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Research Article

Hypoxia Preconditioning Enhances Self-Renewal and Multi-Differentiation Potential of Tendon-Derived Stem Cells

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Abstract

Recently, Tendon-Derived Mesenchymal Stem Cells (TMSCs) have been successfully isolated and shown that they possess self-renewal potential and multilineage differentiation capacity which serves them as a promising cell source for tendon tissue engineering.

However, long-term culture *in vitro* may alter the biology of adult MSCs and result in significant changes on their primitive characteristics. Given that oxygen concentrations *in vivo* are significantly less at tissue level, it is conceivable that many cells would function more normally *in vitro* at oxygen concentrations lower than 20%.

To determine the hypoxic effects on rabbit Tendon-Derived Mesenchymal Stem Cells (TMSCs), they were cultured in normoxia (21% O_2) *vs.* hypoxia (5% O_2) for up to passage 5 (P_5), and their differentiation potential, stem cell marker expression and proliferation rate were compared at early and late passages. We found that TMSCs at 5% O_2 significantly increased in proliferation compared to those at 20% O_2. Moreover, the expression of two stem cell marker genes, Nanog and Oct-4, was upregulated in the cells cultured at 5% O_2. Similarly, more TMSCs expressed three stem cell markers including SSEA-4, nucleostemin and Nanog. The total collagen production released by these stem cells was highly expressed in normoxia group compared to hypoxia group at each passage. In addition to these, higher expression of markers for adipogenesis, osteogenesis and chondrogenesis were observed by qRT-PCR and western blotting.

We conclude that hypoxia conditions have a beneficial effect on TMSCs for their maintenance of stem cell properties and improvement of multi-differentiation potential. Oxygen tension does play a critical role in the niche of TMSCs *in vitro*.

Keywords: Tendon-derived stem cells; Hypoxia conditions; Normoxia conditions; Self-renewal; Multi-differentiation potential

Abbreviations

TMSCs: Tendon-Derived Mesenchymal Stem Cells; BMSCs: Bone Marrow Stem Cells; MSCs: Mesenchymal Stem Cells; ESCs: Embryonic Stem Cells; IACUC: Institutional Animal use and Care committee; PBS: Phosphate Buffered Saline; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal Bovine Serum; PDT: Population Doubling Time; SSEA-4: Stage-Specific Embryonic Antigen-4; GAG: Glycosaminoglycans; FACS: Flow Cytometeryl; FITC: Fluorescein Isothiocyanate; FITC: Fluorescein Isothiocyanate; PE: Phycoerythrin; PPARγ: peroxisome proliferators-activated receptor γ; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; SD: Standard Deviation; ANOVA: Analysis Of Variance; PLSD: Predicted Least-Square Difference

Introduction

Injured or degenerative tendon demonstrates limited capacity for spontaneous repair. The absence of vasculature prevents reparative cells from penetrating the tissue and maintaining its integrity. Thus the restoration of damaged tendon still remains an ongoing challenge. In recent years, tendon regenerations using stem cell-based therapies and tissue engineering techniques have been attempted, which provide a promising alternative for repair of tendon rupture and tendinopathy. Of particular interest is the use of Bone Marrow-Derived Mesenchymal Stem Cells (BMSCs) to regenerate functional tendons, however, unfortunately, several reports have shown that ectopic bone formation is observed after transplantation [1-3]. Furthermore, it has also been verified that tumor can be induced by undifferentiated BMSCs in some specific circumstances [4]. Recently, a rare cell population from tendons called Tendon-Derived Mesenchymal Stem Cells (TMSCs) has been successfully isolated and confirmed that they possess several universal criteria of stem cell, including clonogenicity, self-renewal and multipotent differentiation capacity, indicating that TMSCs may be an appealing cell source for tendon tissue engineering [5].

Although Mesenchymal Stem Cells (MSCs) show high cell renewal potential, they are also vulnerable to replicative senescence.

The bottleneck of any sources of MSCs for cell therapy is the low survival rate after transplantation of cells. In addition, long-term culture *in vitro* may alter the biology of adult MSCs and result in significant changes in cell cycle kinetics [6]. There is an increasing body of evidence that MSCs may become senescent during protracted culture as indicated by their decreased differentiation potential, morphologic alterations and reduced telomerase activity [7,8]. More work in these areas needs to be pursued before the clinical application of MSCs.

It is well known that self-renewal and multilineage differentiation are the marked abilities for all kinds of stem cells [9]. These are maintained within a niche composed of various factors, including cytokines, growth factors, adhesion molecules, and extracellular matrix [10]. Apart from these, a hypoxic environment is also denoted as an important regulator which plays a role in the maintenance of multipotency and extension of survival [11,12].

It has been accepted that the oxygen tension in most *in vitro* settings is considerably higher than that found in most mammalian tissues. These tensions correspond to an oxygen concentration of approximately 4-7% [13,14]. Thus, it is conceivable that many cells would function more normally *in vitro* at oxygen concentrations lower than 20%. Low O_2, therefore, appears to be a niche component for tendon-derived mesenchymal stem cells. As we all know, Oxygen Can Generate Reactive Oxygen Species (ROS), and exposure to aberrant levels of ROS may induce senescence dysfunction in stem cells [15]. Thus, we have sufficient reason to speculate that it is advantageous for TMSCs to localize within a hypoxic niche where the ROS source O_2 is limited.

However, the degree and duration of hypoxia described in the literature vary greatly and may result in opposite effects on the proliferation and differentiation capacities of MSCs [16-18]. So far only one study described the effect of low O_2 tension (2%) on the *in vitro* expansion and maintenance of undifferentiated stem cell characteristics of human TMSCs and showed higher clonogenicity, cell proliferation but lower differentiation potential [19]. In the present study we investigated the effect of reduced oxygen (5%) *in vitro* on rabbit patella Tendon-Derived Mesenchymal Stem Cells (TMSCs), this oxygen tension approximates that of tendon *in vivo*, as described above. Rabbit TMSCs were cultured in normoxia (21% O_2) versus hypoxia (5% O_2) for up to passage 5 (P_5) and their differentiation potential, stem cell marker expression and proliferation rate were compared at early and late passages. We observed that hypoxia was conducive to maintenance of the stem-cell characteristics and enhancement of the multi-differentiation capabilities during their expansion *in vitro*.

