Abstract
HYPOXIA AND SERUM DEPRIVATION IN HUMAN MESENCHYMAL STEM CELLS, AND THE PROTECTIVE ROLE OF THYMOSIN β-4
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Background: Current literature suggests nutrient deprivation and hypoxia affect human mesenchymal stem cell (hMSC) survival after cell transplantation. Wound-healing protein Thymosin β-4 (Tβ4) has been shown to improve hMSC proliferation and prevent apoptosis. This study examines the effects of nutrient deprivation and hypoxia on hMSC survival, and the pro-survival effect of pre-treatment with Tβ4. **Methods:** Early hMSC cultures, at 48 hours, were grown under nutrient deprivation (1.5% FBS media), a 24-hour, 1.0 μg/mL Tβ4 pre-treatment, and 24 hours of hypoxia (1% O_2). Proliferation of hMSCs was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, and intracellular stress was examined by a Reactive Oxygen Species (ROS) assay. In addition, hMSCs were grown for 2 weeks in complete media (16.5% FBS), then nutrient-deprived (1.5% FBS media) with a 24-hour, 1.0 μg/mL Tβ4 pre-treatment, followed by 24 hours of hypoxia (1% O_2). Proliferation was measured with immunocytochemistry using a Ki-67 antibody. Protein expression of hypoxia inducible factor-1 alpha (HIF-1α), Vascular Endothelial Growth Factor- alpha (VEGF-α), caspase-3, B cell lymphoma-2 (Bcl-2), and Connexin 43 (Cx43) were measured with western blot. **Results:** In 48-hour cell cultures, nutrient-deprived hMSCs had significantly increased ROS production ($p \leq .01$), and decreased proliferation

compared to hMSCs in complete media; hypoxia had no effect. Tβ4-treated hMSCs showed decreased ROS production and increased proliferation compared to non-treated hMSCs. In 2-week hMSC cultures, nutrient deprivation and hypoxia resulted in elevated caspase-3 protein expression. Hypoxia increased HIF-1α and VEGF-α expression in complete media and nutrient-deprived media. Additionally, Tβ4-treated hMSCs showed increased HIF-1α and VEGF-α expression under normoxia and hypoxia. In Tβ4-treated hMSCs, expression of Bcl-2 increased under normoxia, and Cx43 expression increased under hypoxia. **Discussion:** These findings suggest that nutrient deprivation is the predominant factor affecting hMSC survival in early cultures. However, Tβ4 may promote hMSC survival in a nutrient-deprived environment in early and expanded cultures. Further studies regarding Tβ4 effectiveness will shed insight into its potential as a therapeutic agent in hMSC transplantation.

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Bcl-2: B-cell Lymphoma-2

BSA: Bovine Serum Albumin

Cx43: Connexin43

DAPI: 4',6-diamidino-2-phenylindole

DCF: 2'7'-dichlorofluorescein

DCFH-DA: 2',7'-dichlorfluorescein-diacetate

FBS: Fetal Bovine Serum

HIF-1α: Hypoxia-Inducible Factor-1 alpha

hMSC: Human Mesenchymal Stem Cell

HRP: Horseradish Peroxidase

LDS: Lithium Dodecyl Sulfate

LV: Left Ventricle

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NAC: N-acetyl-cysteine

PBS: Phosphate-buffered Saline

PI3: Phosphatidylinositol 3-kinase

Tβ4: Thymosin beta-4

TBS: Tris-buffered Saline

VEGF-α: Vascular Endothelial Growth Factor-alpha

INTRODUCTION

Nearly 5 million people in the U.S. have congestive heart failure (CHF), wherein the heart is unable to provide sufficient blood supply throughout the body (Fitzgerald, 2011). This weakened condition of the heart results over time due to underlying factors such as hypertension, sedentary lifestyle, smoking, diabetes, and atherosclerosis. Some suggest that 50% of patients diagnosed with CHF will die within 5 years. Currently, medication, bypass surgery, pacemaker insertion, and organ transplantation are therapies used to treat CHF, however none of these efforts are long-term solutions to heart failure.

A highly researched field is human stem cell transplantation, which appears to have long-term potential to stop or reverse the progression of tissue damage. Stem cells can be extracted from an individual, allowed to proliferate in culture, and reinjected into the same individual with minimal risk of rejection by the immune system. Sources of stem cells for transplantation include the bone marrow, peripheral blood, and umbilical cord blood. Bone marrow-derived human mesenchymal stem cells (hMSCs) have been the choice of many studies due to their ease of extraction, self-renewal, and multipotency, as shown by their ability to differentiate into osteoblasts, chondrocytes, myocytes, and adipocytes (Potier et al., 2007). However, problems arise when the ischemic (nutrient-deprived and hypoxic) microenvironment of damaged tissue is unsuitable for long-term survival and engraftment of stem cells (Chacko et al., 2010).

The first goal of this project was to begin to explore the effects of nutrient deprivation and hypoxia on hMSC behavior *in vitro*. Studies suggest poor nutrient supply, not hypoxia, to be the primary factor influencing hMSC survival after transplantation (Zhu, Chen, Cong, Hu, and

Chen, 2006; Potier et al., 2007; Chacko et al., 2010). In fact, hypoxia-induced reactive oxygen species (ROS), although traditionally associated with cell death stimuli, have been shown to increase pro-survival transcription factor hypoxia-inducible factor-1α (HIF-1α), thereby extending short-term longevity (Oh and Moon, 2010). Furthermore, short-term hypoxia (24- 48 hours) has been shown to stimulate growth factor production, specifically vascular endothelial growth factor (VEGF), thereby playing a role in therapeutic angiogenesis (Abdollahi et al., 2011). Pre-conditioning hMSCs is a potential solution to minimize cell death in an ischemic microenvironment post-transplantation. In this case, cells may be pre-exposed to hypoxia or other treatments in an effort to prepare the cells for upcoming environmental stress.

Researchers are now adding growth factors and protein pre-treatments to hMSC cultures in efforts to improve hMSC survival. This research project analyzed the effects of a hMSC pre-treatment with the wound-healing protein Thymosin beta-4 (Tβ4). Previously, the Muller-Borer laboratory studied a hMSC Tβ4 pre-treatment, and reported enhanced survival and engraftment in Tβ4-treated hMSCs injected in the mouse heart compared to non-treated hMSCs (Byrum, 2008). In addition, intercellular communication and gap junction protein Connexin 43 (Cx43) was increased in cardiomyocytes, while no change in hMSC Cx43 protein expression was detected. Wang et al., (2010) found Cx43 to promote survival of mesenchymal stem cells in the ischemic heart. Consequently, there is ongoing interest to further examine the direct effect of Tβ4 on hMSC survival.

Tβ4 is a naturally occurring, g-actin sequestering polypeptide originally discovered in the thymus gland. It is expressed in numerous cell types and can be cross-linked to proteins including collagen and fibrin to facilitate wound healing (Oh et al., 2008). In addition, Tβ4

shows a range of effects when added to the medium surrounding cells in culture (Moon, Im, Ryu, and Kang, 2010; Phil and Kleinman, 2010; Smart et al., 2010). During oxidative stress, co-administration of Tβ4 has been shown to prevent apoptosis. Cell survival is thought to be promoted by Tβ4 through the lowering of ROS levels; as hypoxia-induced ROS can trigger apoptosis. It is considered unlikely that Tβ4 exerts all these effects solely through intracellular sequestration of g-actin. The diverse activities related to tissue repair may depend on interactions with intra- or extracellular receptors. Tβ4 is proposed to act through activation of the PI3/Akt pathway, reducing apoptotic caspase-3 activation and maintaining anti-apoptotic bcl-2 regulation (Sosne, Siddiqi, and Kurpakus-Wheater, 2004; Zhang et al., 2009; Shrivastava, Srivastava, Olson, DiMaio, and Bock-Shrivastava, 2010). Oh and Moon (2010) found that Tβ4 also stabilizes HIF-1α, further promoting cell survival during hypoxia. Based on this background and our preliminary data, a follow-up Tβ4 pre-treatment investigation was conducted.

