Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through downregulation of E2A-p21 by HIF-TWIST

Running title: Hypoxia inhibits senescence and promotes differentiation properties

Chih-Chien Tsai2,4, Yann-Jang Chen3, Tu-Lai Yew4, Ling-Lan Chen4, Jir-You Wang1,4, Chao-Hua Chiu1,6, Shih-Chieh Hung1,2,4,5

1Institute of Clinical Medicine, 2 Institute of Pharmacology, Faculty of Medicine, 3Institute of Genome Sciences, Department of Life Sciences, National Yang-Ming University, Taipei 112, Taiwan. 4Stem Cell Laboratory, Department of Medical Research and Education, 5Orthopaedics and Traumatology, 6Chest Medicine, Taipei Veterans General Hospital, Taipei 112, Taiwan

*Correspondence and who should receive reprint requests: Shih-Chieh Hung, M.D, and Ph.D.
Department of Medical Research and Education, Veterans General Hospital-Taipei 201, Sec. 2, Shih-Pai Road, Taipei, 11217, Taiwan
Tel +886-2-28757557 ext 118, Fax +886-2-28265164
E-mail: hungsc@vghtpe.gov.tw
Abstract

Though low-density culture provides an efficient method for rapid expansion of human mesenchymal stem cells (MSCs), MSCs enriched by this method underwent senescence and lost their stem cell properties, which could be preserved by combining low-density with hypoxic culture. The mechanism was mediated through direct downregulation of E2A-p21 by HIF-1α-TWIST. Expansion under normoxia induced E2A and p21 expression, which were abrogated by overexpression of TWIST, whereas siRNA against TWIST upregulated E2A and p21 in hypoxic cells. Furthermore, siRNA against p21 in normoxic cells enhanced proliferation and increased differentiation potential, whereas overexpression of p21 in hypoxic cells induced a decrease in proliferation and a loss of differentiation capacity. More importantly, MSCs expanded under hypoxic conditions by up to 100 population doublings exhibited telomerase activity with maintained telomere length, normal karyotyping and intact genetic integrity, and do not form tumors. These results support the method of low-density hypoxic culture for efficiently expanding MSCs without losing stem cell properties or increasing tumorigenicity.

Keywords: mesenchymal stem cells, hypoxia, HIF, TWIST, E2A, p21, senescence
Introduction

Human multipotent stromal cells or mesenchymal stem cells (MSCs), capable of self-renewal and differentiating into various mesenchymal tissues,\(^1\) have emerged as a promising tool for clinical applications in, for example, cell-based therapy for osteogenesis imperfecta\(^2\) and tissue engineering in cartilage and bone.\(^3\) MSCs are also applied in cardiac therapeutics since they prevent deleterious remodeling and improve recovery.\(^4\) However, the variations in the isolation techniques, growth media, and culture conditions cause a remarkable difference in their proliferation and differentiation capacity.\(^5\) Further, many studies have consistently noticed a senescent tendency of MSCs upon expansion.\(^6, 7\) Thus, the difference in stem cell properties and the senescence encountered during expansion hinder the clinical applications of MSCs.

Hypoxia has been known to regulate several cellular processes and signal transductions via the expression of Hypoxia Inducible Factor-1 (HIF-1), a heterodimer consisting of the constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT) and the hypoxic response factor HIF-1\(\alpha\). HIF-1\(\alpha\) is regulated by the cellular \(O_2\) concentration and determines the transcriptional activity of HIF-1.\(^8\) Most of the effects of HIF-1\(\alpha\) were investigated on cancer cells. HIF-1\(\alpha\), induced during ischemia that occurred in the course of tumor progression or after
treatment, stimulates proliferation,\textsuperscript{9} induces VEGF expression and angiogenesis.\textsuperscript{9}

Hypoxia has also been reported to enhance proliferation, survival, and dopaminergic differentiation of CNS precursors.\textsuperscript{10} In parallel, hypoxia also determines cell fate of neural crest stem cells.\textsuperscript{11} These findings suggest neural stem cells may exhibit a conserved response to reduced oxygen levels. Since human and mouse cells differ in oxygen sensitivity for acquiring replicative senescence,\textsuperscript{12} it is not clear whether the benefits of hypoxic culture on mouse neural stem cells could be observed in neural or other stem cells of human origin.

TWIST, a basic helix-loop-helix (bHLH) transcription factor, promotes tumor metastasis by inducing epithelial-mesenchymal transition (EMT).\textsuperscript{13} TWIST cooperates with N-myc to induce tumorigenic transformation.\textsuperscript{14} TWIST and Snail, another inducer of EMT, were proved to increase cells with cancer stem cell properties when overexpressed in breast cancer cells.\textsuperscript{15} Further, TWIST can overcome oncogene-induced senescence to complete oncogenic transformation.\textsuperscript{16} Recently, the HIF-TWIST axis has been demonstrated in head and neck cancer and is involved in tumor metastasis.\textsuperscript{17} Stem cells and cancer cells share a lot of similarities in gene expression, cellular processes and signal transductions, however there are few, if any, studies researching the effects of HIF-TWIST on normal stem cells.

The bHLH transcription factor E2A is essential for the differentiation of
B-lymphoid lineage via inducing p21 expression. \(^{18}\) p21 is also induced by p53 and acts as a suppressor in cell cycle progression. \(^{19}\) After p21 activation, cells undergo senescence and induce apoptosis. Although p21 expression and senescence have been reported to impair the efficiency of somatic cell reprogramming, \(^{20}\) the roles of p21 in regulating pluripotency and stem cell properties of stem cells have not been elucidated.