Materials and Methods

Control of hypoxic and normoxic culture conditions

We used a tri-gas incubator to achieve hypoxic culture conditions (Thermo Scientific Heracell 150i, Thermo Scientific, Pittsburgh, PA). In the tri-gas incubator, the concentration of oxygen was precisely controlled by two gas controllers and one oxygen sensor. The supply of nitrogen and carbon dioxide gases was achieved by using a nitrogen gas controller and a carbon dioxide gas controller which were connected to two nitrogen tanks and two carbon dioxide

tanks, respectively. The gas tank could be automatically switched to another when the gas in one tank ran out. To avoid extra air was brought into the incubator by opening the door, the incubator was separated into three isolation chambers and each chamber was sealed by double doors. The oxygen in the incubator was further controlled by an oxygen sensor. With these control devices in place, the oxygen concentration in the incubator was kept at the constant level of 5% during our cell culture experiments.

For normoxic culture conditions, a regular tissue culture incubator (Thermo Scientific) was used, where 95% air and 5% carbon dioxide were fed into the incubator and as a result, a 20% O_2 concentration inside the incubator was achieved.

Isolation of rabbit TMSCs

Five female New Zealand white rabbits (8-10 week-old, 3.0-4.0 kg) were used in all experiments. The protocol for use of the rabbits was approved by the IACUC of University of Pittsburgh. TMSCs were isolated from rabbit patellar tendons. The procedures for isolation of TMSCs were similar to our previously published protocol [20].

Cell culture

Rabbit TMSCs were seeded in 6-well plate at a density of 1.5×104/well and cultured with 3 ml of 20% FBS-DMEM/well in the tri-gas incubator as described above to achieve a 5% O_2 culture condition, or in the regular incubator to realize a 20% O_2 culture condition. When changed medium for the cells cultured in the tri-gas incubator, we placed the replacement medium inside the tri-gas incubator for 30 min before being used. The medium was changed every 3 days under both hypoxic and normaxic conditions. The cell proliferation was determined by cell counting at each time point according to the method published previously [21].

Cellular production of total collagen

Rabbit TMSCs were seeded in 6-well plates at a density of 4.5×104 per well and grown in growth medium in the tri-gas or regular incubator for 5 days. The assay of collagen production was performed when the cell culture became confluent at 80%. After the cell-conditioned medium was collected, cells were detached by trypsinization. Cell numbers were then counted using auto cellometer (Nexcelom Bioscience LLC). To measure total soluble collagen in cell-conditioned media, we used a Sircol collagen assay (Biodye Science, Biocolor Ltd, Carrickfergus, Northern Ireland and UK). Briefly, the cell-conditioned medium was mixed with Sircol dye reagent on an orbital shaker for 30 minutes. This solution was then centrifuged to obtain a collagen-dye complex pellet, which was solubilized with an alkali reagent. A microplate reader (Spectra MAX 190, Molecular Devices, Sunnyvale, California) was used to measure absorbance of the samples at a wavelength of 540 nm. A standard curve for calculating collagen concentration was obtained using a manufacturer-supplied acid soluble type I collagen calibration standard solution. Finally, to compare the hypoxia group with the normoxia group at different passages, we normalized the amounts of collagen with the total cell number produced by each group.

Expression of stem cell markers

Immunocytochemical assay was used for the expression of the following stem cell markers: nucleostemin, Nanog, stage-specific embryonic antigen-4 (SSEA-4) on TMSCs. To perform

Table 1: Primers used for qRT-PCR analysis.

Gene	Size(bp)	Primers	Type	Tm
PPARγ	200	5'-TGG GGA TGT CTC ATA ATG CCA-3'	Forward	59°c
		5'-TTC CTG TCA AGA TCG CCC TCG-3'	Reverse	
Collagen II	84	5'-TGG GTG TTC TAT TTA TTT ATT GTC TTC CT-3'	Forward	63°c
		5'-GCG TTG GAC TCA CAC CAG TTA GT-3'	Reverse	
Sox9	79	5'-AGT ACC CGC ACC TGC ACA AC-3'	Forward	59°c
		5'-CGC TTC TCG CTC TCG TTC AG-3'	Reverse	
Runx2	70	5'-TGA TGA CAC TGC CAC CTC TGA-3'	Forward	58°c
		5'-GCA CCT GCC TGG CTC TTC T-3'	Reverse	
GAPDH	107	5'-ACT TTG TGA AGC TCA TTT CCT GGT A-3'	Forward	63°c
		5'-GTG GTT TGA GGG CTC TTA CTC CTT-3'	Reverse	
Nanog	382	5'-CCCAGCTGTGTGTGCTCAA-3'	Forward	52°c
		5'-CCAGGCTTGGGAGTACCAGG-3'	Reverse	
Oct4	575	5'-CTCGGCGCAGCGCACGCCCTGGAG-3'	Forward	66°c
		5'-CAGCTGGTCGCGCAGCGGGCCCAG-3'	Reverse	
osteocalcin	70	5'-GAAGCCCAGCGGTGCA-3'	Forward	59°c
		5'-CACTACCTCGCTGCCCTCC-3'	Reverse	