The aims of this study were to investigate (i) the effects of nutrient deprivation and hypoxia on hMSC survival and (ii) the effect of Tβ4 pre-treatment on hMSC survival during nutrient deprivation and hypoxia *in vitro*. In concert with previous findings, we hypothesized that nutrient deprivation would impact cell death at a greater magnitude than hypoxia (Potier et al., 2007). Due to the protective effects of Tβ4, hMSCs pre-treated with Tβ4 were expected to show higher rates of survival than those without treatment.

METHODS

Human Mesencymal Stem Cell (hMSC) Culture

At passage 2, hMSCs were thawed and counted via trypan blue method using a hemocytometer. Then, hMSCs were divided into groups based on nutrient deprivation, hypoxic microenvironment, and Tβ4 pre-treatment (Table 1). The Tβ4 treatment was only given to nutrient-deprived groups since hMSCs in damaged tissue are expected to be nutrient-deprived, with varying amounts of oxygen. A preliminary study analyzed the effects of Tβ4 on hMSCs at concentrations of 0.1, 1.0, and 10.0 μg/mL. The 0.1 μg/mL dosage was insufficient in yielding significant differences in hMSC proliferation or survival when compared to untreated hMSCs. Changes in these parameters were noted at 1.0 μg/mL Tβ4, and these differences did not significantly vary when hMSCs were treated with Tβ4 at 10.0 μg/mL (Byrum 2008). Therefore, a concentration of 1.0 μg/mL Tβ4 was chosen as the optimal dosage for this experiment. The hMSCs were plated at 100 cells/cm^2 in T-75 flasks, and placed in a 37^oC and 5% CO_2 incubator. The question whether or not to thaw stem cells and immediately transplant them into tissue, or to allow the stem cells to proliferate in culture and then transplant them has not been answered. Therefore, we evaluated hMSCs in early and expanded cultures. To evaluate early cultures, immediately after thawing, hMSC Groups 3-8 were nutrient-deprived, i.e., media with 1.5% fetal bovine serum (FBS), and Groups 5 and 6 received a 24-hour, 1.0 μg/mL Tβ4 (S-1298, Bachem) pre-treatment. After Tβ4 pre-treatment, Groups 2, 4, 6, and 8 were placed in a hypoxic microenvironment (1% O_2) for 24 hours, and Groups 1-8 were placed in the cell culture incubator. Forty-eight hours after Tβ4 treatment began, proliferation and reactive oxygen species (ROS) assays were conducted. Since the plate readers used to analyze these two assays

examined a small surface area with minimal hMSCs, different assays were used to assess proliferation and protein expression after 2 weeks of hMSC proliferation. Protein expression was not evaluated in early cultures due to small sample size, i.e. too few cells to harvest protein. As hMSCs are likely to be transplanted only after expansion in culture, we investigated the effect of Tβ4 on protein expression in expanded cultures. To evaluate expanded cultures, hMSCs were initially cultured in normal nutrients (media with 16.5% FBS) under normoxia (20% O_2) for 2 weeks. Media was refreshed every third day, and hMSCs were grown to 80% confluency. After reaching confluency, i.e. 2 weeks, Groups 3-8 were nutrient-deprived (media with 1.5% FBS) for 24 hours, and Groups 5 and 6 received a 24-hour, 1.0 μg/mL Tβ4 pre-treatment. After 24-hour Tβ4 pre-treatment, Groups 2, 4, 6, and 8 were placed in a hypoxic microenvironment (1% O_2) for 24 hours, and Groups 1-8 were placed in the cell culture incubator. Forty-eight hours after Tβ4 treatment, hMSCs were harvested and evaluated for proliferation via immunocytochemistry and protein expression via western blot. Figure 1 illustrates the experimental protocol.

Table 1. hMSC culture groups

Treatments	1	2	3	4	5	6	7	8
Complete media	+	+	-	-	-	-	-	-
Normoxia	+	-	+	-	+	-	+	-
Tβ4	-	-	-	-	+	+	-	-
NAC (control for ROS assay)	-	-	-	-	-	-	+	+

