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Effect of Hypoxia on Self-Renewal Capacity and Differentiation in Human Tendon-Derived Stem Cells

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Hypoxic conditions play roles in functioning of human tendon-derived stem cells (hTSCs). The goal of this study was to investigate the effect of various hypoxic conditions in self-renewal capacity and differentiation of TSCs.

Material/Methods: hTSCs was obtain from supraspinatus tendon donors. Colony formation and cell proliferation assay were used to assess the self-renewal of hTSCs. qRT-PCT and Western blot analysis were used to examine stemness and multi-differentiation potential of hTSCs.

Results: We found that culturing at 5% O_2 is more beneficial for the self-renewal of hTSCs than the other 3 culture conditions, with larger colony size and numbers. The proliferation of hTSCs in 5%, 10%, and 20% O_2 cultures increased after seeding. The number of cells in the 5% O_2 condition was higher than that in other culture; however, self-renewal capacity of hTSCs in 0.5% O_2 was inhibited. The expression levels of stem cell markers, including NS, Nanog, Oct-4, and SSEA-4, were highest in 0.5% O_2 culture. Furthermore, hTSCs cultured in 20% O_2 exhibited significantly higher expression of the 3 markers (PPAR-γ, Sox-9, and Runx-2).

Conclusions: Hypoxic condition of culture encouraged self-renewal capacity of hTSCs, but inhibited their multi-differentiation potential, compared to normoxic condition of culture. Moreover, excessively low oxygen concentration impaired the capacity of hTSCs.

MeSH Keywords: Adult Stem Cells • Antigens, Differentiation • Cell Hypoxia

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Background

Tendons are fibrous connective tissues connecting muscle to bone to facilitate joint movement and to maintain skeletal stability [1]. Tendons are mainly composed of extracellular matrix (ECM) containing 68% water, 30% collagen, and 2% elastin, in which a very low density of tenocytes are embedded [2]. As the main cell population in tendons, tenocytes are responsible for synthesizing and maintaining tendon ECM [3]. Tenocytes were long considered as the only cell type in tendons. Tendon-derived stem cells (TSCs) were first discovered in 2007 in the tendons of many species, including humans, mice, rats, and rabbits [4,5]. Like other stem cells, TSCs have self-renewal capacity with multi-differentiation potential to change into tenocytes, chondrocytes, osteocytes, and adipocytes under specific conditions [5].

Due to being subjected to large mechanical loads, tendons are easily injured. After tendon injuries, patients often have a long, complex healing process with the formation of a fibrotic scar [6]. As a result, the pattern of collagen fibers and fibrils are changed in tendons with fibrotic scarring; therefore, these tendons have inferior mechanical strength compared to normal tendon tissue, resulting in significant dysfunction and disability. Recently, many laboratory studies have shown promising outcomes of tendon repair treated with stem cells [7–10] because of various proliferation and differentiation advantages of using stem cells. Moreover, many studies demonstrated that TSCs not only retained multi-differentiation potentials like other stem cells, but also were more prone to transform into tenocytes than other stem cells. Therefore, TSCs might become a novel cell source for tissue engineering, attracting increasing attention from experimental and clinical researchers.

The self-renewal capacity and differentiation of stem cells are influenced by different environments, such as ECM composition, pH value, oxygen tension, and mechanical loading. TSCs expansion is necessary to collect sufficient numbers of cells for tendon repair. However, in the expansion process TSCs are predisposed to differentiate quickly, causing stemness loss under regular culture conditions of 95% air and 5% CO_2. *In vivo*, tendons are collagen-rich, avascular structures; therefore, the oxygen level in tendons is relatively lower than in vascular-rich organs and tissues [11]. Therefore, hypoxia might favor TSCs.

A previous study has demonstrated that TSCs could better maintain their stemness under hypoxic conditions. However, the role of oxygen concentrations in differentiation potential of TSCs remains unclear. Therefore, we performed the present study to investigate the effect of different hypoxic conditions in self-renewal capacity and differentiation of human TSCs (hTSCs).

Material and Methods

Cell culture

All tendon tissues were collected from supraspinatus tendons of 6 young adult donors, with approval from the Research Ethics Committee of the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (Wenzhou, China). hTSCs isolation was performed according to the method previously introduced by Lee et al. [12]. A condition of 37°C with 5% CO_2 in a humidified incubator was applied for cell culture, as previously described (13). Cells from passages 4 to 6 were used in all experiments.