immunostaining, the cells were seeded in two 12-well plates at a density of 1.5×10^4/well with 1.5 ml medium and cultured in either 5% O_2 or 20% O_2 conditions for 3 days. All of them were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and treated with 0.1% Triton X-100 for 30 min for Nanog and nucleostemin staining. After washing the cells with PBS, either mouse anti-Nanog (1:350, Santa Cruz Biotechnology, Inc., cat. # sc-33759, Santa Cruz, CA) or goat anti-nucleostemin (1:400, Neuromics, Cat. # GT15050, Edina, MN) was applied for 2 h at room temperature. The cells were washed with PBS for three times, and either Cy-3-conjugated goat anti-mouse IgG antibodies (1:500 for Nanog, Millipore, Cat. # AP124C, Billerica MA) or Cy3-conjugated donkey anti-goat IgG antibodies (1:500 for nucleostemin, Millipore, Cat. # AP180C, Billerica, MA) was applied for 1 h at room temperature. In order to stain for SSEA-4, fixed cells were incubated with mouse anti-human SSEA-4 antibodies (1:500, Invitrogen, Cat. # 414000, Frederick, MD) for 2 hours at room temperature. After washing the cells with PBS, cells were treated with Cy3-conjugated goat anti-mouse IgG antibodies (1:500, Millipore, Cat. # AP124C, Billerica MA) for 1h at room temperature. To quantify the expression of stem cell markers, the stained samples were examined using an inverted fluorescence microscope and images were taken with a 20 × objective using a CCD camera.

Multilineage differentiation potential

Multilineage differentiation potential was tested *in vitro* for adipogenesis, chondrogenesis, and osteogenesis, respectively. The cells at passage 2 were seeded in 6-well plates at a density of 2.4×10^5 cells/well in basic growth medium (DMEM plus 10% FBS) and cultured either under 5% O_2 or 20% O_2 tension. To test adipogenic potential, cells were cultured in adipogenic induction medium (Millipore, Billerica, MA) consisting of basic growth medium added with dexamethasone (1 mM), insulin (10 mg/ml), indomethacin (100 mM), and isobutylmethylxanthine (0.5 mM). As a test of chondrogenic potential, they were cultured in basic growth medium supplemented with proline (40 mg/ml), dexamethasone (39 ng/ml), TGF-β3 (10 ng/ml), ascorbate 2-phosphate (50 mg/ml), sodium pyruvate (100 mg/ml), and insulintransferrin-selenious acid mix (50 mg/ml) (BD Bioscience, Bedford, MA). Finally, the osteogenic potential was tested by culturing cells in osteogenic induction medium (Millipore, Billerica, MA) consisting of basic growth medium supplemented with dexamethasone (0.1 mM), ascorbic 2-phosphate (0.2 mM), and glycerol 2-phosphate (10 mM). After culturing for 21 days, Oil red O assay, Safranin O assay, and Alizarin red S assay, as descried previously [21], were used to assess adipogenesis, chondrogenesis, and osteogenesis of TMSCs when grown in 5% O_2 and 20% O_2 culture conditions.

Semi-quantification of the extent of cell differentiation

The stained samples were examined using an inverted microscope and images were taken with a 20 × objective using a CCD camera. A total number of eight views from each well were randomly chosen. The areas of positive staining were identified manually and computed by a SPOT imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI). The ratio of positive staining was calculated by dividing the stained area by the view area. The values of all views from three duplicate wells (24 views in total) were averaged to obtain the percentage of positive staining, which represented the extent of cell differentiation in the respective induction medium.

Quantitative real-time PCR (qRT-PCR)

To measure the stemness and multipotential of TMSCs under hypoxic and normaxic culture conditions, we performed qRT-PCR. Total RNA was extracted using an RNeasy Mini-Kit with an on-column DNase I digest (Qiagen). First-strand cDNA was synthesized in a 20 μl reaction of 1μg total RNA through reverse transcription with Super-Script II (Invitrogen). The conditions for the cDNA synthesis were: 65°C for 5 min and cooling for 1 min at 4°C, then 42°C for 50 min, and finally 72°C for 15 min. The qRTPCR was carried out using QIAGEN QuantiTect SYBR Green PCR Kit (Qiagen) [22]. In

a 50 μl PCR reaction mixture, 2 μl cDNA (total 100 ng RNA) were amplified in a Chromo 4 Detector (MJ Research). Rabbit-specific primers were used for stem cell genes expression, including Oct-4 and Nanog. For differentiated TMSCs, rabbit-specific primers were used for collagen type II, peroxisome proliferators-activated receptor γ (PPAR γ), Sox9, osteocalcin, and Runx2. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an internal control. The forward and reverse primer sequences and the resultant products were designed according to published methods, and are listed in (Table 1) [23-26]. All primers were synthesized by Invitrogen (Carlsbad, CA). The relative gene expression levels were calculated from 2-ΔCT, where ΔCT was determined by the formula: ΔCT = (Cttarget-CTGAPDH) differentiation-(Cttarget-CTGAPDH) control. In the formula, CTtarget and CTGAPDH are the cycle thresholds of target gene and GAPDH gene, respectively, for each RNA sample. The Standard Deviation (SD) of the ΔCT was determined from at least three parallel tests.