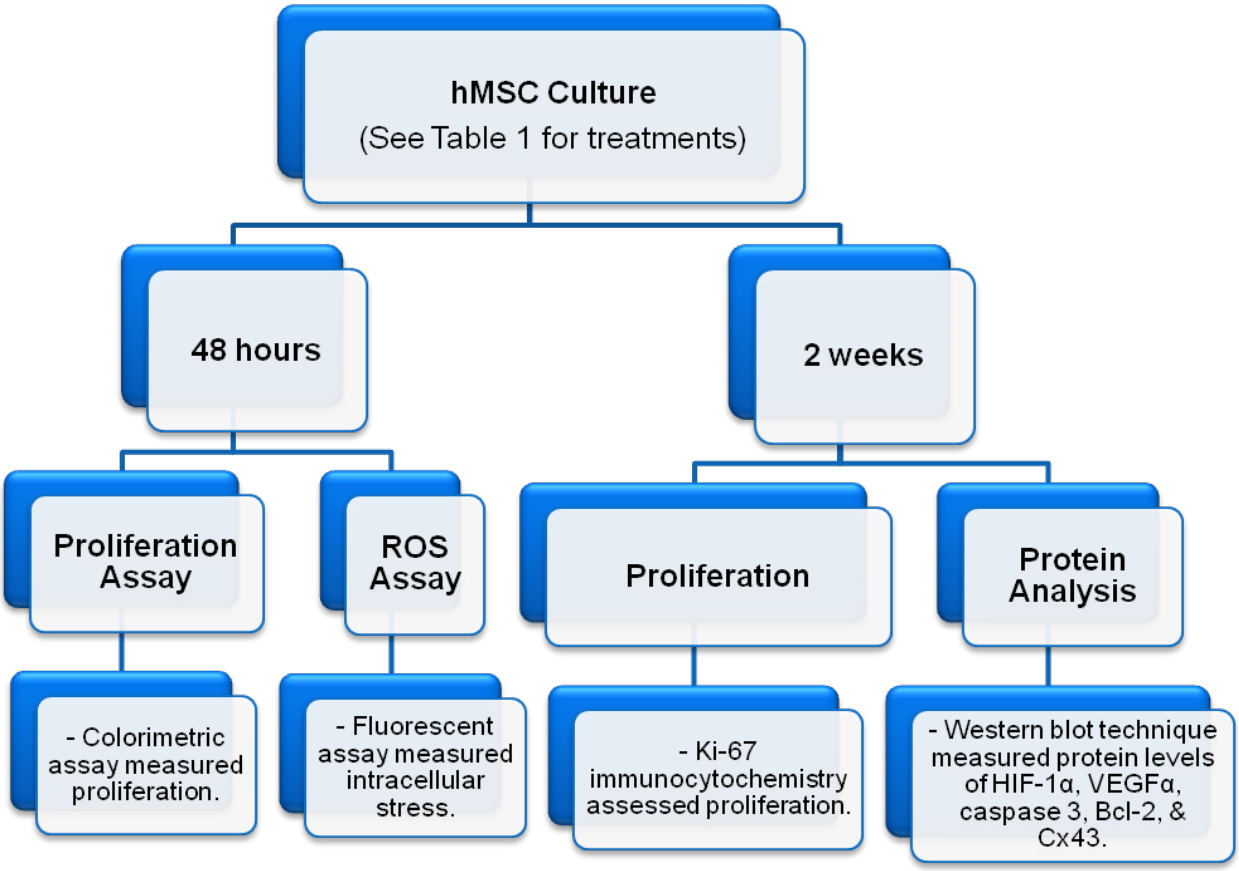


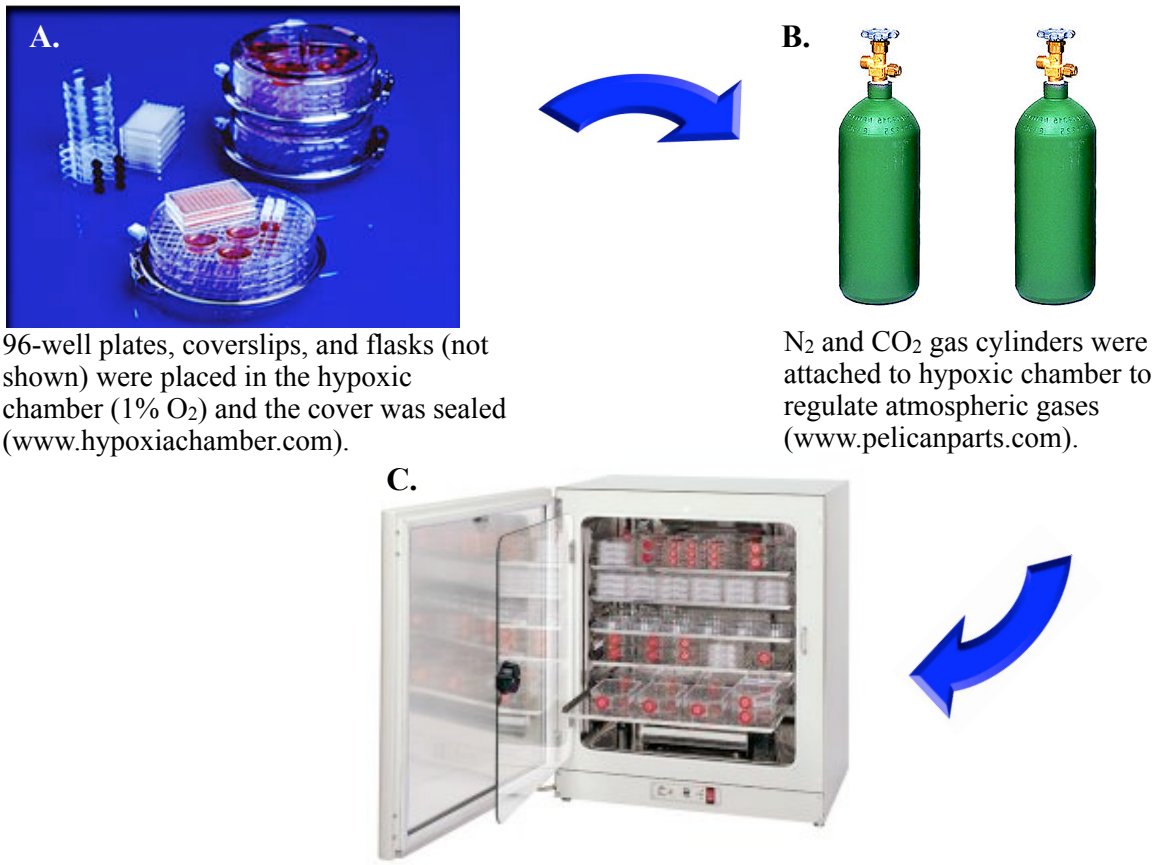
Figure 1. Experimental protocol.

Nutrient Levels

All hMSCs were grown in Dulbecco's Modified Eagle Medium+GlutaMAX low glucose medium (10567, GIBCO) with 16.5% Fetal Bovine Serum (SH30071, Hyclone) and 1% Penicillin-Streptomycin (15140, GIBCO), i.e. complete media, prior to nutrient deprivation, hypoxia, and Tβ4 pre-treatment. For nutrient-deprivation analysis, FBS concentration of the media was reduced to 1.5%.

Hypoxia

Hypoxic conditions (1% O_2) were simulated using a hypoxic chamber (Billups-Rothenberg, Del Mar, CA). The CO_2 and O_2 within the hypoxic chamber was sampled with an Illinois Instrument Gas Analyzer. Cell cultures were placed in the hypoxic chamber and the atmosphere was flushed for 10 minutes with a 95% N_2, 5% CO_2 mix. The hypoxic chamber was sealed and placed in a 37°C and 5% CO_2 cell culture incubator for 1 hour. After 1 hour, the atmosphere was flushed again with the same N_2 /CO_2 mix for 10 minutes to remove residual O_2. A flow meter attachment was used to regulate the N_2, set at 70 mmHg, and CO_2, at 5 mmHg. The hypoxic chamber was sealed and placed in the cell culture incubator for 24 hours (Figure 2).



96-well plates, coverslips, and flasks (not shown) were placed in the hypoxic chamber (1% O_2) and the cover was sealed (www.hypoxiachamber.com).

N_2 and CO_2 gas cylinders were attached to hypoxic chamber to regulate atmospheric gases (www.pelicanparts.com).

Place hypoxic chamber in cell culture incubator at 37 °C, 5% CO_2 (www.cooler-store.com).

Figure 2. Schematic illustration of hypoxia setup. **A.** Billups Rothenberg hypoxic chamber. **B.** N_2 and CO_2 gas cylinders. **C.** Cell culture incubator.

Proliferation Assay

Forty-eight hours after hMSC plating, proliferation was measured via a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. An early indication of cellular damage is a reduction in metabolic activity. This assay measures mitochondrial activity, wherein only metabolically active cells convert MTS into a colored formazan product. The quantity of formazan product measured at 490 nm absorbance is directly proportional to the number of viable cells in culture. The hMSCs were divided into groups based on nutrient deprivation, hypoxic microenvironment, and Tβ4 pre-treatment (Table 1). hMSCs were plated at 15,000 cells/well in 96-well plates (Figure 3). Groups 1 and 2 were grown in complete media (16.5% FBS), and Groups 3-6 in 1.5% FBS media; Groups 5 and 6 were pre-treated with 1.0 μg/mL Tβ4 for 24 hours before hypoxia. After the 24-hour, Tβ4 pre-treatment, Groups 2, 4, and 6 were placed in the hypoxic chamber for 24 hours and Groups 1-6 were placed in the cell culture incubator. After removal from the hypoxic environment, a MTS reagent (G3582, Promega) was added to all cells (Groups 1-8). Groups 2, 4, and 6 were returned to the hypoxic chamber and Groups 1-6 were placed in the cell culture incubator for 2 hours. Mitochondrial activity was determined by measuring the intensity of formazan with a 96-well plate reader at wavelength 490 nm.

Plate A. No Hypoxia

	1	2	3	4	5	6	7	8	9
A	Group 1		Group 3		Group 5		Group 7		Blank
B	Group 1		Group 3		Group 5		Group 7		Blank
C	Group 1		Group 3		Group 5		Group 7		Blank
D	Group 1		Group 3		Group 5		Group 7		
E	Group 1		Group 3		Group 5		Group 7		

Plate B. Hypoxia

	1	2	3	4	5	6	7	8	9
A		Group 2		Group 4		Group 6		Group 8	Blank
B		Group 2		Group 4		Group 6		Group 8	Blank
C		Group 2		Group 4		Group 6		Group 8	Blank
D		Group 2		Group 4		Group 6		Group 8	
E		Group 2		Group 4		Group 6		Group 8	

Figure 3. Group organization in 96-well plates. Each rectangle represents a well in the plate. Groups were plated with 5 repeats to ensure efficiency and accuracy in pipette technique.

Reactive Oxygen Species (ROS) Assay

Forty-eight hours after hMSC plating, intracellular stress was measured via a reactive oxygen species (ROS) assay. Over-production of ROS is traditionally a cell death signal. The generation of ROS was measured using 2',7'-dichlorfluorescein-diacetate (DCFH-DA), a cell-permeable probe that is oxidized by ROS intracellularly, and is converted to fluorescent 2'7'-dichlorofluorescein (DCF). ROS production and DCF fluorescence intensity are proportional. N-acetyl-cysteine (NAC), a natural ROS scavenger, was used as a control to ensure effectiveness of the assay. The hMSCs were divided into groups based on nutrient deprivation, hypoxic microenvironment, Tβ4 pre-treatment, and NAC treatment (Table 1). First, hMSCs were plated in black-wall, clear bottom, 96-well plates at 15,000 cells/well. A background measurement was obtained using nutrient-deprived or complete media without cells. Groups 1 and 2 were plated in complete media (16.5% FBS), and Groups 3-8 were plated in 1.5% FBS media; Groups 5 and 6 were pre-treated with 1.0 μg/mL Tβ4. Groups 1-8 were placed in the cell culture incubator for 24 hours. After the 24-hour, Tβ4 pre-treatment, 500 μM NAC (A9165, Sigma-Aldrich), was

added to Groups 7 and 8. After 30 minutes, DCFH-DA (D6883, Sigma-Aldrich) was added to all cells, and the plates were returned to the cell culture incubator for 1 hour. Next, the DCFH-DA was removed, hMSCs were washed once with 1.5% FBS media, and media was refreshed: Groups 1 and 2 received complete media and Groups 3-8 received 1.5% FBS media; Groups 5 and 6 were maintained with 1.0 μg/mL Tβ4, and Groups 7 and 8 were treated with 500 μM NAC. Groups 2, 4, 6, and 8 were placed in the hypoxic chamber. All groups were placed in the cell culture incubator for 24 hours. After removal from incubation, DCF fluorescence was recorded with a multi-well plate reader and a fluorescein 485 nm filter.

