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Modulatory Role of Severe Hypoxia in Orofacial Mesenchymal Stem Cell (OFMSC) Plasticity

Temitope T. Omolehinwa (nee Odukoya)

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Abstract

Osteoradionecrosis (ORN) of the jaw is a late and major complication of radiation therapy for head and neck cancers. The pathogenesis of ORN is yet to be fully clarified, which has put limitations on effective prevention and treatment of this condition. Radiation induced tissue hypoxia has been associated with development of ORN based on the previously proposed 'hypoxia-hypocellularhypovascular' theory of ORN pathophysiology. This indicates that radiation cycles during head and neck cancer therapy cause sustained hypoxia leading to death of bone cells. Therefore, we hypothesized that severe hypoxia tested at 0.1% oxygen tension alters plasticity of jaw mesenchymal stem cells, the essential osteoprogenitors vital for bone healing.

We assessed the effect of severe hypoxia (0.1% oxygen) on responsiveness of human orofacial mesenchymal stem cells (hOFMSCs) isolated from the jaw based on post-hypoxic survival and in vitro/in vivo multilineage differentiation of surviving cells. The effects of hypoxia inducible factor-1 alpha (HIF-1α) and Endoplasmic reticulum stress response (ERSR) signaling pathways on activation of vascular endothelial growth factor (VEGF) by OFMSCs in response to severe hypoxia were also assessed.

We found that OFMSCs succumbed to severe hypoxia because hypoxia depleted osteoprogenitor pools of OFMSCs. However residual surviving OFMSCs retained appreciable multilineage differentiation capacity. We also found that activation of both HIF-1α and ERSR signaling pathways in response to severe hypoxia coregulate downstream activation of VEGF to support recovery actions of residual OFMSCs from severe hypoxia. These results indicate that hypoxia plays a role in the pathogenesis of ORN and that resilience of OFMSCs to severe hypoxia can be further explored for tissue regeneration in irradiated jaw bone.

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MODULATORY ROLE OF SEVERE HYPOXIA IN OROFACIAL MESENCHYMAL STEM CELL (OFMSC) PLASTICITY

Temitope T. Omolehinwa (nee Odukoya) BDS

A DISSERTATION

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2017

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STEM CELL (OFMSC) PLASTICITY
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DEDICATION

I have made decisions in life and at the time the decisions were being made, I was not so sure what path of life those decisions would lead me to. Enrolling in the DScD program was one of those defining times in my life. I dedicate this thesis to the Lover of my soul, who has always guided my decision making and worked me through the process of achieving all I have so far in life.

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To our wonderful department- Oral Medicine Department, School of Dental Medicine, University of Pennsylvania, thank you all so much both faculty and staff for all your encouragement.

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ABSTRACT

MODULATORY ROLE OF HYPOXIA IN OROFACIAL MESENCHYMAL STEM CELL PLASTICITY

Temitope T. Omolehinwa (nee Odukoya)

Sunday O. Akintoye

Osteoradionecrosis (ORN) of the jaw is a late and major complication of radiation therapy for head and neck cancers. The pathogenesis of ORN is yet to be fully clarified, which has put limitations on effective prevention and treatment of this condition. Radiation induced tissue hypoxia has been associated with development of ORN based on the previously proposed 'hypoxia-hypocellular-hypovascular' theory of ORN pathophysiology. This indicates that radiation cycles during head and neck cancer therapy cause sustained hypoxia leading to death of bone cells. Therefore, we hypothesized that severe hypoxia tested at 0.1% oxygen tension alters plasticity of jaw mesenchymal stem cells, the essential osteoprogenitors vital for bone healing.

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endothelial growth factor (VEGF) by OFMSCs in response to severe hypoxia were also assessed.

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CHAPTER ONE

1.0   INTRODUCTION

Head and neck cancers rank 6th among the top ten common neoplasms (Allegra,E. 2012; Graziano,A. 2008). They have a prevalence of 3 - 5.5% in the United States of America (USA) (Dandekar, Mitali 2017; Argiris, A. 2003) and 10% worldwide (Argiris, A. 2003). Head and neck cancers can affect the hypopharynx, nasopharynx, oropharynx (which involves the lingual and palatine tonsils), larynx, oral cavity (including the tongue and salivary glands), the paranasal sinuses and the nasal cavity (Figure 1) (Marur, S. 2016; Argiris,A. 2008).

Although neoplasms in the head and neck can develop in the hard or soft tissues (Purgina,Bibianna 2017), carcinomas of squamous cell origin are the most prevalent (Argiris, A. 2008). They make up 90% of all head and neck cancers (Marur, Shanthi 2008) while the incidence of sarcomas in the body is less than 1% (Sturgis, E.M. 2003). Of this 1%, 5-15% occur in the head and neck region (Sturgis, E.M. 2003).

Several risk factors have been associated with squamous cell cancers of the head and neck. These include smoking, alcohol consumption, chewing of betel quid/areca nut, human papilloma virus (HPV), Epstein Barr virus (EBV), smokeless tobacco, cigar smoking and marijuana (Argiris, A. 2003; Marur, S. 2016).