Control of hypoxic and normoxic culture conditions

The hypoxic and normoxic culture conditions was controlled according to the procedure described by Zhang et al. [14]. Hypoxic conditions (0.5%, 5%, 10% O_2) in the present study were achieved using a dedicated tri-gas incubator. A regular tissue culture incubator was used to maintain normoxic culture conditions via feeding 95% air and 5% CO_2. During all experiments, oxygen concentration in all incubators were kept at a constant level. Therefore, in the present study, hTSCs cultured under normoxic culture conditions were divided into a control group (20% O_2 group), and hTSCs cultured under the other 3 conditions were divided into 3 experimental groups (0.5%, 5%, and 10% O_2).

Colony formation and cell proliferation assay

hTSCs were seeded into a culture dish at a seeding density of 50 cells/cm^2 for 14 days. Subsequently, all cells were stained using PBS method for counting cell colonies. Colonies of more than 50 cells under a microscope were counted. Triplicate experiments were used to ensure accuracy. We determined cell proliferation using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) at days 1, 2, 6, and 12 after seeding, as previously described [15].

Quantitative real-time PCR (qRT-PCR)

Total RNA extraction from hTSCs was conducted using Trizol reagent (Invitrogen, Carlsbad, CA). We reverse-transcribed 1 µg RNA to synthesize first-strand cDNA with the RevertAid RT-PCR system (Fermentas, Pittsburgh, PA). qRT-PCR was carried out using the Maxima SYBR Green qPCR Master Mix (Applied Biosystems, Carlsbad, CA) in a Chromo 4 Detector (MJ Research) following the manufacturer's instructions. We synthesized gene-specific primers for nucleostemin (NS), Nanog, Oct-4, SSEA-4, Runx-2, PPAR-γ, and Sox-9 based on previously published sequences (16). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. At least 3 replicates were performed for each experiment in the study.

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Multi-differentiation assays

The adipogenic, osteogenic, and chondrogenic differentiation capabilities of hTSCs in hypoxic and normoxic culture conditions were evaluated as previously described [13,14,17]. To perform differentiation potential assay, cells were cultured for up to 14 days in 3 different mediums – adipogenic induction medium (DMEM, 20% FBS, 100 mM indomethacin, and 0.5 mM isobutylmethylxanthine), osteogenic induction medium (DMEM-low glucose, 20% FBS, 50mg/ml ascorbic 2-phosphate, 100 mg/ml sodium pyruvate, and 50 mg/ml insulin-transferrin-selenious acid mix), and chondrogenic induction medium (DMEM, 20% FBS, 0.2 mM ascorbic 2-phosphate, 10 mM glycerol 2-phosphate) – according to the manufacturer's instructions. Oil Red O (Millipore) staining was used to assess adipogenesis. Alizarin Red solution (Millipore) was used to examine calcium deposition for osteogenesis. Alcian Blue (Millipore) staining was used to measure chondrogenesis.

Western blot analysis

Western blotting was performed to examine Nanog, NS, Oct-4, and SSEA-4 protein. After being cultured under hypoxic or normoxic conditions for 3 days, hTSCs were collected and protein was obtained. The protein was separated by 12% SDS-PAGE, transferred to PVDF membranes, and subsequently blocked in 5% fat-free milk for 2 h, following by incubation with primary antibodies at 4°C overnight. All primary antibodies were from Novus Biologicals, Inc. (Littleton, CO). Secondary antibody (Dako, Carpentaria, CA) conjugated with horseradish peroxidase was then applied. Finally, protein bands were detected with chemiluminescence (Beyotime, Shanghai, China). The expression levels of proteins assessed in this study were normalized to GAPDH. All experiments were repeated 3 times.

Statistical analysis

Statistical analysis, including one-way analysis of variance (ANOVA), *t* test, and Tukey's HSD post hoc test, was conducted using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). All data are presented as the mean ±SEM. $P<0.05$ was considered to be statistically significant.

Results

We first assessed the effects of hypoxia treatment on self-renewal capacity of hTSCs. Similar numbers of hTSCs were cultured in 0.5%, 5%, 10%, and 20% O_2 cultures. We found that 5% O_2 culture is more beneficial for the self-renewal of hTSCs than the other 3 cultures, with larger colony sizes and numbers (Figure 1A–1C). In addition, proliferation of hTSCs in 5%, 10%, and 20% O_2 cultures increased after seeding (Figure 1D). The number of cells in the concentration of 5% of O_2 was higher than that in other culture; however, self-renewal capacity of hTSCs in 0.5% O_2 was inhibited.