Western blot

The cells at passage 2 were seeded in 6-well plates at a density of 6×10^4 per well and cultured separately with adipogenic, osteogenic and chondrogenic induction media under different oxygen tension for 21 days. Then they were lysed using a mammalian protein extraction reagent cocktail (Pierce, Rockford, Illinois) containing 1.5% protease inhibitors (Sigma-Aldrich). After centrifugation at 12,000 rpm for 10 minutes, the protein concentrations of the supernatants were determined using a BCA Protein Assay (Pierce). Equal amounts of total protein were run on 12% SDS polyacrylamide gels (Bio-Rad) at a constant voltage of 100 V for 60 minutes. Proteins were blotted to a nitrocellulose membrane using a Semi-Dry transfer module (Bio-Rad) at 200 mA for 90 minutes. The membrane was blocked in a 5% dry milk/TBS-Tween 20 solution for 1 hour at room temperature and then probed for 5 hours with a mouse monoclonal anti-human adiponectin antibody (Millipore; Cat # MAB3604) at a dilution of 1:1000; mouse monoclonal anti-human osteocalcin (Abcam; Cat # ab13418) at a dilution of 1:1000 for 5 hours; and mouse anti-human collagen II (Millipore; Cat # MAB8887) at a dilution of 1:500 in a 1% dry milk/PBS-Tween 20 solution. Incubation with the primary antibody was followed by a horseradish peroxidase-conjugated goat anti-mouse antibody (Millipore; Cat #12-349) at a dilution of 1:2000 in a 1% dry milk/PBS solution. The targeted protein bands were detected using an ECL (enhanced luminol-based chemiluminescence) detection kit (Amersham Biosciences, Piscataway, New Jersey), followed by exposure of the membrane to x-ray film. Membranes were also re-probed for mouse anti- glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology; Cat # sc-66163) to verify equal protein loading in the gels. The quantification of protein bands were performed by the Image J software (http://rsb.info.nih.gov/nih-image/) and normalized by respect GAPDH using the following formulae:

Protein Expression (target-hypoxia) = Intensity (target-hypoxia) /Intensity (GAPDH-hypoxia)

Protein Expression (target-normoxia) = Intensity (target-normoxia) /Intensity (GAPDH-normoxia)

Statistical analysis

Data is presented as mean plus and minus Standard Deviation
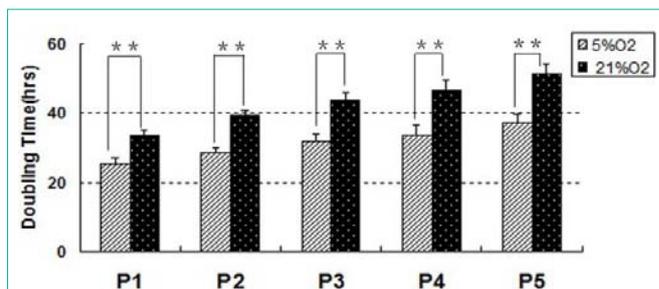
Figure 1: Proliferation of TMSCs cultured under hypoxic and normaxic conditions. Rabbit TMSCs were cultured either in 5% O$_2$ or 20% O$_2$ for 5 passages during total 35 days. The medium in each condition was changed every three days. Cell proliferation was assessed by measuring the Population Doubling Time (PDT) of the TMSCs in each culture. As indicated, the PDT of TMSCs grown in 20% oxygen tension was much longer than that in 5% oxygen tension at each passage, demonstrating that the latter proliferated faster than former (¨P<0.01).
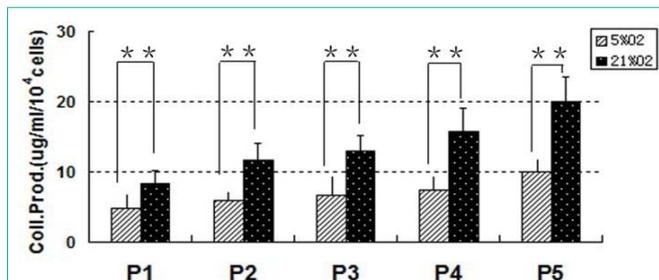

Figure 2: Collagen production in the media of TMSCs cultured in hypoxic and normxic conditions. After 5 days culture, the collagen production in the media produced by rabbit TMSCs grown under hypoxic condition was markedly decreased when compared to those grown under normoxic condition. These findings indicated that hypoxia prevent TMSCs from differentiation by default (¨¨P<0.01).

(SD). At least three replicates for each experimental condition were performed, and the presented results are representative of these replications. One-way Analysis Of Variance (ANOVA), followed by either Fisher's Predicted Least-Square Difference (PLSD) for multiple comparisons or two tailed student t-test wherever applicable, were used for statistical analysis. Differences between two groups were considered significant when the p-value was less than 0.05.
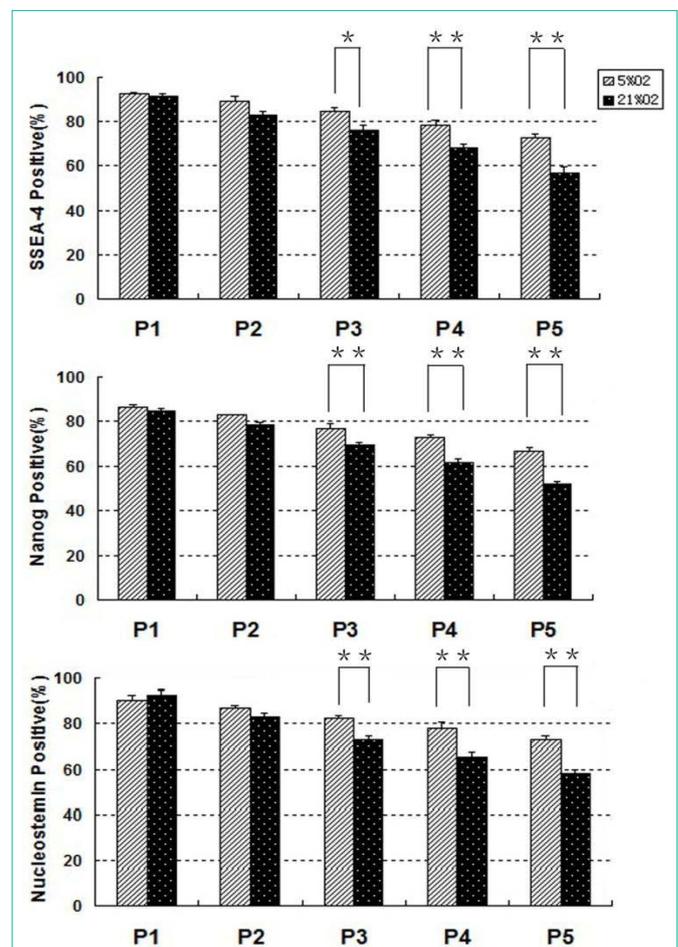
Results and Discussion