Squamous cell carcinoma of the head and neck region has a predilection for Caucasian males of approximately 60 years of age. Due to the increasing smoking habits of females, the incidence in females is on the increase (Argiris, A. 2003; Argiris 2008). Interestingly,

the non-smoking population of Caucasian males is associated more with HPV-positive head and neck cancer.

About 50-60% of all cases of head and neck cancers are diagnosed at an advanced stage of the disease (Marur, S. 2016, Guizard, A.N. 2017), which has a high impact on the outcomes of therapy.
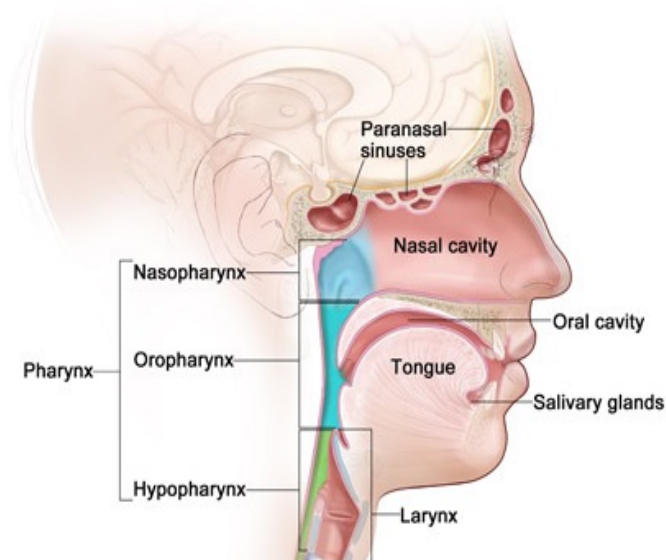
Figure 1. Sites of head and neck cancer

Management of head and neck cancers is based on several treatment approaches. Unfortunately, recurrence occurs in about 10% of patients due to the activity of cancer stem cells (CSC) (Allegra, E. 2012; Graziano, A. 2008). Early diagnosis with prompt commencement of treatment is vital to reducing morbidity and mortality.

1.1 TREATMENT OF HEAD AND NECK NEOPLASMS

There are several treatment modalities for management of head and neck cancers based on the site and stage of the primary tumor (Marur, S. 2016). Typically, early-stage head and neck cancers are treated with surgery or radiation therapy. Advanced stages however, are often treated with combination of both therapies. In addition to surgery and radiotherapy, other treatment methods include: chemotherapy (Marur, S. 2016; Marur, Shanthi 2008) and immunotherapy/biologic agents (Ye, X. 2017; Moreira, J. 2017).

Surgery

Surgical options include use of lasers, robotic surgery for resectable tumors or conventional open surgery (Marur, S. 2016). What is most important is that the tumor margin following surgery should be free of tumor tissues to avoid recurrence.

Radiation therapy

Radiation doses of 50-70 Gy is often used to treat head and neck cancers (Marur, S. 2016).

Radiation therapy can be used alone or in combination with other treatment modalities. About 0.9-35% of patients with head and neck cancer receive radiation therapy either as the main treatment or as an adjunct therapy (McCaul, James Anthony 2014; Cheriex, K.C. 2013). Conventional radiotherapy or intensity modulated radiation therapy are the main treatment options in this group of patients. For non-surgically resectable tumors, a combination of surgery and radiation and/or chemoradiotherapy is employed.

Chemotherapy and Immunotherapy (Systemic treatment)

Chemotherapeutic agents like cisplastin, 5-fluorouracil have been in use for several years to treat head and neck cancer. Combination of radiation with chemotherapy (chemoradiation) is highly effective in the management of locally advanced head and neck cancers, (Jeremic, Branislav 2000; Blasco, Michael A. 2017).

An Immunotherapeutic agent - cetuximab (a monoclonal antibody that targets epidermal growth factor receptors) has also been used successfully. This was the first chemotherapeutic agent to be approved by the Food and Drug Administration (FDA) after thirty (30) years of wide acceptance and use of cisplastin and 5-fluorouracil (Blasco, Michael A. 2017). Success has been reported with using cetuximab either as lone therapy or in combination with radiation therapy in treatment of patients with head and neck cancer (Bonner, J.A. 2006). Other immunotherapeutic medications used in head and neck cancer are monoclonal antibodies that target programmed cell death/apoptosis. Examples are pembrolizumab and nivolumab. They are especially used in the treatment of recalcitrant, recurring or metastatic head and neck cancers (Blasco, Michael A. 2017).

Stem cell transplant

Stem cell transplant is a more established treatment for hematopoietic cancers like leukemia and lymphoma. In recent years, stem cell transplant has been used successfully to treat non-hematopoietic diseases. Animal studies in pigs have been carried out, using an osteoradionecrosis model. Successful outcomes have been reported with transplantation of autologous bone marrow stem cells into osteoradionecrotic bony defects in the jaw (mandible) with noted vascular and bony regeneration (Xu, Junji 2012).

