Hypoxia Mediated Efficient Expansion of Human Tendon-Derived Stem Cells (hTDSCs) in vitro

INTRODUCTION:
Tendons regenerate and repair slowly and inefficiently after injury. Tendon-Derived Stem Cells (TDSCs) have been isolated recently and have been shown to promote tendon repair. The ability to achieve sufficient number of cells for transplantation is essential for their clinical applications. In this study, we aimed to study the effect of low oxygen tension (2%) on the clonogenicity, metabolic rate, DNA incorporation, population doubling time, β-galactosidase activity, immunophenotypes, multi-lineage differentiation potential, and tenocyte-like properties of human TDSCs (hTDSCs).

METHODS:
The study was approved by the Clinical Research Ethics Committee of the authors’ institution. Human patellar tendons were collected from three patients undergoing anterior cruciate ligament reconstruction or bone-patellar tendon bone autograft after getting their consent. hTDSCs were isolated according to our well-established protocol.

For the studies of clonogenicity, metabolic rate, DNA incorporation, population doubling time, β-galactosidase activity and immunophenotypes, the cells were sub-cultured, incubated at 37°C, normoxia (20% O₂ and 5% CO₂) overnight and then cultured either at normoxic or hypoxic (2% O₂ and 5% CO₂) conditions. For the study of multi-lineage differentiation potential and expression of tendon-like properties of hTDSCs under 20% or 2% oxygen tension, the cells were cultured at 20% oxygen tension until confluence, and then subjected to induction / treatment at either 20% or 2% oxygen tension. To study the reversibility of the effect of hypoxia on the multi-lineage differentiation potential and expression of a tendon-related marker in hTDSCs, the cells were pre-conditioned in either 20% or 2% oxygen tension for at least 14 days and then induced / treated at 2% or 20% oxygen tension.

The clonogenicity of hTDSCs was assessed by counting the number of cell colonies after staining with crystal violet. The number of hTDSCs was determined by direct cell counting. The doubling time of hTDSCs at log phase was calculated. The proliferation rate, the metabolic rate and the senescence-associated β-galactosidase activity of hTDSCs were determined using flow cytometry. The amounts of collagenous and non-collagenous proteins produced by hTDSCs were measured by Sirius Red F3BA and Fast Green FCF assays. The osteogenic, adipogenic and chondrogenic differentiation of hTDSCs upon induction were done using standard assays. The expression of tendon-related markers in hTDSCs in basal completed medium was measured by qRT-PCR.

RESULTS:
HTDSCs were characterized by their adherence to plastic, colony-forming ability, multi-lineage differentiation potential, high expression level of CD44, CD73, CD 90, and CD105, but low in CD34, CD45, CD146, and Stro-1 at 20% oxygen tension. Low oxygen tension increased DNA incorporation but not metabolic rate of hTDSCs (not shown). It increased cell number by 25% (Figure 1) and the number of colonies (Figure 2) but reduced the osteogenic (Figure 3), adipogenic (Figure 4) and chondrogenic (Figure 5) differentiation potential of hTDSCs. The reduction in differentiation potential was associated with decreased mRNA expression ratios of some lineage-related markers, including BGLAP, ALP, C/EBPα, PPARγ2 and ACAN and SOX9. However, the expression of a tendon-related marker, TNMD, was increased (not shown). There was no significant difference in the production of collagenous to non-collagenous protein ratio (not shown); the immunophenotypes (not shown) and β-galactosidase activity (not shown) were similar at 2% and 20% oxygen tension. Hypoxia pre-conditioned hTDSCs could successfully differentiate at 20% oxygen tension as shown by the reversal increase in the mRNA expression ratios of lineage-related markers compared to pre-incubation of cells at 20% oxygen tension and subsequent induction / treatment of cells at 2% oxygen tension (Figure 6).

DISCUSSION AND SIGNIFICANCE:
Hypoxia is advantageous for efficient expansion of hTDSCs in vitro for tendon tissue engineering.

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Figure 1. Boxplots showing the fold change in the number of hTDSCs incubated at 20% or 2% oxygen tension at (A) low plating density (5000 cells/cm²) and at (B) high plating density (5000 cells/cm²). (C) Boxplot comparing the doubling time of hTDSCs at the exponential phase at low plating density at 20% and 2% oxygen tension. # indicated p ≤ 0.01 and ## indicated p ≤ 0.001 for comparing 2% versus 20% oxygen tension groups at each time point.

Figure 2. Boxplot showing the number of colonies upon incubation at 20% or 2% oxygen tension at day 14 and day 21. * indicated p ≤ 0.05 and ## indicated p ≤ 0.001 for comparing 2% versus 20% oxygen tension.

Figure 3. Photographs showing the formation of calcium nodules in hTDSCs at either (A, B) 20% or (C, D) 2% oxygen tension in (A, C) basal or (B, D) osteogenic media for 21 days as indicated by Alizarin red S staining. (E) Boxplot showing the relative mRNA expression ratios of BGLAP, SPP1 and ALP in hTDSCs maintained osteogenically induced at 20% or 2% oxygen tension for 14 days. Arrow: calcium nodules; Scale bar: 50μm. # indicated p ≤ 0.05, ## indicated p ≤ 0.01 and ### indicated p ≤ 0.001 for comparing 2% versus 20% oxygen tension groups. * indicated p ≤ 0.05 and ** indicated p ≤ 0.01 and *** indicated p ≤ 0.001 for comparing the induction group versus basal group.

Figure 4. Photographs showing the formation of oil droplets in hTDSCs at either (A, B) 20% or (C, D) 2% oxygen tension in (A, C) basal or (B, D) adipogenic media for 21 days as indicated by Oil red O staining. (E) Boxplot showing the relative mRNA expression ratios of PPARγ2 and CEBPα in hTDSCs adipogenically induced at 20% or 2% oxygen tension for 14 days. Arrow: oil droplets; Scale bar: 25μm. # indicated p ≤ 0.05, ## indicated p ≤ 0.01 and ### indicated p ≤ 0.001 for comparing 2% versus 20% oxygen tension groups. * indicated p ≤ 0.05 and ** indicated p ≤ 0.001 for comparing the induction group versus basal group.

Figure 5. Photographs showing the production of proteoglycans in hTDSCs pellets incubated at either (A, B) 20% or (C, D) 2% oxygen tension in (A, C) basal or (B, D) chondrogenic media for 21 days as indicated by Safranin O (SO) / fast green staining. (E) Boxplot showing the relative mRNA expression ratios of COL2A1, ACAN and SOX9 in hTDSCs maintained at 20% oxygen tension chondrogenically induced at 20% or 2% oxygen tension for 14 days. Arrow: SO-stained area; Scale bar: 100μm. # indicated p ≤ 0.05 for comparing 2% versus 20% oxygen tension groups.

Figure 6. Boxplots showing the relative mRNA expression ratios of (A) ALP, (B) PPARγ2, (C) SOX9 in hTDSCs maintained at 2% or 20% oxygen tension for at least 14 days and subsequently induced in (A) osteogenic, (B) adipogenic or (C) chondrogenic medium at 2% or 20% oxygen tension for 14 days. (D) Boxplot showing the mRNA expression of TNMD in hTDSCs maintained at 2% or 20% oxygen tension for at least 14 days and subsequently incubated at 2% or 20% oxygen tension for 7 days. * indicated post-hoc p ≤ 0.05 with overall p < 0.05, & indicates post-hoc p ≤ 0.05 with overall p < 0.01.