The proliferation of rabbit TMSCs was investigated by Population Doubling Time (PDT) calculated through cell number counting using cytometry. Our data showed that PDT of TMSCs cultured at 20% O$_2$ was much longer than that at 5% O$_2$ at each passage, demonstrating that the latter proliferated much faster than former. Great difference was found between two groups at each passage (Figure 1, p<0.01). Also, the amount of collagenous proteins provided by TMSCs which was pre-cultured under different oxygen tension was measured. The total collagen production released by these stem cells was lower in hypoxia group compared to that in normoxia group at each passage. Moreover, with the passage increasing, the discrepancy between two groups became more and more apparent (Figure 2, p<0.01). Immunocytochemical staining of these cells showed that both hypoxia group and normoxia group in culture for more than one month could express SSEA-4, nucleostemin and Nanog despite their decreasing numbers compared to passage 1 (Figure 3). Although
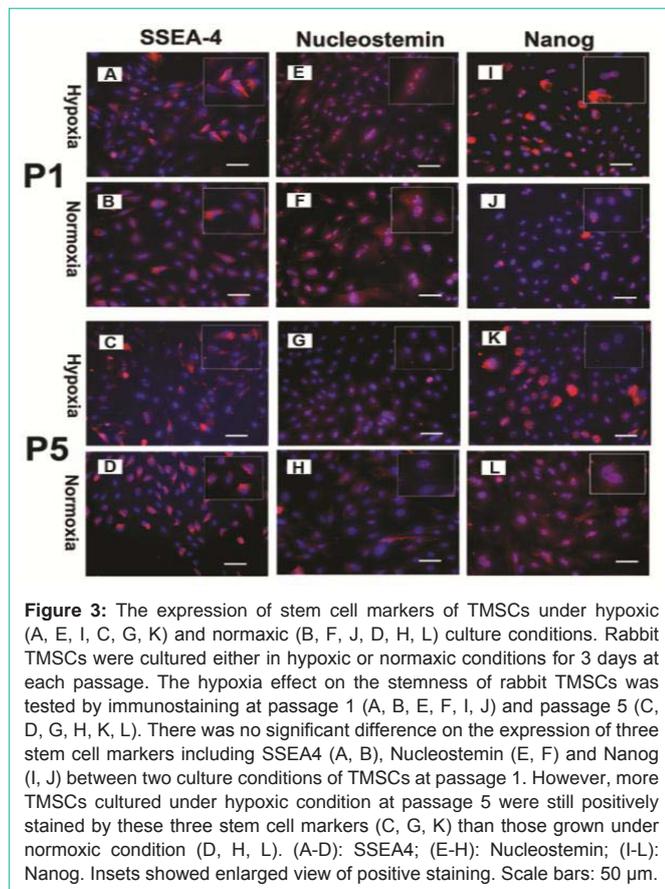
Figure 3: The expression of stem cell markers of TMSCs under hypoxic (A, E, I, C, G, K) and normaxic (B, F, J, D, H, L) culture conditions. Rabbit TMSCs were cultured either in hypoxic or normaxic conditions for 3 days at each passage. The hypoxia effect on the stemness of rabbit TMSCs was tested by immunostaining at passage 1 (A, B, E, F, I, J) and passage 5 (C, D, G, H, K, L). There was no significant difference on the expression of three stem cell markers including SSEA4 (A, B), Nucleostemin (E, F) and Nanog (I, J) between two culture conditions of TMSCs at passage 1. However, more TMSCs cultured under hypoxic condition at passage 5 were still positively stained by these three stem cell markers (C, G, K) than those grown under normoxic condition (D, H, L). (A-D): SSEA4; (E-H): Nucleostemin; (I-L): Nanog. Insets showed enlarged view of positive staining. Scale bars: 50 μm.

Figure 4: Semi-quantification of staining results for three stem cell markers on TMSCs cultured under hypoxic and normoxic conditions. Rabbit TMSCs specifically stained for SSEA4, Nanog and nucleostemin by immunostaining at each passage in each culture condition were analyzed by semi-quantification. There was no significant difference on the expression of these three stem cell markers between two culture conditions of TMSCs at earlier passages. However, from passage-3, the expression of these three stem cell markers was decreased in the TMSCs cultured under normoxic condition (*P < 0.05). As indicated, significantly higher percentages of TMSCs at passages 4 and 5 cultured under 5% O_2 conditions expressed the stem cell markers (SSEA-4, nucleostemin and Nanog) compared to those cultured under 20% O_2 conditions (¨P<0.01).

semi-quantification measurement of immunostaining results showed that cells cultured at 5% O_2 tension expressed higher levels for these three stem cell markers than that at 20% O_2 tension, there was no great difference between the two groups at P1 and P2. However, from the beginning of passage 3, the percentages of positive staining for the three markers decreased sharply in the normoxia group and significant difference exhibited (Figure 4, p<0.05 for SSEA4 at passage-3; P<0.01 for Oct-4 and Nanog at passages 3 to 5). Similar situation occurred in gene analysis. Our results demonstrated that the expression levels of both Nanog and Oct-4 genes were significantly up-regulated at 5% O_2 tension than they were at 20% O_2 tension after passage-3 (Figure 5, p<0.05 for passage-3, p<0.01 for passages 4 and 5), whereas non significantly higher expressions of the two stem cell genes displayed between the two groups at P1 and P2 (Figure 5).