Since bone marrow, the original environment of MSCs, is hypoxic with the oxygen tension around 1 to 7%, \(^{21}\) we hypothesized that hypoxic culture provides more benefits than normoxic culture. Our results provide evidences for proposing a general protocol for rapid and efficient expansion of MSCs by combining low density culture with hypoxic culture. We found hypoxic culture not only prevented senescence that was noted in expanded MSCs but also increased differentiation efficiency. The underlying mechanism mediating the increase in stem cell properties by hypoxic culture of MSCs occurred through downregulation of E2A-p21 by the HIF-TWIST pathway.

\section*{Methods}

\section*{Cells and Preparation of hypoxic cultures}
Primary MSCs from three normal human volunteers were obtained from the Tulane Center for Distribution of Adult Stem Cells (wolfe@tulane.edu) and were prepared as described previously. The cells were seeded at 50 to 4×10^3 cells per cm^2 and grown in complete culture medium [CCM: α-MEM (α-minimal essential medium; Gibco-BRL, Gaithersburg, MD), supplemented with 16.6% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine] with medium change twice per week. For hypoxic culture, cells were cultured in a gas mixture composed of 94% N\textsubscript{2}, 5% CO\textsubscript{2}, and 1% O\textsubscript{2}. For maintenance of the hypoxic gas mixture, an incubator with two air sensors, one for CO\textsubscript{2} and the other for O\textsubscript{2}, was used; the O\textsubscript{2} concentration was achieved and maintained utilizing delivery of nitrogen gas (N\textsubscript{2}) generated from a liquid nitrogen tank or a tank containing pure N\textsubscript{2}. If O\textsubscript{2} percentage rose above the desired level, N\textsubscript{2} gas was automatically injected into the system to displace the excess O\textsubscript{2}.

**In vitro and in vivo differentiation**

For in vitro differentiation into osteoblasts, adipocytes and chondrocytes, cells were induced with osteogenic induction medium [OIM: α-MEM supplemented with 16.6% FBS, 50 μg/mL ascorbate-2 phosphate), 10^{-8} M dexamethasone and 10 mM β-glycerophosphate], adipogenic induction medium [AIM: α-MEM supplemented
with 16.6% FBS, 50 μg/mL ascorbate-2 phosphate, 10^{-7} M dexamethasone, 50 μM indomethacin, 0.45 mM 3-isobutyl-1-methyl-xanthine and 10 μg/mL insulin], and chondrogenic induction medium [CIM: cell pellets in serum-free α-MEM supplemented with ITS+ (GIBCO) and 10 ng/mL TGF-β1 (Preprotech, Rocky Hill, NJ)], respectively. After the appearance of morphologic features of differentiation, cells treated with OIM and AIM, and then stained with Alizarin Red S (ARS) and Oil-red O, respectively. Cells induced with CIM were prepared for Alcian Blue staining and immunohistochemistry. For immunohistochemistry, paraffin sections were initially incubated with blocking serum, probed with a monoclonal antibody against human type II collagen (Chemicon; CA), then reacted with an alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody, and finally processed for AP-Vector Red staining (Vector; CA). For in vivo osteogenic differentiation, 10^6 cells delivered in ceramic cube were induced with OIM. One week after induction, the cell-containing constructs were transplanted s.c. into the immunodeficient mice by surgical procedures. The specimens were analyzed by Mallory Trichrome staining 4 weeks later. For in vivo chondrocyte differentiation, cells were encapsulated with alginate as described before\textsuperscript{23} and then induced with CIM for 1 week. One week after induction, the alginate-encapsulated cells were transplanted s.c. into the immunodeficient mice by surgical procedures. The specimens were analyzed by
Alcian blue and Type II collagen immunohistochemistry staining 4 weeks later. The images were saved and analyzed with Image-Pro Plus 4.5 software (Media Cybernetics; Silver Spring, MD) using histogram-based quantification. For in vivo adipocyte differentiation, 1μg/mL bFGF in 100μL matrigel (BD Biosciences, CA,) was mixed with or without (serves as control) 1 × 10^6 cells, and injected immediately into the subcutaneous layer of immunodeficient mice. The specimens were analyzed by Sudan IV staining of frozen sections 3 weeks later.

**β-galactosidase staining**

Cells were washed with PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde for 5 min at room temperature. After washing, the cells were incubated at 37°C for an appropriate time with fresh senescence-associated β-Gal (SA-β-Gal) chromogenic substrate solution [1 mg/mL 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal, Cell Signaling Technology), 40 mM citric acid (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150mM NaCl, and 2 mM MgCl₂]. The experiment was repeated for 3 times and the mean percentage of cells expressed β-galactosidase was calculated.

**RT-PCR and real-time PCR**
Total RNA was extracted using TRIzol kit (Invitrogen, CA). RNA was reverse
transcribed in a final volume of 20 µL using 0.5 µg of oligo dT and 200 U Superscript
III RT (Invitrogen) for 30 min at 50°C, followed by 2 min at 94°C to inactivate the
reverse transcriptase. PCR amplification of the resulting cDNAs was performed under
the following conditions: 35 cycles of 94°C for 30 s, 58°C for 45 s, and 68°C for 45 s,
in which the 68°C step was increased by 5 s every cycle after 10 cycles. The reaction
products were resolved by electrophoresis on a 1.5% agarose gel and visualized with
ethidium bromide. For real-time PCR, the amplification was carried out in a total
volume of 25 µL containing 0.5 µM of each primer, 4 mM MgCl₂, 12.5 µL of
LightCycler™, FastStart DNA Master SYBR green I (Roche Molecular Systems,
Alameda, CA) and 10 µL of 1:20 diluted cDNA. PCR reactions were prepared in
duplicate and heated to 95°C for 10 min followed by 40 cycles of denaturation at
95°C for 15 sec, annealing at 60°C for 1 min, and extension at 72°C for 20 sec.
Standard curves (cycle threshold values versus template concentration) were prepared
for each target gene and for the endogenous reference (GAPDH) in each sample. The
quantification of the unknown samples was performed using the LightCycler Relative
Quantification Software version 3.3 (Roche). The sequences of primers are listed in
supplemental Table 2.
Western blotting

Cell extracts were prepared with M-PER (Pierce, IL) plus protease inhibitor cocktail (Halt™; Pierce) and protein concentrations were determined using the BCA assay (Pierce). Aliquots of protein lysates were separated on SDS–10% polyacrylamide gels, transferred onto PVDF membranes, blocked with 5% blotting grade milk (Bio-Rad, Hercules, CA) in TBST (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 1% Tween 20), probed with the indicated primary antibodies then reacted with corresponding secondary antibodies, and detected using a chemiluminescence assay (Millipore). Membranes were exposed to X-ray film (Amersham Pharmacia Biotech, NJ) for visualization.