Then, we examined the stemness of hTSCs using qRT-PCR and Western blot analysis. We found that the expression levels of stem cell markers, including NS, Nanog, Oct-4, and SSEA-4, were highest in 0.5% O_2 culture. Furthermore, the environment with 0.5% O_2 or 20% O_2 inhibited the stemness of hTSCs (Figure 2).

Finally, the multi-differentiation potential of hTSCs was determined through the analysis of PPAR-γ (adipogenic marker), Runx-2 (osteogenic marker), and Sox-9 (chondrogenic marker). Oil Red O (Millipore) staining for adipogenesis, Alizarin Red solution (Millipore) for osteogenesis, and Alcian Blue (Millipore) staining for chondrogenesis were used. During the 14-day period of differentiation, hTSCs cultured in 20% O_2 exhibited significantly higher expression of the 3 markers (PPAR-γ, Sox-9, and Runx-2), suggesting that the 20% O_2 environment promoted the differentiation of hTSCs (Figure 3).

Discussion

Tendon disorders are a serious health problem involving over 30% of musculoskeletal injury. Tendon injuries include chronic tendinopathy and acute tendon rupture. Surgical options for tendon injuries during clinical therapy are limited, with different implantations, including autografts and allografts. However, instead of complete regeneration, tendon healing after surgical treatment is accompanied by poor results, with scarring formation and adhesion, leading to partial tendon dysfunction [2]. Moreover, tendon injuries have a slow recovery process and high healthcare costs. An increasing number of investigators are engaged in fundamental basic science studies aimed at understanding the exact mechanism of tendon injury and healing [18]. The identification of TSC started a new epoch in understanding the pathology of and developing novel strategies for tendon injury. Increasing animal studies have shown the outstanding effect of TSCs for the repair of tendon injuries [19]. However, effective measures to regulate the fate of TSCs remain limited. The present study is the first to investigate the effect of different hypoxic concentrations on hTSCs.

Our findings suggest that environmental oxygen is an important factor for the growth and proliferation of hTSCs, and that a hypoxic environment is promotes TSCs to form effective engineering tissue for injured tendon repair. Moreover, hypoxia should be kept within a certain range, because concentrations of O_2 that are too low influence the capacity of hTSCs. Our results also indicated that the hypoxic condition of 5% O_2 can improve TSCs self-renewal to achieve sufficient numbers of TSCs necessary for tissue engineering. Larger numbers of

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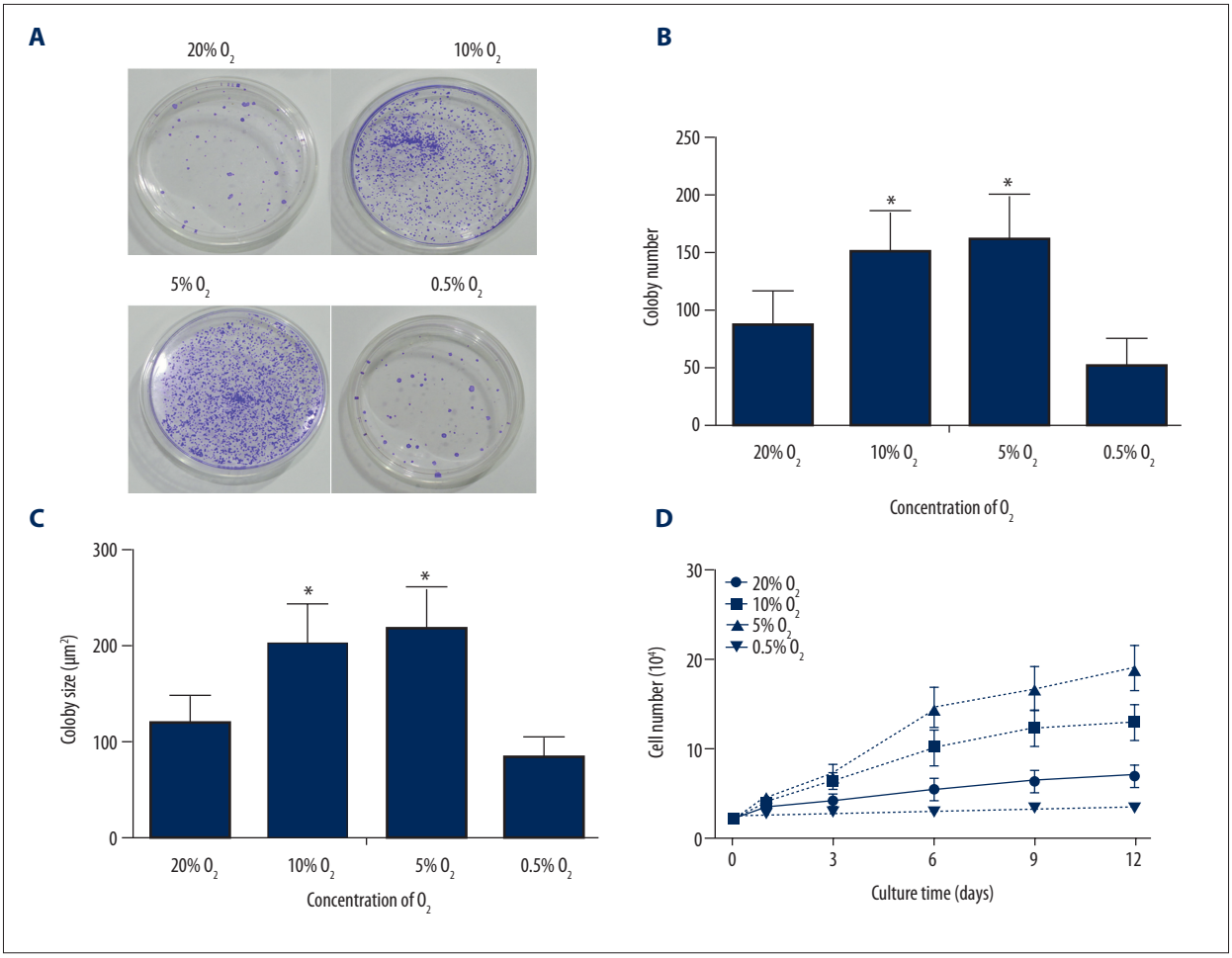