After 21 days in respective induction media, the multi-differentiation potential of rabbit TMSCs towards adipogenesis, osteogenesis and chondrogenesis in different oxygen conditions were determined through special staining and qRT-PCR. The data showed that the degree of adipogenesis, chondrogenesis, and osteogenesis of rabbit TMSCs were all more extensive at 5% O_2 conditions compared to that at 20% O_2 conditions (Figure 6). Regarding adipogenesis, both groups exhibited lipid droplets, an indicator of adipogenesis which can be detected by Oil red O staining. Semi-quantification, by calculating stained area, showed that more than 48% of cells were positively stained at 5% oxygen tension whereas only 32% were stained positively at 20% oxygen tension (Figure 6 G, p<0.05). Similarly, the gene expression level of PPARγ in hypoxia group

was upregulated 1.75 times compared to that in normoxia group (Figure 7, p<0.05). For osteogenesis, more than 51% positive areas were found by Alizarin Red S assay in hypoxia group compared to 36% in normoxia group (Figure 6 G, p<0.05). Meanwhile, the gene expression of osteogenic markers osteocalcin and Runx-2 were all significantly higher in hypoxia group than that in normoxia group (Figure 7, p<0.05). For chondrogenesis, Safranin O assay showed that 82% positive staining under hypoxia condition appeared while only 43% under normoxia condition (Figure 6 G, p<0.01). Equally, the expression levels of collagen type II and Sox9 were upregulated 3.82 and 3.94 times at 5% tension in comparison to 20% tension (Figure 7, p<0.01). Western blotting was performed to quantify the level of specific protein expression in the two groups. It was evident that adiponectin (1.3 times, p=0.021), osteocalcin (1.79 times, p=0.046) and collagen type II (3.49 times, p=0.028) were expressed higher in
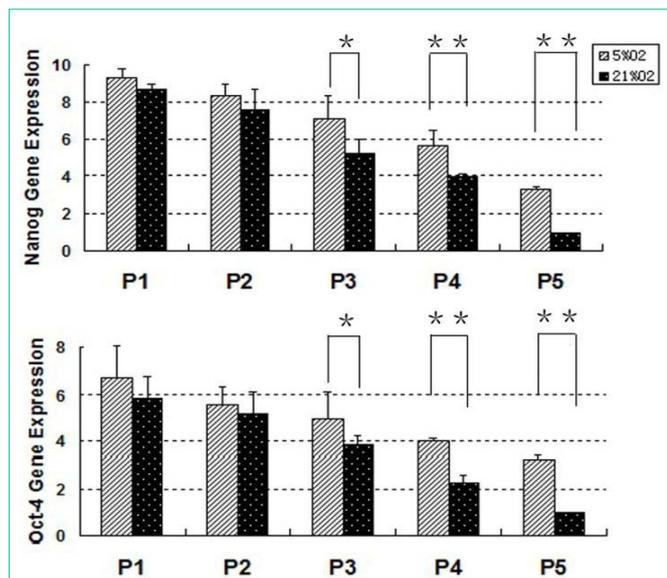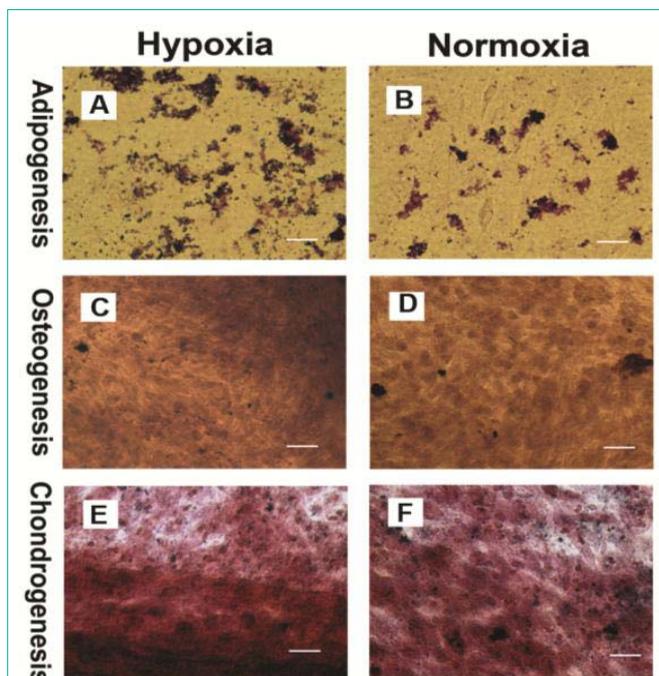
Figure 5: Stem cell marker gene expression by rabbit TMSCs cultured in hypoxic and normoxic conditions. Rabbit TMSCs cultured either in hypoxic or normoxic conditions for 7 days at each passage were used for gene analysis by qRT-PCR. Total RNA was extracted from these cells and tested by rabbit specific primers to Nanog and Oct-4. There was no significant difference on the expression of these two stem cell marker genes between two culture conditions of TMSCs at earlier passages. However, from passage-3, the expression of these two stem cell marker genes was decreased in the TMSCs cultured under normoxic condition (*P < 0.05). Both Nanog and Oct-4 genes were all expressed at significantly high levels by TMSCs at higher passages under hypoxia conditions in contrast with normoxia conditions (¨P<0.01). Note that for real time RT-PCR analysis, the gene expression levels were normalized to GAPDH, obtained from at least three independent experiments.
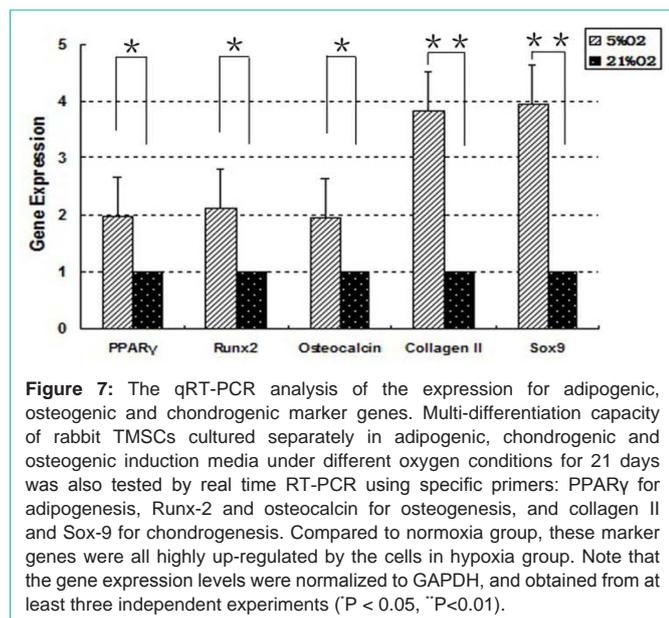