Immunocytochemistry

Two weeks after hMSC plating, immunocytochemistry was performed to determine hMSC proliferation via nuclear protein Ki67. Ki-67 is present during all active phases of the cell cycle, excluding G_0. In previous experiments, the Muller-Borer laboratory established the Ki-67 antigen as an effective marker of hMSC proliferation. hMSCs were divided into groups based on nutrient deprivation, hypoxic microenvironment, and Tβ4 pre-treatment. First, hMSCs were grown in complete media (16.5% FBS) on 9.6 cm^2 coverslips at 100 cells/cm^2 for 2 weeks. Next, hMSCs in groups 3-6 were subjected to 24 hours of nutrient deprivation (1.5% FBS media) and groups 5 and 6 were pre-treated with 1.0 μg/mL Tβ4. After the 24-hour nutrient deprivation and Tβ4 pre-treatment, hMSCs in groups 2, 4, and 6 were placed in a hypoxic environment for 24 hours. All groups were placed in the cell culture incubator. Next, hMSCs were removed from the incubator and immediately fixed in cold 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes, washed in 1X PBS (2 x, 5 minutes), permeabilized with 0.1% Triton X-100 for 10 minutes, washed with 1X PBS (2 x, 5 minutes), and blocked in 3% bovine serum

albumin (BSA) for 1 hour to prevent non-specific binding of the antibodies. Next, hMSCs were washed with 1X PBS (1 x, 5 minutes) and incubated with a mouse anti-Ki-67 antibody (1:100) (Dako) in 3% BSA in 1x PBS for 1 hour. The hMSCs were washed with 1X PBS (3 x, 6 minutes), and incubated with secondary antibody, donkey anti-mouse (1:1000) Alexafluor 488 (A11055, Invitrogen), in 1X PBS for 45 minutes to allow Ki-67 visualization. Coverslips were washed with 1X PBS (3 x, 6 minutes), nuclear-counterstained with 4'-6-Diamidino-2-phenylindole (DAPI) for 5 minutes, and washed with 1X PBS (2 x, 5 minutes). Coverslips were dipped in distilled water 2 times to remove PBS salts and mounting media was added to adhere the coverslips to slides. All slides were covered with foil to provide protection from light exposure, and slides were allowed to dry overnight. Fluorescent images were acquired within 24 hours of preparation using a Zeiss fluorescent microscope. Proliferation was quantified by counting the number of Ki-67 positive hMSCs among the total number of Dapi positive hMSCs using ImagePro software. Results were reported as the ratio of Ki-67 positive cells/Dapi positive cells. Ten fields were counted per coverslip, and 1 coverslip was analyzed per group for each experiment (n=3).

Protein Expression

Cell Lysis for Protein. Two weeks after plating, protein expression was analyzed in hMSCs. The hMSCs were harvested, i.e. media was removed, trypsin was used to detach adherent cells, FBS was added to stop the trypsin, and the flasks were observed under an inverted microscope to ensure confluency. The hMSCs were collected in 50 mL conical tubes and spun to pellet in a centrifuge at 1600 rpm for 5 minutes. To prepare hMSC protein for western blot analysis, media was removed and the hMSCs were washed twice with tris buffered saline (TBS).

The cell suspension was transferred to a 1.5 mL microfuge tube and centrifuged at 1600 rpm for 5 minutes to pellet. The supernatant was discarded and ice-cold lysis buffer containing TBS/ 0.5% Triton X-100/Protease Inhibitor Cocktail (P8340, Sigma) was added to the cell pellet, and the hMSCs were vortexed vigorously and left on ice for 15 minutes. Next, hMSCs were centrifuged at 16,000 rpm at 4 ^{o}C for 5 minutes to remove insoluble debris. The supernatant, i.e., protein, was transferred to a fresh, labeled, cold microfuge tube, and stored at -80 ^{o}C.

Protein Normalization. All protein samples were quantitated and normalized to the lowest concentrated sample using the Bradford method (Bio-Rad DC Protein assay). Comparison to a standard Bovine Serum Albumin (BSA) curve corresponded to relative protein concentration. The standard curve was constructed using serial dilutions: 0.9 mL of distilled water to 0.1 mL of BSA standard (2.5 mg/mL). A 250 µL lysis buffer (previously described) aliquot was added to 5 microfuge tubes and the BSA standard was serial diluted into each tube at concentrations: 1.25, 0.625, 0.312, 0.156, 0.00 mg/mL. Aliquots (5 µL) of each sample were placed in a 96-well plate in triplicate, and 25 µL of Reagent A/S (500-0113, 500-0115, Bio-Rad) was added to each well, followed by 200 µL of Reagent B (500-0114, Bio-Rad). The reagents were mixed for 10 seconds, then incubated for 15 minutes at room temperature (21^{o} C). Absorbances were recorded at 650 nm on a platereader (31420050, Perkin-Elmer Victor). All samples were normalized (diluted with lysis buffer) to the lowest concentrated sample (3.17 µg).

Electrophoresis/Western Blotting. Survival of hMSCs was quantitated with: caspase-3, Bcl-2, HIF-1α. The effect of Tβ4 treatment on VEGF-α and Cx43 were also measured. These proteins were chosen based on previous hMSC studies with Tβ4 treatment: Caspase-3 is an apoptotic end path protein, and expression was expected to be down-regulated. Bcl-2 is an anti

apoptotic protein. Bcl-2 acts through the same pathway (PI3) that Tβ4 is thought to regulate, and expression was expected to be improved. HIF-1α is a pro-survival factor. Expression of HIF-1α was expected to be up-regulated during hypoxia. VEGF-α is an angiogenesis protein. We anticipated VEGF-α expression to be up-regulated by HIF-1α. Cx43 is a gap junction protein that was examined in a preliminary Tβ4 study. Cx43 expression was expected to be up-regulated. For this analysis, jurkat cell protein was used as a control for caspase-3, Bcl-2, HIF-1α (did not show) and VEGF-α. Human heart left ventricle (LV) protein (BioChain) was used as a control for Cx43. Protein samples (58 μg) with 4X LDS sample buffer (NP0007, Invitrogen) and Reducing agent (NP0004, Invitrogen) were separated on a bis-tris (4-12%) gel (345-0118, Bio-Rad) at 175 V for 1 hour, then transferred to a nitrocellulose membrane at 60 V for 1 hour and 15 minutes. The membrane was blocked in a 5% milk/tris-buffered saline solution (TBS) for 1 hour at room temperature, and washed in TBS/0.1%Tween (3 x, 5 minutes) at room temperature. The membrane was incubated for 1 hour at room temperature with the antibodies listed in Table 2. Next, the membrane was washed with TBS-Tween (3 x, 5 minutes) and a secondary antibody was applied for 1 hour incubation at room temperature. The membrane was washed with TBS-Tween (3 x, 5 minutes), and chemiluminescent substrate (Pierce) was added and incubated for 5 minutes. Protein bands were detected by exposure to audoradiography film (248300, RPI). The film was developed and Gel-Pro Analyzer software was used for densitometry analysis.

Table 2. Western blot antibodies		
Protein	**Primary**	**Secondary**
HIF-1α	mouse anti-HIF-1α (120 kDa, BD Biosciences) at 1:500	goat anti-mouse-HRP (MP 00852) at 1:4000
VEGF-α	rabbit anti-VEGF-α (16 kDa, Millipore) at 1:500	goat anti-rabbit-horseradish peroxidase (HRP) (MP 07264) at 1:5000
Caspase-3	rabbit anti-caspase-3 (20 kDa, Millipore) at 1:1000	goat anti-rabbit-HRP
Bcl-2	mouse anti-Bcl-2 (26 kDa, Invitrogen) at 1:1000	goat anti-mouse-HRP
Cx43	mouse anti-Cx43 (43 kDa, Millipore) at 1:200	goat anti-mouse-HRP

Statistical Analysis

All statistical analysis was conducted using a multivariate fit model and analysis of variance with JMP software (SAS Institute Inc.). Variability between experiments contributed to a lack of statistical significance for some measurements. Results were consulted with the biostatistics department.