Chromatin immunoprecipitation assay (ChIP)

To demonstrate the binding of TWIST protein to the E2- and E5-box of E2A promoter, the ChIP assay was performed using a commercial kit (Upstate Biotechnology, NY) according to the manufacturer’s protocol with minor adjustments. The MSCs and 293T transfected with pFLAG-TWIST were grown to confluence, treated with 1% formaldehyde for 20 min at 37°C, washed in ice-cold PBS containing protease inhibitors, then lysed on ice for 10 min in lysis buffer [10 mM Tris HCl, pH 8.0, 1% SDS] containing phosphatase and protease inhibitors. DNA-protein complexes were
sonicated to 200 and 600 bp. One aliquot of the soluble chromatin was stored at -20°C for use as input DNA, and the remainder was diluted 10 times in immunoprecipitation (IP) buffer [10 mM Tris HCl, pH 8.0, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, and 150 mM NaCl] containing phosphatase and protease inhibitors, and incubated overnight (4°C) with anti-human TWIST polyclonal antibody (H-81, sc-15393) (Santa Cruz Biotechnology). DNA–protein complexes were isolated on salmon sperm DNA/protein A agarose beads and then eluted with 1% SDS with 0.1 M NaHCO₃.

Cross-linking was reversed by incubation at 65°C for 5 h. Proteins were removed with proteinase K, and DNA was extracted with phenol/chloroform, redissolved and PCR-amplified with specific primer for E box on E2A promoter. All resulting precipitated DNA samples were also quantified with quantitative RT-PCR. Data were expressed as the percentage of input DNA.

**Calvarial defect animal models**

The animal research protocol was reviewed and approved by the animal center committee of National Ying-Ming University. The calvarial defects were created in 8-week-old NOD-SCID mice under general anesthesia using xylazine and ketamine. Full thickness skin flaps were raised and the left and right parietal bones were exposed. Defect diameter of 4 mm in the center of parietal bones was generated using
a hand drill trephine burr with constant saline irrigation. Collagen scaffold seeded with $1 \times 10^5$ cells was implanted into the defect. The skin was resealed. The procedure was performed under sterile conditions. Animals were sacrificed at 6 weeks after implantation for radiographic and histological analyses.

**Microfocal CT (micro-CT) measurements**

Micro-CT images were acquired using a cone-beam micro-CT imaging system. The scanner generates a cone beam at 110-$\mu$m spot size and operates at 50 keV. A region of 404 slices was imaged at 65-$\mu$m isotropic resolution and reconstructed in the size of 540×540. Reconstructed images were analyzed by threshold selection method.

**Dual energy X-ray absorptiometry (DEXA)**

The bone mineral density (BMD) of specimen was measured by Norland Dual Energy X-ray Absorptiometry (pDEXA; NORLAND Medical System, Inc. USA). The instrument was calibrated with a phantom of known mineral content. Each scan was performed at a speed of 5 mm s$^{-1}$ and scanning resolution was 0.1×0.1 mm. DEXA measurement was analyzed by system’s most recent software, version 3.9.4, and the region of interest for each sample was 0.1×0.1 cm.
Results

Hypoxic culture increases expansion efficiency and decreases senescence

For the rapid expansion of MSCs with maintained properties, the expansion efficiency at a variety of seeding densities was first analyzed. The increase in seeding density caused a decrease in expansion efficiency (Table S1). Increase in cell number by up to 170-250 folds for each passage was seen for low density (~50 cells/cm$^2$) culture, whereas high density (~1000 to 4000 cells/cm$^2$) culture showed a 5-fold increase for each week. However, we observed a decrease in expansion efficiency when cells were continuously expanded at low density (Table S1). Accumulatively, low density culture increased cell number by more than $10^{10}$ fold within 60 days, according to a 1000 fold of the increase in high density culture. We then examined whether hypoxic culture (1% O$_2$, if not indicated otherwise) could prevent low density culture-induced decrease in expansion efficiency. The expansion rate was the same for both normoxic (21% O$_2$) and hypoxic culture at the earliest passages, but was significantly less under normoxic conditions than hypoxic conditions after passage 2 with up to 10-fold difference at passage 6 or 7 (Figure 1A). The population doubling time (PDT) increased by up to 2-fold with the increase in passage number at passage 6 or 7 under normoxic conditions, however, under hypoxic conditions, the PDT remained the same as the earliest passages (Figure 1B). To elucidate the factors causing the dramatic
difference under normoxic and hypoxic conditions, the proliferation capacity and the expression of senescence markers were evaluated. The BrdU incorporation rate was significantly higher in hypoxic cells compared to normoxic cells (Figure 1C). Increased cell growth was noted in O₂ between 1% to 7% (Figure S1). Further, the decrease in cellular proliferation of normoxic cells was also associated with an increase in cell size with broader morphology (Figure 1D), a representative picture for cellular senescence in MSCs. 22 In addition, senescence as assayed by the expression of senescence-associated β-galactosidase (β-gal) revealed a significant increase in normoxic cells (Figure 1E). Western blotting of cells in late-passage MSCs under normoxic conditions also revealed a decrease in Senescence marker protein-30 (SMP-30), which was down-regulated with senescence and aging (Figure 1F), 25 suggesting hypoxic culture prevented MSCs from replication-induced senescence and aging. RT-PCR showed a higher expression of Apol and p21, markers of senescence, in normoxic cells than in hypoxic cells (Figure 1G). Cells under normoxic conditions began to cease proliferation and were difficult to subculture after passage 6 to 7, whereas cells under hypoxic conditions could be further expanded without significant loss of proliferation capacity. Similar results were also demonstrated with MSCs derived from two other donors. Taken together, these data suggest low density culture expanded MSC with a decrease in proliferation capacity and an increase in
senescence; however, combining low density and hypoxic culture expanded MSCs with preserved proliferation capacity without inducing senescence.