Figure 6: Histochemical staining of differentiated cells. Multi-differentiation capacity of rabbit TMSCs cultured separately in adipogenic, chondrogenic and osteogenic induction media under different oxygen conditions for 21 days was tested by histochemical staining using Oil red O for adipogenesis, Alizarin red S for osteogenesis and Safranin O for chondrogenesis. Rabbit TMSCs cultured either at 5% O_2 or 20% O_2 were able to differentiate into adipocytes (A, B), osteocytes (C, D) and chondrocytes (E, F), as shown by the accumulation of lipid droplets, calcium deposits and proteoglycans on cell surfaces. However, the extent of differentiation under hypoxia conditions was greater than that under normoxia conditions. Scale bars: 50 μm.

rabbit TMSCs grown at 5% tension than those at 20% tension (Figure 8).

Tendon is a dynamic tissue which transmits force from muscle to bone, thus allowing limb and joint movement possible. Tendon is frequently target of injury from trauma in sports and aging. Damaged tendon tissue heals very slowly and rarely attains the structural integrity and mechanical strength of normal tendon [27]. Recently, a tissue engineering approach has been sought to restore the damaged tendon using MSCs due to their capability for self-renewal and multilineage differentiation potential [28-30]. In addition to these, the discovery of tendon-derived stem cells that possess regenerative capabilities opens new possibilities for treating damaged tendon tissue. Therefore, TMSCs may be an optimal cell source for effective tissue engineering of injured tendons.

Unfortunately, therapeutic potential of MSCs is always hampered by an incomplete understanding of *in vitro* culture parameters that can maintain their stem-cell phenotype and multi-differentiation capabilities during expansion. It is well known that TMSCs tend to differentiate and lose their stemness quickly under regular culture conditions that use 95% air and 5% CO_2. It has been shown that MSCs and other stem cell populations continuously grown *in vitro* for 10 passages are subject to the replicative senescence known as Hayflick limit [7,31]. In addition, traditional cell-culture techniques can facilitate billion-fold expansion of MSCs, but result in a gradual loss of their primitive characteristics and self-renewal properties [32]. Furthermore, MSCs can undergo spontaneous transformation

to malignant cells during extended culture *in vitro* [33]. Hence the application of MSCs therapy for tendon regeneration remains a great challenge because the role and niche of Mesenchymal Stem Cells (MSCs) in tendon have not been firmly established [34].

In order to obtain sufficient numbers of tendon stem cells for cell therapy of injured tendons, simpler and faster approaches to improve the survival of stem cells, maintain their self-renewal and promote their multi-lineage potential need to be pursued.

It is well known that oxygen concentrations *in vivo* at tissue level are significantly less than that supplied (20%) in normal cell culture. In this study we focused on hypoxia preconditioning as a powerful tool through which the stemness of TMSCs can be maintained and their multipotency can be enhanced in expansion. This is the first time to investigate the effect of reduced oxygen tension on Tendon-Derived Stem Cells (TMSCs) cultured at different passages during expansion *in vitro*. The results showed that with the passages increasing, both hypoxia and normoxia groups exhibited descending trend in proliferative potential, however, the cells cultured at 5% oxygen conditions grew much more quickly compared to the cells at 20% oxygen conditions.

Because there is no specific stem cell marker for TMSCs, we used general stem cell markers (SSEA-4, Nanog and nucleostemin) to characterize the stemness of TMSCs under both hypoxic and normaxic conditions. It was noteworthy that the cells from rabbit