The experiments were analyzed by evaluating (i) the effects of nutrient deprivation and hypoxia and (ii) the effect of Tβ4 treatment. Experiments were performed in triplicate, and then averaged as a mean percent of control. For the nutrient deprivation and hypoxia experiment, hMSCs in complete media (16.5% FBS) under normoxia (20% O_2) were used as the control. For the Tβ4 treatment experiment, non-treated hMSCs in nutrient deprivation (1.5% FBS media) under normoxia were used as the control. All groups were analyzed based on the control for that study. Error bars were calculated as a standard error of the mean. Results were considered statistically significant at $p \leq .05$.

RESULTS

Analysis of Early hMSC Cultures

Proliferation. The Proliferation Assay showed an overall decrease in proliferation in nutrient-deprived hMSCs (Figure 4). No differences were found in hMSC proliferation due to 24 hours of hypoxic exposure. With Tβ4 treatment, hMSC proliferation increased compared to non-treated hMSCs. Differences in proliferation were not statistically significant (Table 3).

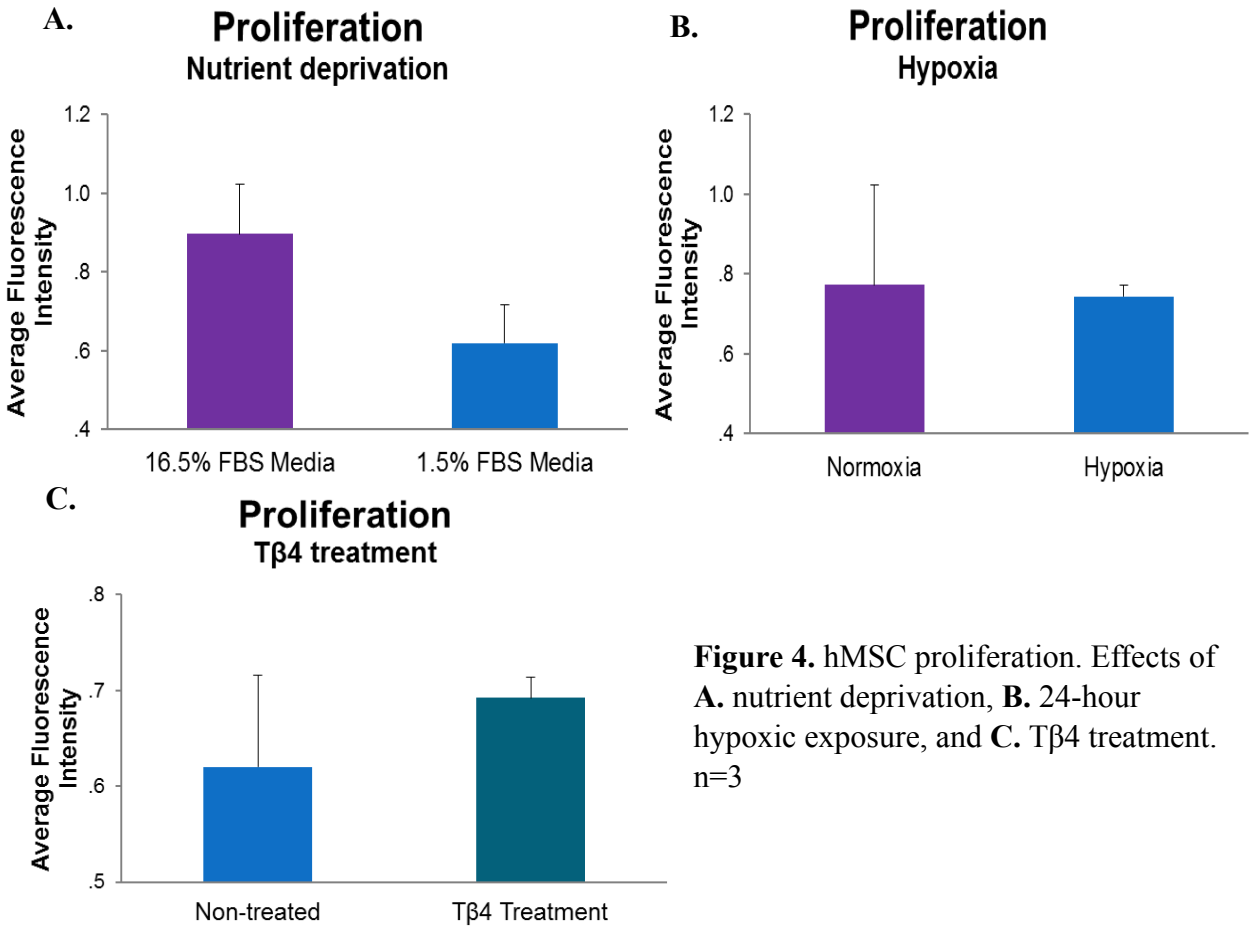


Figure 4. hMSC proliferation. Effects of **A.** nutrient deprivation, **B.** 24-hour hypoxic exposure, and **C.** Tβ4 treatment. n=3

Table 3. Statistical analysis of hMSC proliferation	
	P-values
Complete media vs. nutrient deprivation	0.1089
Normoxia vs. hypoxia	0.6849
Non-treated vs. Tβ4 treatment	0.4586

*Proliferation Assay. n=3

Reactive Oxygen Species (ROS) Production. The ROS assay revealed a significant increase in ROS production in nutrient-deprived hMSCs compared to hMSCs in complete media ($p \leq .01$) (Figure 5). The 24-hour hypoxic exposure did not impact ROS production. With Tβ4 treatment, hMSC ROS production decreased only under normoxia compared to non-treated hMSCs. P-values are listed in Table 4 for reference.