**Hypoxic culture maintains mesenchymal stem cell properties**

We then examined the expansion insults on the stem cell properties of MSCs under normoxic and hypoxic conditions. Cells grown under both conditions had the same surface CD marker profiles; they were consistently positive for CD44, CD73, CD90, 105 and CD166, putative markers of MSCs, but negative for CD34 and CD133, the markers of haematopoietic stem cells, and CD45, the marker of haematopoietic cells (Figure 2A). Stem cell properties of MSCs could be assayed by the potential to differentiate along the osteogenic, adipogenic and chondrogenic lineages. Interestingly, MSCs expanded at a low density under normoxic conditions showed declining differentiation potential especially at late passage, while cells under hypoxic conditions preserved the same potential for versatile differentiation as the earliest passage cells (Figure 2B-C). Taken together, MSCs cultured at low density lost their differentiation potential, and hypoxic culture increased MSC properties compared to normoxic culture.
Hypoxic culture bypasses senescence by increasing cells in the S phase and suppressing p21 expression

To clarify the anti-senescence effects of hypoxic culture, we compared the proliferation and apoptosis of MSCs grown in normoxia and hypoxia by analyzing the cell cycle phase distribution. FACS analysis of hypoxic cells revealed a marked reduction of cells in the G0/G1 phase and a compensatory increase of cells in S and G2/M phases compared with normoxic cells (Figure 3A), suggesting hypoxic culture increased cell proliferation. Since cell cycle regulatory proteins and their inhibitors are involved in cellular senescence of human fibroblasts, we therefore examined their involvement in the replicative senescence of MSCs. The expression level of p53 was not detectable under both hypoxic and normoxic conditions (data not shown). No obvious difference in CDK4 and CDK2 between hypoxic and normoxic culture was observed at passage 2 and 5 (Figure 3B). In addition, the expression levels of cyclin D1, and MDM2 were slightly decreased in hypoxic culture compared with normoxic culture. Interestingly, the p21 protein level was induced at the late passage compared with the early passage of normoxic culture, while its expression was slightly downregulated at late passage of hypoxic culture (Figure 3B). p21 is highly expressed in senescent cells and its disruption in normal human fibroblasts can bypass senescence.26 Thus, these data suggest replicative senescence in MSCs under
normoxic conditions and the bypass of replicative senescence in hypoxic culture may be mediated by regulating the p21 protein.

**HIF-TWIST inhibits p21 expression by downregulation of E2A**

The transcription factor E2A plays important roles in suppressing cell growth by transcriptionally activating the $p21$ gene.\(^{18}\) Interestingly, MSCs expanded under normoxic conditions exhibited a marked increase in E2A protein level, whereas E2A level was slightly downregulated under hypoxic conditions (Figure 3C). Likewise, the $E2A$ mRNA level was increased in normoxic cells compared with hypoxic cells (Figure 3D). Therefore, p21-induced senescence in normoxic cells is mediated by upregulating E2A expression and activity.

HIF-2$\alpha$, one of the regulators of cellular response to hypoxia, induces OCT-4 expression and transcriptional activity in embryonic stem cells.\(^{27}\) To elucidate the upstream signaling of hypoxia-mediated E2A inhibition and MSC properties maintenance, we first examined whether HIF-2$\alpha$ was upregulated in MSCs under hypoxic conditions. HIF-2$\alpha$ expression level was the same in both normoxic and hypoxic MSCs (data not shown), suggesting HIF-2$\alpha$ was not involved in hypoxia-mediated effects. We then analyzed the expression of HIF-1$\alpha$, the master regulator induced by hypoxia, and TWIST, which is upregulated by HIF-1$\alpha$\(^{17}\) and
generates cells with stem cell properties by EMT. Interestingly, normoxic cells expressed a very low level of HIF-1α, a basal level of TWIST and downregulation of TWIST was noted after expansion, whereas hypoxic cells exhibited increased HIF-1 and TWIST expression and expansion under hypoxia did not induce a loss of TWIST expression (Figure 3E). An increase in HIF-1 and TWIST expression was observed at O2 between 1% to 7% (data not shown). We then examined whether TWIST plays a negative role in the regulation of E2A expression. Ectopic expression of TWIST under normoxia inhibited the expression of E2A and p21 (Figure 3F), and siRNA against TWIST under hypoxia induced E2A and p21 expression (Figure 3F). Together, these data all suggest HIF-TWIST inhibited E2A and p21 expression.

