemical-induced hypoxic condition. DFX and CoCl_2 are usually employed as chemical-induced hypoxic condition as they are able to stabilize HIF-1 α , the main transcriptional factor in hypoxic condition [9,15]. Previous reports suggest different potency of DFX and CoCl_2 . Both chemical agents enhanced HIF-1 α accumulation. DFX is known as an iron chelator that competes with endogeneous iron, resulting in the inhibition of prolyl hydroxylation activity [22]. CoCl_2 replaces iron in an iron-binding domain of prolyl hydroxylase enzyme, thus inhibiting its activity [22]. CoCl_2 also inhibits a binding between HIF α and VHL protein, resulting HIF stabilization [23]. CoCl_2 could also promote HIF stabilization via other mechanisms, including ROS production as well as PI-3K and MAPK pathway [24]. However, DFX, but not CoCl_2 , enhanced HIF-1 α accumulation via COX-2 activity [25]. Further, DFX promoted higher VEGF expression and lesser inhibitory effect on cell proliferation and protein synthesis in human periodontal ligament cells and human gingival fibroblast [26]. In the present study, DFX supplementation enhanced osteogenic marker gene expression similar to those cells treated in hypoxic chamber. However, CoCl_2 did not significantly alter osteogenic gene expression, except *Dlx5*. Further, DFX promoted mineral deposition as early as day 3. CoCl_2 enhanced mineralization at day 7. These results suggest the different regulatory mechanism of DFX and CoCl_2 regarding osteogenic induction in miPS.

The results from the present study demonstrated the marked increase of osteogenic marker gene expression in growth medi-

um upon culturing cells in hypoxic or hypoxic mimicking condition. Although, slight increase of osteogenic related mRNA levels was observed in hypoxic/osteogenic inductive condition compared to the normoxia/osteogenic condition, the difference was not dramatic. This may be due to the influence of osteogenic inductive agents. In other words, hypoxic condition may promote osteogenic marker gene expression by RA-miPS but its effect was not synergist with the osteogenic induction medium.

In summary, the present study demonstrated that hypoxic condition enhanced osteogenic differentiation in miPS cells. In addition, RA-treated embryoid body culture prior to osteogenic induction may be a crucial technique for osteogenic induction in miPS cells. Further, the osteogenic inductive effect of DFX in RA-miPS similar to those in hypoxic chamber. Thus, DFX might be an alternative and practical approach to promote osteogenic differentiation in miPS.

Supplementary Materials

The online-only Data Supplement is available with this article at <http://dx.doi.org/10.1007/s13770-016-9127-9>.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There is no experiment on human and animal subjects in this article.

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