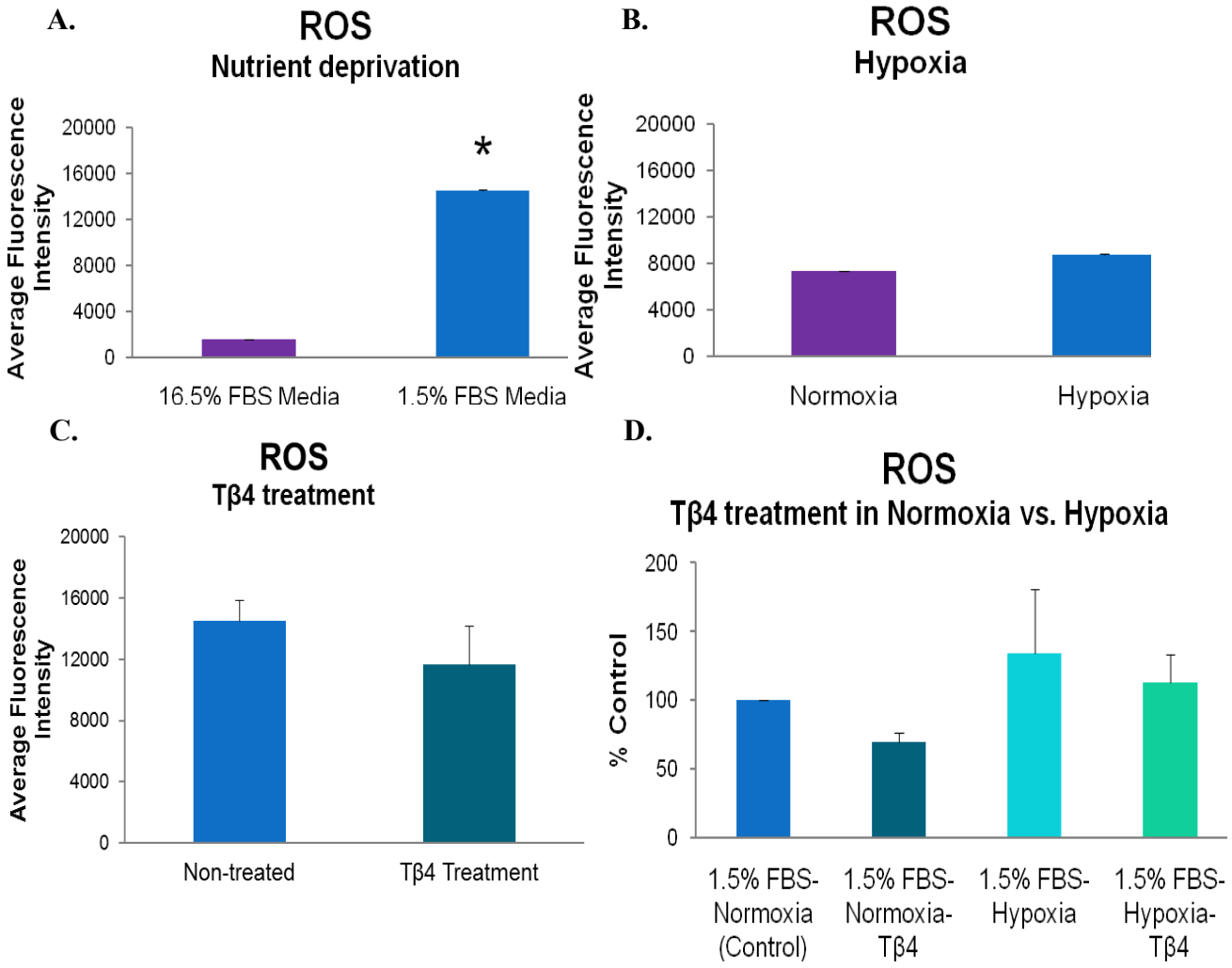


Figure 5. hMSC ROS production. Effects of **A.** nutrient deprivation ($p \leq .01$) **B.** 24-hour hypoxic exposure, **C.** Non-treated vs. Tβ4 treatment, and **D.** Tβ4 treatment under normoxia vs. hypoxia.. n=3

Table 4. Statistical analysis of hMSC ROS production	
	P-values
Complete media vs. nutrient deprivation	0.0036*
Normoxia vs. hypoxia	0.6063
Non-treated vs. Tβ4 treatment	0.3437

*ROS Assay. n=3

Analysis of Expanded hMSC Cultures

Proliferation. Ki-67 immunofluorescence revealed no differences in hMSC proliferation as a result of nutrient deprivation or 24-hour Tβ4 treatment in normoxia or hypoxia (Tables 5 and 6). Dapi-stained nuclei are shown in blue. Ki-67-stained nuclei, an indicator of proliferating cells, are shown in green by arrow (Figure 6).

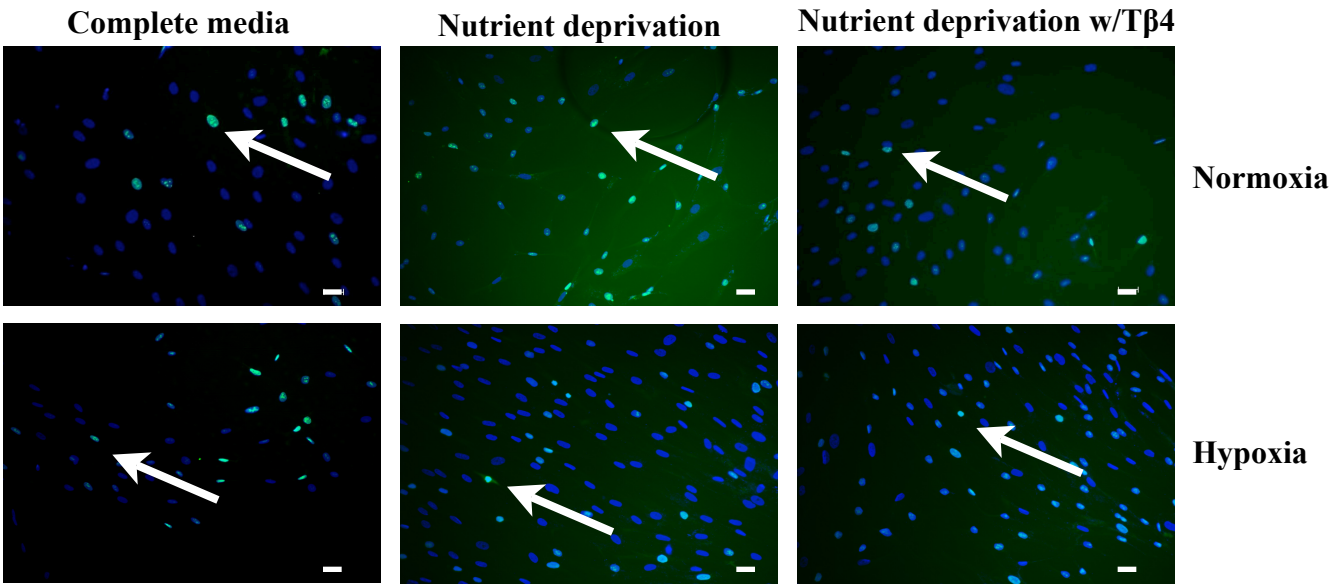


Figure 6. hMSC proliferation at 2 weeks. Representative images of hMSCs acquired under fluorescent microscopy with dapi-stained nuclei (blue) and Ki-67 (green, indicated by arrow). n=3. Scale bar= 50 μm.

Table 5. Statistical analysis of hMSC proliferation at 2 weeks

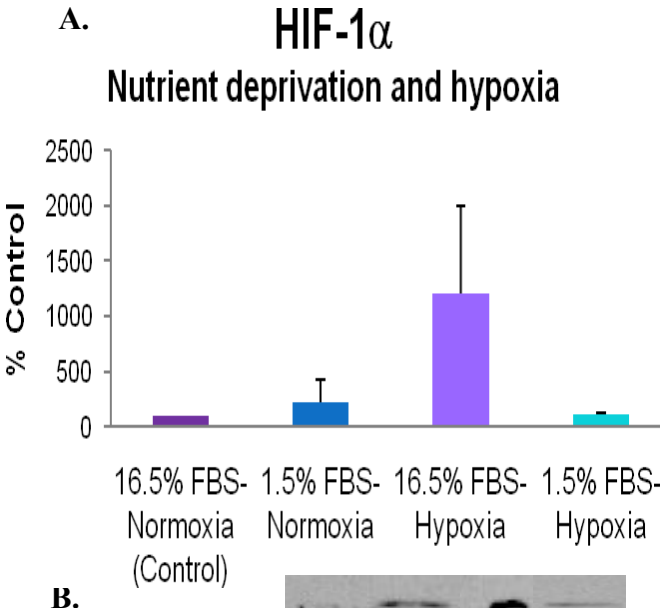
	P-values
Complete media vs. nutrient deprivation	0.4075
Normoxia vs. hypoxia	0.5035
Non-treated vs. Tβ4 treatment	0.3523

*Immunocytochemistry. n=3

Table 6. Immunocytochemistry hMSC counts

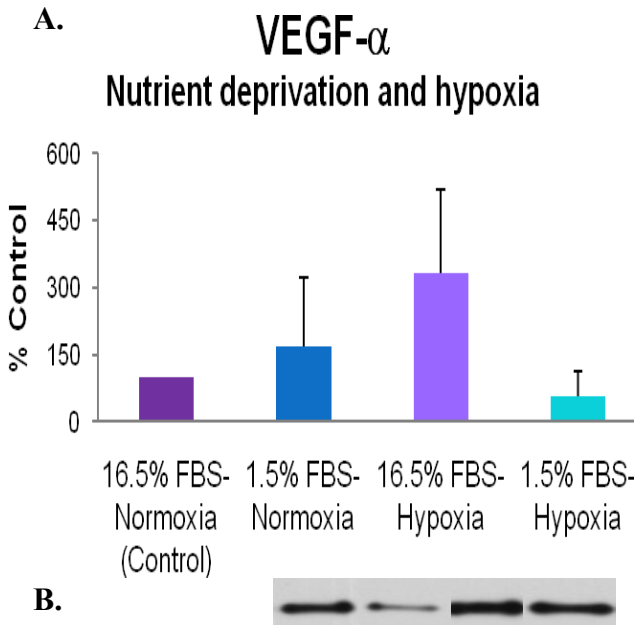
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
hMSCs	101	131	78	126	159	187
Ki67+	57	32	22	44	37	39
Complete media	+	+	-	-	-	-
Normoxia	+	-	+	-	+	-
Tβ4	-	-	-	-	+	+

Protein Expression. The effects of nutrient deprivation, hypoxia, and Tβ4 treatment were analyzed separately, however hMSC protein expression varied based on their interaction. In hMSCs exposed to 24 hours of hypoxia, HIF-1α and VEGF-α expression increased only in complete media (Figures 7 and 8). Both nutrient deprivation and hypoxia resulted in increased cleaved caspase-3 expression (Figure 9). Neither nutrient deprivation nor hypoxia significantly altered Bcl-2 or Cx43 protein expression in hMSCs (data not shown). The LV control expressed a Cx43 band at 43 kDa, and the jurkat control expressed caspase-3 and Bcl-2 bands at 20 and 26 kDa verifying the antibody efficiencies (data not shown). HIF-1α and VEGF-α were not expressed in control tissue, however their protein bands appeared in our data at the expected kDa weights.