**TWIST directly inhibits E2A transcription by binding to the E-box motif in E2A promoter**

To determine whether E2A was directly regulated by TWIST, the human E2A promoter activity was measured by luciferase reporter assay (Figure 4A). TWIST inhibited E2A promoter activity in a dose-dependent manner both in immortalized MSCs (Figure 4B) and 293T cells (Figure 4C). To investigate whether TWIST is directly associated with the E2A promoter via binding to the putative TWIST binding sites, E-boxes (CANNTG), chromatin immunoprecipitation (ChIP) was performed.
PCR amplification (Figure 4D, left panel) and real time PCR (Figure 4D, right panel) showed that the fragments containing the second (E2, -1118~-1112) and fifth (E5, -513~-507) boxes (Figure 4D), but not other E-boxes (Figure S2A) were immunoprecipitated with TWIST antibody in the immortalized MSCs overexpressing TWIST. Similar results were obtained in primary MSCs cultured under hypoxia for 12 days (Figure S2B). To further confirm the functional significance of the TWIST/E-box binding site, E2A promoter constructs with mutated E2-, E5-, or E2E5 box were co-transfected with the TWIST vector into 293T cells. Consistent with the ChIP results, the mutant E2A promoter reporter constructs did not show significant promoter repression in TWIST expressing cells (Figure 4E), indicating that both binding sites are required for maximal repression of E2A by TWIST. To determine the E-boxes binding domain in TWIST, a construct with 316-492 a.a bHLH truncated, pFLAG-tbTWIST, was transfected into 293T cells. Truncation of the bHLH domain abrogated the inhibition of E2A promoter activity by TWIST (Figure 4F). Therefore, TWIST downregulated E2A promoter activity by direct binding through the bHLH domain to the distal E2- and E5-boxes in the E2A promoter.

Hypoxia or HIF-TWIST increases mesenchymal stem cell properties through suppressing E2A and p21
Since HIF-TWIST inhibits E2A-induced p21 expression, we therefore examined whether hypoxia enhanced expression of these factors through suppressing E2A and p21. siRNA against E2A in normoxic cells suppressed the expression of p21 (Figure 5A) and siRNA against p21 in normoxic cells not only stimulated cell growth (Figure 5B), but also increased differentiation potential to osteoblasts, adipocytes and chondrocytes (Figure 5C). Conversely, overexpression of p21 induced a decrease in proliferation capacity, loss of differentiation capacity, and premature cell growth arrest in hypoxic cells (data not shown). These data suggest hypoxia or HIF-TWIST increases MSC properties through suppressing E2A and p21.

**Hypoxic cells have a normal karyotype and an untransformed phenotype**

MSCs from three individuals that were expanded under hypoxic conditions for more than 70-100 population doublings still proliferated well and could be expanded with low density culture. In addition, hypoxically expanded MSCs had a greater telomere length (Figure S3A) and telomerase activity (Figure S3B) than normoxically expanded MSCs. Similarly, knockdown of p21 in normoxic MSCs also induced an increase in telomere length (Figure S3A) and telomerase activity (Figure S3B). To prove the safety of expanding MSCs under hypoxic conditions, MSCs from three individuals were analyzed by karyotyping (Figure 6A) and comparative genomic
hybridization (supplemental Figure 4C). All of the MSCs had normal chromosome (Figure 6A) and intact genetic integrity (Figure S3C), and more importantly, no tumor was observed at 3 months after transplantation into NOD-SCID mice (data not shown). These data demonstrate the safety of expanding MSCs under low density and hypoxic conditions.

Hypoxic cells increase the differentiation potential in vivo

Since MSCs have been used for clinical therapy of skeleton diseases such as osteoarthritis, we investigated the potential of transplanted MSCs to differentiate into bone, fat and cartilage in vivo. Mallory Trichrome staining for collagen revealed the ceramic cube delivered with hypoxic MSCs after osteogenic induction was darkly stained in multiple layers and increased in collagen deposition compared to that delivered with normoxic MSCs after osteogenic induction (Figure 6B left panel).

Micro-CT analysis (Figure 6B right upper panel) and DEXA measurement (Figure 6B right lower panel) also revealed specimen delivered with hypoxic MSCs increased in trabecular formation and bone mineral density compared to that delivered with normoxic MSCs, respectively. Hypoxic cells also exhibited increase in Oil-red O staining when mixed with basic FGF and transplanted in immunodeficient mice (Figure 6C). Hypoxic cells that were encapsulated in alginate beads and induced in
chondrogenic medium showed increase in Alcian Blue staining and
immunohistochemistry for type II collagen after transplantation into immunodeficient
mice (Figure 6D). These data demonstrate the superior efficiency of hypoxic MSCs for
tskeleton tissue regeneration.

**Hypoxic cells increase the bone repairing capacity in vivo**

To examine whether hypoxic cells increase bone repairing capacity in vivo, an
immuno-compromised mice model of a calvarial defect was created to receive a
collagen scaffold with normoxic or hypoxic cells. Through quantification of the
radiographs of the new bone into acrylic thickness with ImagQuant software, bone
formation was found to be increased in hypoxic cell group at 6 weeks after
transplantation compared to the normoxic cell group (Fig. 7A). Micro-CT imaging
further showed a large amount of newly formed mineralized tissue filling in the
defects of hypoxic cell group, while only a small amount of new bone was seen in the
defects of the normoxic cell group (Fig. 7B). Quantification of newly-formed bones
showed the hypoxic cell group had more newly formed bone volume and had greater
bone mineral content (BMC) and density (BMD) compared to the normoxic cell
group (Fig. 7C). Hematoxylin-eosin and Mallory’s trichrome staining of the calvarial
sections at 6 weeks also showed marked bone and collagen formation within sample
with hypoxic cells implanted (Fig. 7D). These results suggest hypoxic cells increase bone repairing capacity when transplanted in bony defects.

Discussion

Expansion of a great amount of cells preserving the selfrenewal capacity and the differentiation potential is essential for successful use of stem cells in clinical practices. Though the low density culture of MSCs efficiently increases cell numbers at each passage, based on the current data, changes in proliferation capacity, senescence markers, gene expression profile, and differentiation potential were even noted in very early passages. Although, MSCs seeded at a density of about $1 \times 10^3$ to $4 \times 10^3$/cm$^2$ and sub-cultured at a ratio of 1 to 3 or 5, could preserve the proliferation capacity and differentiation potential for many passages but to obtain sufficient cells for cell therapy is long-consuming and delays the treatment. Developing a superior protocol exploiting the advantages of each culture method to expand MSCs has therefore attracted a lot of research interest. The current protocol combining low density with hypoxic culture successfully expanded a great amount of efficient MSCs in a short time, and therefore can be applied in clinical practices. Methods enhancing lifespan or stem cell properties of MSCs include retroviral transduction of the human telomerase gene, HPV16 E6E7, and the combined use of growth factors in a serum
free culture. All these methods either need genetic modification or enrich cells with oncogenic potential. The protocol presented here takes advantages of MSCs over other cells with a hypoxic marrow environment and the privileged ability to survive under hypoxia without aberrant genetic and biological changes. Based on the normal karyotype and intact genetic integrity, and the avoidance of tumors formation upon transplantation, the protocol presented here is safe to expand MSCs for clinical application.