Figure 1. The self-renewal capacity of hTSCs under different concentrations of oxygen culture. (**A**) Colony formation; (**B**) Colony number; (**C**) Colony size; (**D**) proliferation of hTSCs.

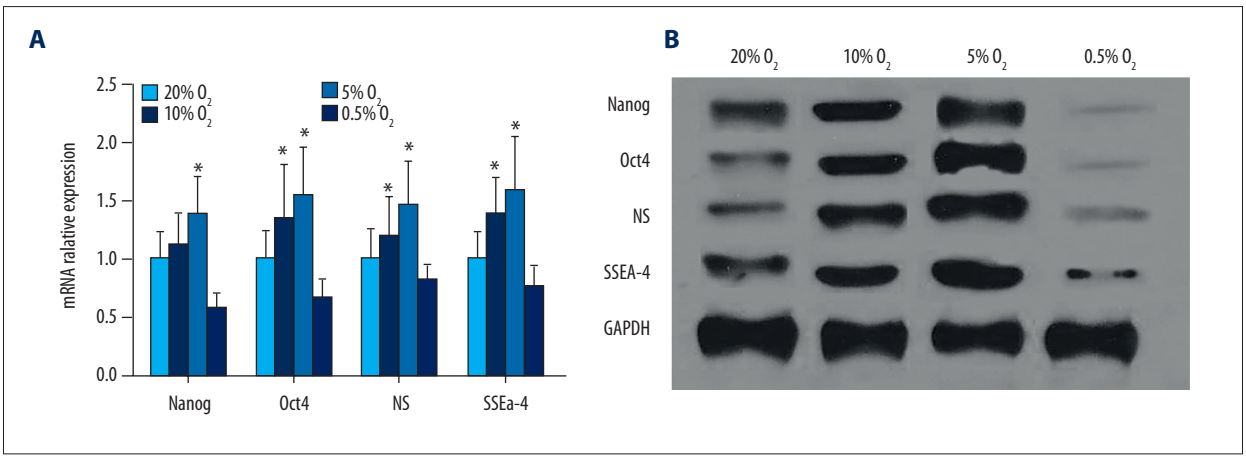

Figure 2. The expression of stem cell markers by hTSCs under different concentrations of oxygen culture conditions by qRT-PCR (**A**) and Western blot (**B**).