Lane	1	2	3	4
Complete media	+	-	+	-
Normoxia	+	+	-	-

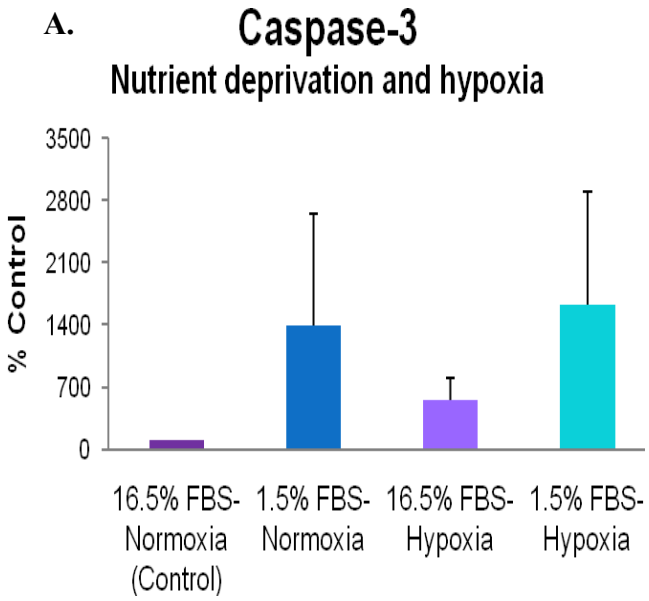
Figure 7. HIF-1α expression in hMSCs. **A.** Results illustrate the effects of nutrient deprivation and hypoxia. **B.** Representative western blot images. n=3



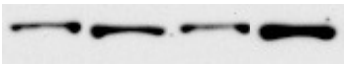
Lane	1	2	3	4
Complete media	+	-	+	-
Normoxia	+	+	-	-

Figure 8. VEGF-α expression in hMSCs. **A.** Results illustrate the effects of nutrient deprivation and hypoxia**. B.** Representative western blot images. n=3

A.



B.

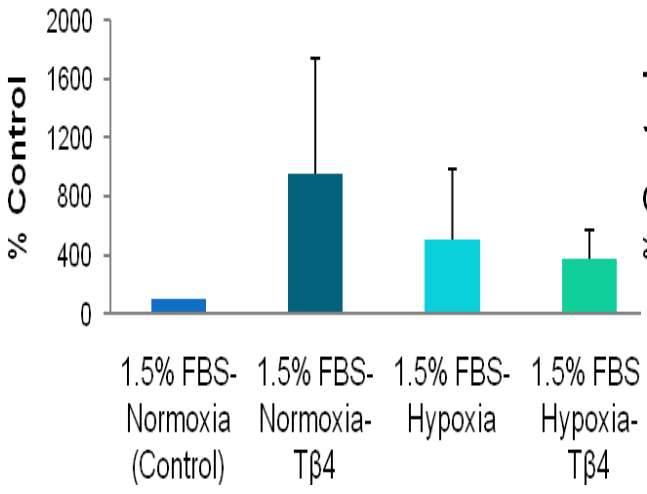


Lane	1	2	3	4
Complete media	+	-	+	-
Normoxia	+	+	-	-

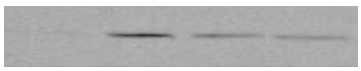
Figure 9. Caspase-3 expression in hMSCs. **A.** Results illustrate the effects of nutrient deprivation and hypoxia. **B.** Representative western blot images. n=3

The effects of Tβ4 were reported in comparison to non-treated hMSCs. Tβ4-treated hMSCs increased in HIF-1α and VEGF-α expression under normoxia and hypoxia (Figures 10 and 11). The degree to which Tβ4 exerted its effects varied according to oxygen concentration, as seen in Figure 10 where Tβ4 had a greater effect on HIF-1α under normoxia, and in Figure 11 where Tβ4 had a greater effect on VEGF-α under hypoxia. In Tβ4-treated hMSCs, Bcl-2 expression increased under normoxia, and Cx43 expression increased under hypoxia (Figures 12 and 13). No changes in caspase-3 expression were observed with Tβ4 treatment (data not shown). P-values from all protein analyses were recorded in Table 7.

A.



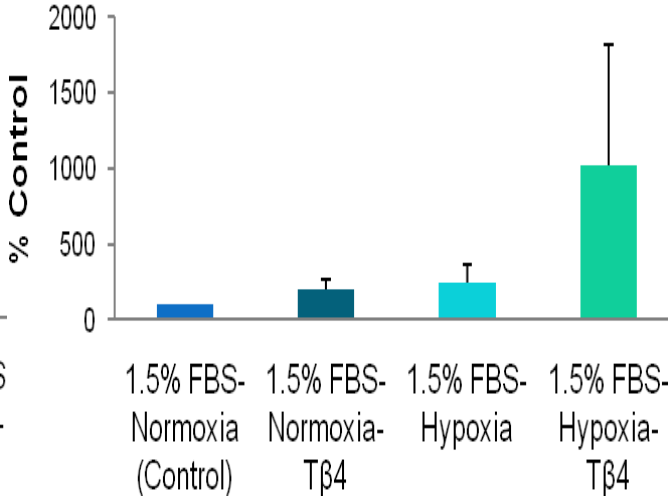
B.



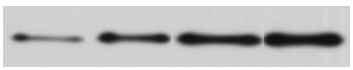
Lane	1	2	3	4
Complete media	-	-	-	-
Normoxia	+	+	-	-
Tβ4	-	+	-	+

Figure 10. HIF-1α expression in hMSCs after Tβ4 treatment. **A.** Under normoxia vs. hypoxia. **B.** Representative western blot images. n=3

A.

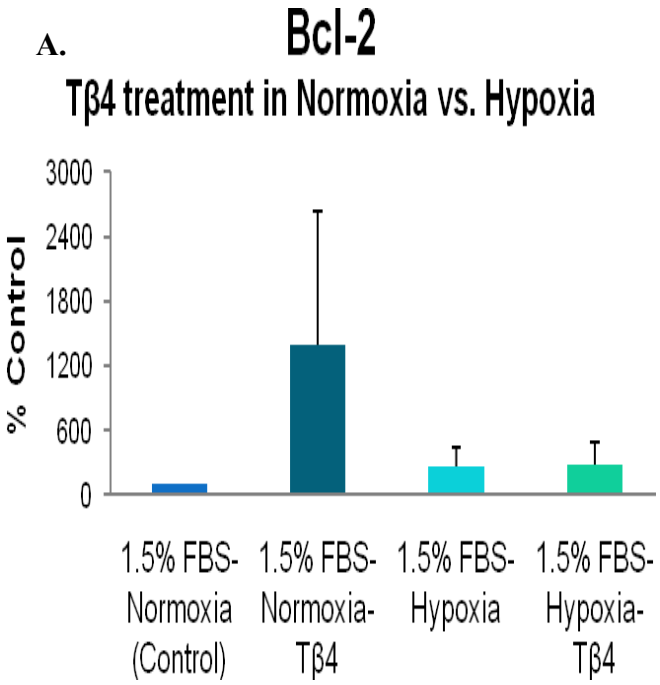


B.