This report further demonstrated hypoxia can prevent proliferation senescence, increase proliferation capacity, lifespan, and maintain stem cell properties of MSCs via HIF-1α-induced TWIST expression. The contribution of HIF-1α to hypoxia-induced prevention of premature senescence has also been demonstrated in embryonic fibroblasts. We also demonstrated that TWIST, through direct binding to E-boxes of E2A promoter, suppressed the E2A transcription to inhibit p21 expression. In addition, knockdown of p21 induced cell growth and increased stem cell properties under normoxia. E2A-p21 pathway plays an important role in B-cell development and induces cell cycle arrest involved in differentiation of various cell types. Previously, senescence of MSCs was found to be closely associated with the expression of the p16INK4A gene. We further demonstrated the upregulation of E2A in expanded MSCs, which in turn induced p21 to cause cell cycle arrest and replicative senescence.
The expansion-induced senescence of MSCs, however, could be reversed under hypoxic conditions, such as bone marrow environment, by a signaling pathway involving the suppression of E2A-p21 by HIF-TWIST. TWIST increases cancer cells with stem cell properties via inducing EMT.\textsuperscript{15,16} No differences were found in the expression of EMT markers between normoxic and hypoxic MSCs (data not shown). To our knowledge, this is the first paper demonstrating the regulation of normal stem cell properties by TWIST via direct regulation of E2A-p21 pathway, rather than modulating EMT.

Importantly, hypoxic MSCs decreased in DNA methylation of the promoter regions of OCT-4 and NANOG and increased in the expression of OCT-4A, NANOG, SOX-2 and SSEA-4 compared to normoxic MSCs. Moreover, MSCs decreased in the mRNA levels of these genes when induced for differentiation. Although the expression levels of these genes in MSCs are lower than in ES cells, they are much higher than in 293T cells. The expression levels were similar with previous reports for human MSCs.\textsuperscript{42} Because the expression of these genes in MSCs or adult stem cells is related to the differentiation status and expansion condition,\textsuperscript{42} therefore, the expression of these genes in MSCs implicates biological relevance. However, these are still correlations and definitively proof awaits further experiments in the future. Aside from, overexpression of p21 under hypoxia reduced the expression of OCT-4A, NANOG and SOX-2, while knockdown of p21 under
normoxia increased the expression of OCT-4A, NANOG and SOX-2. These data suggest p21 plays a suppressive role in the expression of these genes in MSCs under different oxygen concentrations. Currently, the mechanism that p21 mediated to regulate these pluripotency genes still remains unclear. Notably, the level of OCT-4A or NANOG inversely correlated with the level of p21 in cells treated with p21 specific siRNAs, while no stringent correlation was observed between the levels of SOX-2 and p21. One possible explanation is the specific function of SOX-2 which has been first known as a molecular rheostat for the repression of its own transcription and the expression of SOX-2:OCT-3/4 target genes through the binding of its C-terminus to the binding sites for SOX-2 and OCT-3/4.43

Recently, hypoxia has been reported to enhance the undifferentiated status and stem cell properties via the interaction of HIF with Notch intracellular domain to block the activation of Notch-responsive promoters in various stem and precursor cell populations.36 In the current study, HIF-1α activated TWIST to suppress specific signaling pathways such as E2A-p21 and promote the expression of transcription factors such as OCT-4A, NANOG and SOX-2 that control stem cell self renewal and multipotency. Recently, the putative role of HIF-TWIST pathway in tumorigenesis and tumor metastasis has been recognized in several cancers, including breast cancer and head and neck cancer.15,17 This study identified a beneficial role of HIF-TWIST
pathway in increasing lifespan, enhancing expression of embryonic transcription factors and promoting stem cell properties in stem cells.

With the conflict of HIF-TWIST in tumorigenesis of tumor and lifespan or stem cell properties of stem cells, emphasis should be placed on explaining why the activation of HIF-TWIST pathway did not induce tumorigenic transformation of MSCs. TWIST is constantly overexpressed in N-Myc-amplified neuroblastomas, where TWIST overexpression is responsible for inhibiting the ARF/p53 pathway involved in the Myc-dependent apoptotic response. The oncogenic cooperation of these two key regulators of embryogenesis causes cell transformation and malignant outgrowth. The fact that neither amplification of N-Myc nor changes in the p53 level and apoptotic response have been noted between hypoxic and normoxic conditions or HIF-TWIST activation may help to explain why HIF-TWIST did not induce tumor formation in MSCs. Overexpression of TWIST has also been reported to generate tumor cells with properties of stem cells by inducing EMT, or help oncogenic transformation by inhibiting senescence, induced by mitogenic oncoproteins, such as Ras or ErbB2, and inducing EMT. The fact that no changes in EMT expression were observed between hypoxic and normoxic conditions can help to explain why HIF-TWIST did not induce tumor formation in MSCs. For future application of hypoxic culture and HIF-TWIST pathway in increasing the stem cell
properties of stem cells, we need to further clarify the underlying mechanisms of HIF-TWIST-mediated pathway.