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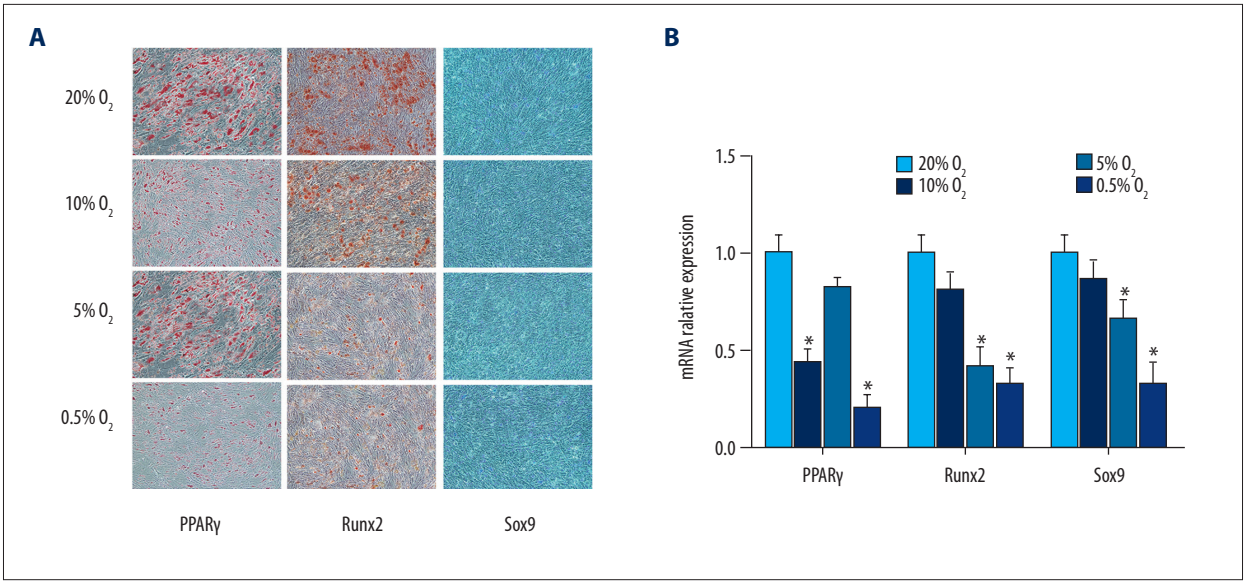

Figure 3. The differentiation potential of hTSCs under different concentrations of oxygen culture conditions. (**A**) At day 14 after differentiation induction, lipid accumulation, cartilage matrix formation, and calcium deposition were assessed by Oil Red O, Alcian Blue, and Alizarin Red S staining, respectively. (**B**) mRNA levels of the adipogenic marker PPAR-γ, the chondrogenic marker Sox-9, and the osteogenic marker Runx-2 were measured by qRT-PCR.

stem cells are more competitive for tissue repair or regeneration when using stem cells to treat tendon injury [20].

Many studies have demonstrated the potent suppressing effect of hypoxia on mitochondrial oxidation [21] and promoting stemness of several stem cell types [22–24]. The mitochondrial oxidative metabolism status has been indicated to play an important role in stem cells [25,26]. A recent study reported that it was easier to induce mouse embryonic fibroblasts to transform into pluripotent stem cells (iPSCs) under hypoxic conditions [25]. Some undifferentiated stem cells with lower levels of mitochondrial mass, such as iPSCs, and bone marrow mesenchymal stem cells (BMSCs), were reported to utilize non-oxidative glycolysis for energy [27–29]. Accumulating studies have demonstrated the effects of different hypoxic conditions on stem cells. Lavrentieva et al. reported that hypoxia promotes self-renewal of human mesenchymal stem cells (MSCs) compared with normoxic condition [30]. Lennon et al. indicated that rat MSCs cultured in a hypoxic condition of 5% O_2 produced more bone formation than those cultured in a normoxic condition of 20% O_2 [31]. Our results agree with the findings of these studies.

Some limitations associated with this study should be considered. First, the medium using plastic dishes for hTSCs might have a role in promoting differentiation cell. Second, we grew the hTSCs in 2D culture, which is not consistent with cells *in vivo*. Moreover, hTSCs lacked mechanical loading, which is an important factor for tendons due to muscle-bone force-transmission function. Finally, the molecular mechanism was not investigated, and this needs to be determined in future work.

Conclusions

In summary, hypoxic culture encouraged self-renewal capacity of hTSCs, but inhibited their multi-differentiation potential, compared to normoxic condition of culture. Moreover, oxygen concentrations that were too low impaired the capacity of hTSCs. Future studies investigating the mechanism by which TSCs function under low-oxygen conditions are required.

Competing interests

The authors declare that they have no competing interests.

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