Figure 7: The qRT-PCR analysis of the expression for adipogenic, osteogenic and chondrogenic marker genes. Multi-differentiation capacity of rabbit TMSCs cultured separately in adipogenic, chondrogenic and osteogenic induction media under different oxygen conditions for 21 days was also tested by real time RT-PCR using specific primers: PPARγ for adipogenesis, Runx-2 and osteocalcin for osteogenesis, and collagen II and Sox-9 for chondrogenesis. Compared to normoxia group, these marker genes were all highly up-regulated by the cells in hypoxia group. Note that the gene expression levels were normalized to GAPDH, and obtained from at least three independent experiments ($^*P < 0.05$, $^{**}P < 0.01$).
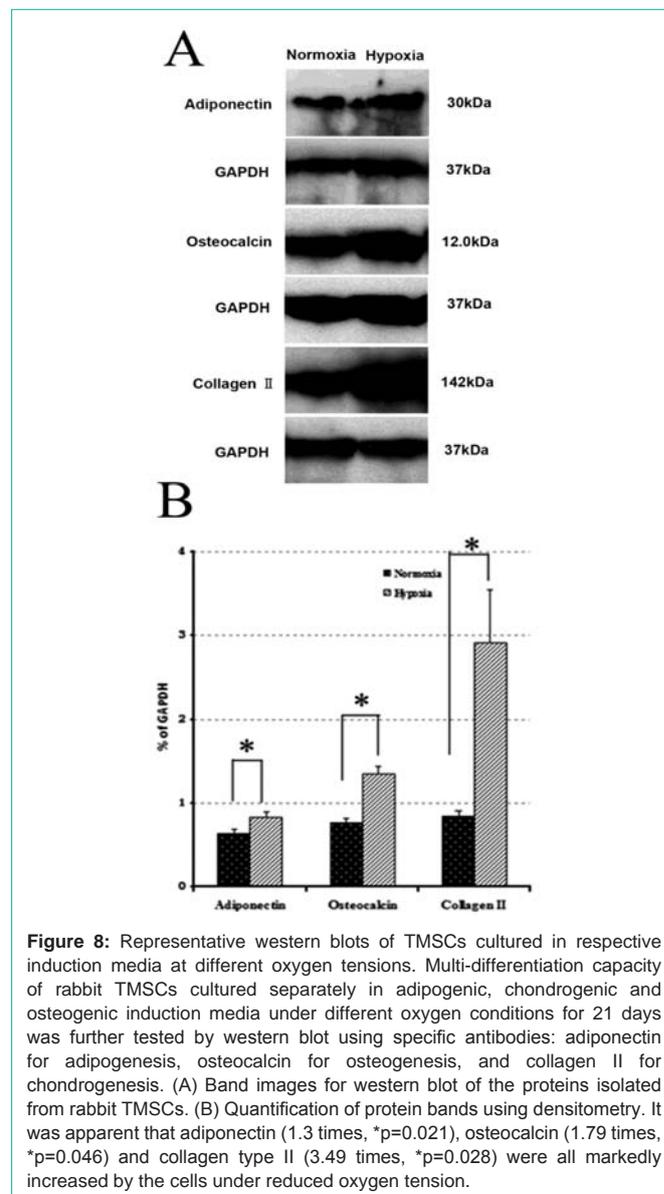
tendons cultured at different oxygen tensions all expressed high levels of these characteristic stem cell markers at the beginning, indicating that they possessed property of stem cells. However, from the passage 3, many cells grown in normoxic conditions lose their "stemness" as evidenced by immunostaining and RT-PCR. As a contrast, higher percentage of TMSCs grown under reduced oxygen conditions even at passages 4 and 5 were still positively stained by these three stem cell markers than those grown in normoxic conditions at the same passages. Consistent with this result, qRT-PCR analysis showed that the expression of the Nanog and Oct-4 genes were all significantly higher in hypoxia group than that in normoxia group after passage 3. These findings indicated that significantly more TMSCs at 5% O_2 tension remained undifferentiated state and preserved self-renewal capability compared with those at 20% O_2 tension during expansion.

Also, as tendon-specific stem cells, TMSCs are always prone to differentiate into tenocytes by default [21], detection of total collagen production in cell-conditioned media can elucidate whether TMSCs undergo differentiation during expansion. Less collagen production in TMSCs at 5% O_2 culture conditions indicated that more tendon stem cells remained undifferentiated state. Namely, hypoxia conditions could maintain the "stemness" of TMSCs better than normoxia conditions.

Finally, we demonstrated TMSCs at hypoxia and normoxia conditions differentiated into adipocytes, chondrocytes, and osteocytes in the respective induction media, evidenced by relevant histochemical staining, qRT-PCR and western blotting. Expression of markers for adipogenesis, osteogenesis and chondrogenesis were all elevated in cultures which had been in low oxygen throughout their cultivation time. Collectively these data suggest that reduced oxygen tension exerted a beneficial influence on promoting multi-differentiation potential of tendon-derived stem cells.

In this study, we found that hypoxic condition enhanced proliferation of tendon stem cells, although the mechanism is largely unknown, one of possible reasons is that hypoxic condition provides a mimic *in vivo* niche for tendon stem cell growth. Compared to

Figure 8: Representative western blots of TMSCs cultured in respective induction media at different oxygen tensions. Multi-differentiation capacity of rabbit TMSCs cultured separately in adipogenic, chondrogenic and osteogenic induction media under different oxygen conditions for 21 days was further tested by western blot using specific antibodies: adiponectin for adipogenesis, osteocalcin for osteogenesis, and collagen II for chondrogenesis. (A) Band images for western blot of the proteins isolated from rabbit TMSCs. (B) Quantification of protein bands using densitometry. It was apparent that adiponectin (1.3 times, *p=0.021), osteocalcin (1.79 times, *p=0.046) and collagen type II (3.49 times, *p=0.028) were all markedly increased by the cells under reduced oxygen tension.

vascular-rich organs and tissues, such as lungs, heart, liver and kidneys, the oxygen levels in tendons tissues *in vivo* are very low due to very few blood vessels in tendons. That means tendon stem cells prefer hypoxic conditions. It has been reported that low oxygen tensions in stem cell niches offers a selective advantage that is well suited to their particular biological roles [35].

Although our study provides clear evidence that hypoxia can maintain the TMSCs undifferentiated and in parallel enhance their pluripotency, a few limitations still exist. First, we only cultured rabbit TMSCs under reduced oxygen conditions for five passages. It still remains indeterminate in the long-term culture with more than five passages. Second, oxygen concentration we chose was based on the literatures which have been published, however, which type of oxygen gradient is optimal in keeping properties of TMSCs needs to be further investigated. Finally, the molecular mechanisms that are responsible for enhanced "stemness" in rabbit TMSCs as shown in this study remain unknown. It has been accepted that the transcription

factor complex hypoxia-inducible factor 1 (HIF1) is a key mediator of adaptive responses to changes in cellular oxygen level [36]. Under hypoxic conditions, HIF-1 is stabilized and permits the activation of genes essential to cellular adaptation to low oxygen conditions [36]. Therefore, HIF-1 deserves much attention in the future.

Conclusion

In conclusion, this study has illustrated the dramatic influence of oxygen concentration as a potent regulator of *in vitro* stem cell physiology. Our data indicate that low oxygen tension (5%) provided a mimic *in vivo* environment for tendon cells. Hypoxia condition can significantly remain the TMSCs at an undifferentiated state, increase their proliferation rate and enhance their multi-differentiation potential. Oxygen tension does, therefore, appear to be critical for establishing the tendon-derived stem cell niche *in vitro*.

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