Besides inhibiting senescence and stimulating stem cell properties, hypoxic culture also inhibited serum-deprivation-induced apoptosis in MSCs.\(^ {37}\) In addition, MSCs cultured under hypoxic condition increased their expression of HIF-1\(\alpha\), and its downstream genes, CX3CR1 and CXCR4 and their engraftment in vivo.\(^ {34}\) Therefore, the lifespan, efficiency, and stem cell properties of MSCs can be controlled by modifying the culture conditions or the corresponding signaling pathways. Hypoxic culture or activation of HIF-TWIST pathway may provide a great amount of MSCs with improved efficiency for clinical applications.

**Acknowledgements:** Grants supported by Veterans General Hospital-Taipei (V98C1-009, V98E1-002, V99E1-011); National Science Council (95-2314-B-075-047-MY3; 97-3111-B-010-001-, 98-3111-B-010-001-) and National Yang-Ming University, Ministry of Education. This work is assisted in part by the Division of Experimental Surgery of the Department of Surgery, Taipei Veterans General Hospital.
Author Contribution

CCT: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

TLY: Collection and/or assembly of data

LLC: Collection and/or assembly of data

YJC: Data analysis and interpretation

JYW: Data analysis and interpretation

CHC: Data analysis and interpretation

SCH: Conception and design, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Conflicts of Interest

No potential conflict of interest exists for all the co-authors.

References

4. Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac


Figure Legends

Figure 1. Hypoxic culture increases expansion efficiency and decreases in senescence. Cells were seeded at 50 cells/cm² and cultured under normoxic and hypoxic conditions. After 12 days of cultured, the cells were recovered and reseeded at 50 cells/cm² and cultured under the same conditions. Fold of cell expansion (A) and population doubling time (B) for each passage were calculated. Normoxic culture decreases in cell expansion rate and increases in population doubling time as the increase of passage number (Pn: Passage No). (C) Normoxic and hypoxic cells were incorporated with 5-bromo-2′-deoxyuridine (BrdU) for 18 h and then detected by flow cytometry. Hypoxic cells increase in BrdU incorporation rate compared with normoxic cells. (D) Microscopic photographs of normoxic and hypoxic cells were used to analyze the cell size. Hypoxic cells increase in cell size and become large and flat after expansion compared with hypoxic cells. (E) Cells expanded under normoxic and hypoxic conditions were stained with β-galactosidase (β-gal). Normoxic cells increase in the percentage of β-gal expression compared with hypoxic cells. (F) Western blotting and quantification for senescence marker protein-30 (SMP-30). After expansion, normoxic cells decrease in SMP-30 level, while hypoxic cells increase the expression. (G) RT-PCR (upper panel) and quantitative RT-PCR (lower panel) analysis for markers of senescence. After expansion, the expression of...
senescence-related genes significantly increases under normoxic conditions compared with hypoxic conditions. [Values are mean + SD; *, p<0.05 and **, p<0.01 indicate significant variance (independent t-test) between normoxia (Nor) and hypoxia (Hyp).] Bar = 50 µm.

**Figure 2. Hypoxic culture increases in stem cell properties.** Cells were seeded at 50 cells/cm² and expanded under normoxic and hypoxic conditions. (A) Flow cytometry for detecting surface CD markers. Cells under normoxic and hypoxic conditions have the same profile of surface CD markers. (B & C) Hypoxic cells increase in differentiation potential into osteoblasts, adipocytes and chondrocytes. (B) Normoxic and hypoxic cells at passage 6 were induced to differentiate to osteoblasts and adipocytes for 3 weeks, and stained by ARS and Oil-red O, respectively. Stained dye was extracted and OD values were measured. (C) Normoxic and hypoxic cells at passage 6 were induced to differentiate to chondrocytes for 3 weeks, and Alcian Blue staining and immunohistochemical study for collagen II and X were performed. Quantitative data performed by the computerized image analysis show hypoxic cells increase in Alcian blue staining and IHC for collagen II and X. [Values are mean + SD; *, p<0.05 and **, p<0.01 indicate significant variance (independent t-test) between Nor and Hyp.] Bar = 20 µm.
Figure 3. HIF-TWIST inhibits p21 expression via suppressing E2A protein level and activity. (A) Analysis for cell cycle distribution in normoxic and hypoxic cells by propidium iodide (PI) staining followed by flow cytometric analysis. G0/G1 phase is reduced but S and G2/M phases are increased in hypoxic cells compared with normoxic cells. (B) Western blotting for cell cycle-related proteins. Hypoxic cells decrease in p21 protein level compared with normoxic cells. (C) Western blotting and (D) quantitative RT-PCR for E2A expression. After expansion, normoxic cells increase in E2A protein and mRNA levels compared with hypoxic cells. (E) Western blotting for HIF-1α and TWIST. Expression of HIF-1 and TWIST are decreased in normoxic cells compared with hypoxic cells. (F) Cell lysates were detected by Western blotting. As compared with control vectors, overexpression of TWIST in normoxic cells inhibits the expression of p21 and E2A, and siRNAs against TWIST in hypoxic cells induces p21 and E2A expression. The results shown here are representative of three independent experiments. [Values are mean + SD; *, p<0.05 and **, p<0.01 indicate significant variance (independent t-test) between Nor and Hyp.]