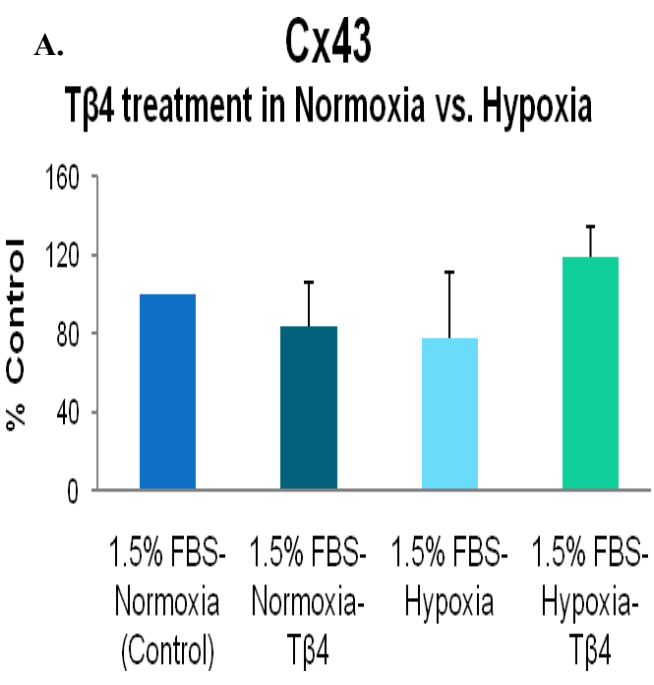
Lane	1	2	3	4
Complete media	-	-	-	-
Normoxia	+	+	-	-
Tβ4	-	+	-	+

Figure 11. VEGF-α expression in hMSCs after Tβ4 treatment. **A.** Under normoxia vs. hypoxia. **B.** Representative western blot images. n=3



Lane	1	2	3	4
Complete media	-	-	-	-
Normoxia	+	+	-	-
Tβ4	-	+	-	+

Figure 12. Bcl-2 expression in hMSCs after Tβ4 treatment. **A.** Under normoxia vs. hypoxia. **B.** Representative western blot image. n=3



Lane	1	2	3	4
Complete media	-	-	-	-
Normoxia	+	+	-	-
Tβ4	-	+	-	+

Figure 13. Cx43 expression in hMSCs after Tβ4 treatment. **A.** Under normoxia vs. hypoxia. **B.** Representative western blot image. n=3

Table 7. Statistical analysis of hMSC protein expression

P-values:	HIF-1α	VEGF-α	Caspase-3	Bcl-2	Cx43
Complete media vs. nutrient deprivation	0.356	0.5113	0.1513	0.3097	0.1858
Normoxia vs. hypoxia	0.2285	0.5345	0.784	0.41	0.9376
Non-treated vs. Tβ4 treatment	0.4018	0.3332	0.7211	0.3567	0.6187

*Western Blot. n=3

DISCUSSION

In order to simulate the environment of damaged tissue, hMSCs were subjected to nutrient deprivation and hypoxia. In early hMSC cultures, nutrient deprivation (1.5% FBS media) was primarily responsible for significantly increased ROS production and decreased hMSC proliferation. The stem cell niche has been found to have low oxygen tensions from 1%-5%, therefore even in this artificial environment, hypoxia was not expected to greatly affect stem cell activity (Abdollahi et al., 2011).

The Tβ4 pre-treatment was given to hMSCs to improve cell survival in an ischemic environment. In the early cell cultures, hMSCs responded to Tβ4 treatment as proliferation was higher under normoxia and hypoxia, and ROS production was lower under normoxia compared to non-treated hMSCs. ROS production increased in Tβ4-treated hMSCs under hypoxia, wherein higher ROS levels may trigger cell death signals. However, earlier research suggests that ROS production may be maintained by Tβ4 under hypoxia in order to enable HIF-1α stabilization (Oh and Moon, 2010), which is necessary to promote cell survival factors.

Protein expression was only evaluated at 2 weeks because hMSC transplantation is expected to be utilized primarily after cell expansion, ensuring a large number of cells for transfer. In expanded hMSC cultures, HIF-1α and VEGF-α proteins showed increased expression under hypoxia compared to normoxia. Other investigations have suggested that HIF-1α may be part of the mechanism involved in Tβ4-induced VEGF-α expression (Jo et al., 2010). With Tβ4 treatment, hMSCs showed increased HIF-1α and VEGF-α expression under both normoxia and hypoxia compared to non-treated hMSCs. Such corresponding trends

support the theory that HIF-1α may be related to VEGF-α expression, and that Tβ4 may operate through HIF-1α (Jo et al., 2010).

Despite beneficial conditions offered by hypoxia and Tβ4 pre-treatment, apoptotic protein caspase-3 expression did not show significant downregulation in these groups. Caspase-3 expression was found to increase during nutrient deprivation and, to a lesser extent, hypoxia. No differences in anti-apoptotic protein Bcl-2 expression were found with nutrient deprivation or hypoxia. However, with Tβ4 treatment, hMSCs showed increased Bcl-2 expression under normoxia. This may be explained as Bcl-2 is regulated by the PI3/Akt pathway, which is thought to be the route where Tβ4 acts to stimulate survival genes. Additionally, hypoxia activates the PI3 pathway, which upregulates Akt and promotes cell survival through either targeting apoptotic proteins or regulating cell metabolism under stress (Chacko et al., 2010). Consequently, stem cell research efforts are now applying hypoxic preconditioning as a strategy to induce anti-apoptotic genes for increased survival and engraftment (Chacko et al., 2010). Chacko et al. (2010) found the expression of proteins involved in pro-survival were upregulated at 24 hours of hypoxic preconditioning.

Although 48 hours of nutrient deprivation and 24 hours of hypoxia did not notably alter Cx43 expression, Tβ4-treated hMSCs showed increased Cx43 expression under hypoxia. Other labs report that significant levels of Cx43 were detected in cultures grown under hypoxic pre-conditioning for long periods (11 days), compared to hMSCs under normoxia (Grayson, Zhao, Bunnell, and Ma, 2007). Since this study measured Cx43 protein expression in hMSCs subjected to 24 hours of hypoxia, it is possible that prolonged hypoxic exposure is necessary to induce significant changes in Cx43 expression. Grayson et al. (2007) have proposed that, under

hypoxia, Cx43 may play a role in continued proliferation, and maintenance of the undifferentiated state of hMSCs.

The present data support evidence that nutrient deprivation is the predominant trigger of mass hMSC death in early cultures, as opposed to hypoxia, and Tβ4 pre-treatment may provide protection to nutrient-deprived hMSCs in early and expanded cultures. In both early and expanded hMSC cultures, Tβ4 elicits pro-survival effects regardless of oxygen tension, and in expanded hMSC cultures, Tβ4 may also promote angiogenesis. Given these findings, the utility for Tβ4 pre-treatment warrants further investigation. Such mediative effects could prove invaluable as an aid in stem cell survival upon engraftment, leading to increased efficiency in tissue repair.

A variety of technical factors may have contributed to data variability. One of the trial cultures was not 80% confluent after 2 weeks, and thus was grown for an another week until reaching confluency- this may have resulted in differences in protein expression. Variability in the hypoxic protocol may have contributed to hMSC cultures receiving slightly under or over 24 hours of hypoxia, as this step was not precisely timed. Also, hMSCs in the cell culture incubator may not have received identical oxygen and moisture circulation as cultures were commonly placed on different incubator shelves. Since the incubator was shared with other lab technicians, it also contained other cell cultures that may have altered the oxygen gradient, and frequent opening and closing of the incubator may have varied the temperature for cultures closer to the incubator door. Lastly, the heterogeneous nature of the hMSCs used presents challenges as hMSC differentiation occurs during cell expansion and may produce a particular lineage that dominates the culture. This heterogeneity may affect reproducibility of results

between repeat trials. Nonetheless, it is becoming increasingly accepted that diseased heart tissue may be best treated with transplantation of stem cells to increase local proliferation and healing. Although clinical trials using hMSCs to treat damaged tissue have been published, the survivability of stem cells in an ischemic microenvironment is low, and rigorous study is needed to enhance transplantation effectiveness.

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