Figure 4. TWIST directly inhibits E2A transcription by binding to E-box motif in E2A promoter
(A) Genomic organization of the region flanking the promoter region of human E2A (upper panel) and the schematic representation of the pGL3-E2A reporter construct. Transcription start site, TSS. Reporter assays showing, in immortalized MSCs (B) or 293T (C), TWIST represses the E2A promoter in a dose dependent manner (n=3). β-galactosidase was used as a control of transfection efficiency. (D) ChIP analysis of immortalized MSCs after transfection of pFLAG-TWIST. The chromatin was incubated either without antibodies, with an anti-TWIST antibody or with isotype IgG antibody. Fragments of the E2-(147bp) and E5-(115bp) box in the E2A promoter were amplified by PCR (left panel) and were also quantified with quantitative RT-PCR (right panel). Input, 2% of total input lysate. Results are shown as the mean ± SD values. (Black bar for E2 box; White bar for E5 box) (E) Mutational analysis of E2-box and E5-box sites in the E2A promoter in 293T cells. Reporter constructs containing wild-type E2A (WT), E2-box (E2M) or E5-box (E5M) mutations, or double mutations (E2E5M) were generated and used to analyze the importance of these sites in mediating repression by TWIST (n=3) (F) Truncation of the bHLH domain (tbTWIST) inhibits TWIST repression of E2A promoter. (n=3). Each ratio was normalized to the control (pGL3 basic vector), and significance was determined by Student’s t-test. (* p<0.05 and ** p<0.01 versus control).
Figure 5. Hypoxia or HIF-TWIST increases stem cell properties via suppressing p21. (A) siRNA against E2A in normoxic cells decreases the expression of p21. (B) siRNA against p21 in normoxic cells increases cell growth. Normoxic cells were cultured 12 days after p21 knockdown in low density culture and cell numbers were counted. (C) Normoxic cells at passage 6 were stably transfected with scrambled or p21 siRNA followed by differentiation into osteoblasts, adipocytes and chondrocytes for 3 weeks, and achievements of differentiation were analyzed by staining with ARS, Oil-red O and Alcian Blue, respectively. O.D. values of ARS and Oil-red O were analyzed for quantifying osteoblast and adipocyte differentiation, respectively. Knockdown of p21 increases the differentiation potential to osteoblasts, adipocytes and chondrocytes. Bar = 50 µm.

Figure 6. Safety and efficiency of hypoxic culture. (A) Karyotyping analysis shows hypoxic MSCs have normal karyotype. (B) For in vivo bone formation, cells were delivered in ceramic cube and induced in osteogenic medium for one week followed by transplantation under beneath the dorsal skin of NOD-SCID mice for 4 weeks. Mallory Trichrome staining (left panel) shows hypoxic cells increase in collagen synthesis. Micro-CT (right upper panel) shows hypoxic cells increase in trabecular formation. DEXA (right lower panel) shows hypoxic cells increase in bone mineral
density. (C) For in vivo fat formation, cells were mixed with basic FGF and
transplanted in NOD-SCID mice for 4 weeks. Oil-red O staining shows hypoxic cells
increase in the accumulation of fat droplets. (D) For in vivo cartilage formation, cells
were encapsulated in alginate beads and induced in chondrogenic medium for one
week followed by transplantation into NOD-SCID mice for 4 weeks. Alcian Blue
staining and immunohistochemistry for type II collagen demonstrate hypoxic cells
increase in the synthesis of proteoglycan and type II collagen. Bar = 50 µm.

Figure 7. Hypoxic cells increase in vivo bone repairing ability

Calvarial defects were implanted with hypoxic (Hyp) or normoxic cells (Nor). (A)
Radiographic images and densitometric analysis were performed at 6 weeks. (B)
Micro-CT 3D reconstruction imaging and (C) quantitation for bone volume, bone
mineral content (BMC), and bone mineral density (BMD) of newly formed tissues
were performed at 6 weeks. The data are expressed as mean ± SEM. Asterisks
indicate significant differences (*p<0.05, **p<0.01, ***p<0.005). (D) Samples were
harvested for histological analysis 6 weeks later. Hematoxylin and eosin staining (H
& E) for morphological evaluation (the rectal angle area in the upper panel is
magnified in the middle panel) and Mallory’s trichrome staining for collagen
deposition in hypoxic and normoxic groups. Arrow indicates bone area (Bar=100 µm)
Figure 1

A: Cell Increase (Folds) over Culture Days for Nor and Hyp conditions.

B: Population Doubling Time for Nor and Hyp conditions.

C: BrdU Incorporation (%) for Nor and Hyp conditions.

D: Cell Size (Pixel) for Nor and Hyp conditions across P1 to P4.

E: β-gal Cell No. / Power-field for Nor and Hyp conditions.

F: Western Blot images and bar graphs for SMP-30 and β-actin expression in Nor and Hyp conditions for P1 to P5.

G: Relative expression to GAPDH of Apol and p21 in Nor and Hyp conditions for P2 and P3.

* indicates statistical significance at p < 0.05.
** indicates statistical significance at p < 0.01.
Figure 2

A

![Bar graph showing CD marker+ cells (Nor vs. Hyp)]

B

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Graphs showing O.D. value for ARS and Oil-red O:
- **ARS**:
  - Nor: ![Graph](image)
  - Hyp: ![Graph](image)
- **Oil-red O**:
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C

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Graph showing % stained area for Alcian Blue, Col II, and Col X:
- **Alcian Blue**:
  - Nor: ![Graph](image)
  - Hyp: ![Graph](image)
- **Col II**:
  - Nor: ![Graph](image)
  - Hyp: ![Graph](image)
- **Col X**:
  - Nor: ![Graph](image)
  - Hyp: ![Graph](image)
Figure 3

A

Cell Percentage (%)

G0/G1  S  G2/M

B

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D

Relative expression of E2A

Nor  Hyp

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Relative expression of TWIST, p21, E2A, and β-actin
Figure 7

A

Nor  Hyp

Acrylic thickness (mm)

Nor  Hyp

B

Nor  Hyp

Bone Volume (mm³)

Nor  Hyp

BMC (g)

Nor  Hyp

BMD (g/mm³)

Nor  Hyp

D

Nor  Hyp

H&E

H&E

Trichrome staining

Bar

Bar
Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through downregulation of E2A-p21 by HIF-TWIST

Chih-Chien Tsai, Yann-Jang Chen, Tu-Lai Yew, Ling-Lan Chen, Jir-You Wang, Chao-Hua Chiu and Shih-Chieh Hung