In the above study, harvested and expanded iliac crest MSCs were transplanted to the jaw of the pig. Another clinical study in Spain, has also reported similar success in a clinical trial. A cocktail of autologous mesenchymal stem cell, platelet-rich plasma, non-platelet-rich plasma and tricalcium phosphate hydroxyapatite scaffold was transplanted successfully into a bony defect in a patient with advanced osteoradionecrosis. Interestingly stem cells in this study was also from non -jaw site (Mendonca, Jose J. 2010). A major concern of stem cell therapy is graft rejection and graft versus host disease.

1.2   COMPLICATIONS OF THERAPY

Despite the successful outcomes of these therapies, patients with head and neck cancer still experience mild to severe treatment complications some of which can be debilitating. Some of these complications occur early, while others present late. Patients presenting with co-morbid conditions can also further complicate these management complications Early complications of head and neck cancer therapy include dysgeusia, chemotherapy and radiation-induced mucositis (which largely affects the ability of the patient to have proper nutrition) and stomatitis; radiation induced xerostomia and trismus. The xerostomia can be transient when the damage to the salivary gland tissue is transient or severe due to irreversible damage to the salivary glands. The common late complication of head and neck cancer therapy are radiation induced caries and osteoradionecrosis (Omolehinwa, T.T. 2016; Rayatt, S. 2007). However, hypothyroidism can result from radiation damage to the thyroid gland and dysphagia can result from damage to the pharyngeal constrictor muscles.

1.4   OSTEORADIONECROSIS

Osteoradionecrosis (ORN) is defined as exposed non-healing necrotic bone, for at least three (3) months in a previously irradiated field, usually with surrounding compromised soft tissue.(Rayatt, S. 2007), in the absence of a recurring, residual or new primary tumor (Madrid,C. 2010; McCaul, James Anthony 2014).

Osteoradionecrosis can occur in any bone in the irradiation field. The incidence is reported to be higher in the jaw bone, with an incidence of 10% among head and neck cancer patients and less than 1% in patients with cancer in non-head and neck sites (Delanian, Sylvie 2011). ORN of the jaws is a major, usually late complication of head and neck cancer radiotherapy (Teng,M.S. 2005; Chrcanovic,B.R. 2010). It occurs in 1-37% of patients (Gal, T.J. 2000; Rayatt, S. 2007; Sciubba, J.J. 2006).

A patient receiving radiation dose greater than 50Gy is at a high risk of developing osteoradionecrosis (Rayatt, S. 2007). Other risk factors for osteoradionecrosis in a head and neck cancer patient receiving radiotherapy include poor oral hygiene, pulpal infections, periodontal diseases, old age with co-morbid conditions such as diabetes, and procedures such as dental extractions and other intraoral surgical procedures carried out before and after radiation (Madrid,C. 2010; Rayatt, S. 2007).

1.4.1 Pathogenesis of osteoradionecrosis

Several theories have been proposed regarding ORN pathogenesis. A widely-accepted theory is development of radiation-induced hypoxic-hypocellular-hypovascular environment (3H) in the irradiated bone leading to ORN. (Delanian, S. 2004; Marx, Robert

E. 1983). The 3H theory is based on the premise that radiation damages bone cells and blood vessels to induce a hypoxic environment within the bone. This led to the use of hyperbaric oxygen (HBO) therapy for the management of ORN (Marx, Robert E. 1983). However, HBO therapy has unpredictable clinical outcomes (Shaw, Richard J. 2011). Another recent theory is the fibroatrophic theory, which attributes osteoradionecrosis to the initiation and dysregulation of fibroblastic processes. The result of this is tissue atrophy and subsequently necrosis. A combination of endothelial dysfunction, inflammation, microvascular thrombosis and production of free radical from reactive oxygen species are implicated (Madrid, C. 2010). The treatment of choice that was proposed based on this theory is 800 – 1200 mg daily pentoxifylline and 1000U of α-tocopherol (McCaul, James Anthony 2014). Pentoxifylline is a vascular dilator which increases endothelial flexibility and increased blood flow, leading to increased vascularization. Pentoxifylline also has anti-tumor necrosis factor alpha (anti-TNFα) effect which decreases the inflammatory cytokine cascade at the irradiated site. Alpha tocopherol also known as Vitamin E, is an antioxidant. It scavenges free oxygen radicals therefore mopping up the reactive oxygen species formed by vascular damage and hypoxia. A combination of Pentoxifylline, alpha tocopherol and clodronate (an aminobisphosphonate drug) has been shown to be effective in resolving soft tissue fibrosis induced by radiation damage and effects of osteoradionecrosis. (Delanian, Sylvie 2011).

1.4.2 Role of stem cells in the pathogenesis of ORN

Based on Robert Marx's 3H theory (Marx, Robert E. 1983), radiation causes vascular damage (hypovascularity) which translates to reduced oxygen tensions in the affected tissues. The combination of hypovascularity and hypoxia activate release of reactive oxygen species causing more vascular damage and increased hypoxia. This continuous cycle of hypoxia and hypovascularity cause cellular death (hypocellularity) (Figure 2). The undifferentiated progenitor cells are believed to be affected by this radiation and hypoxic insult.
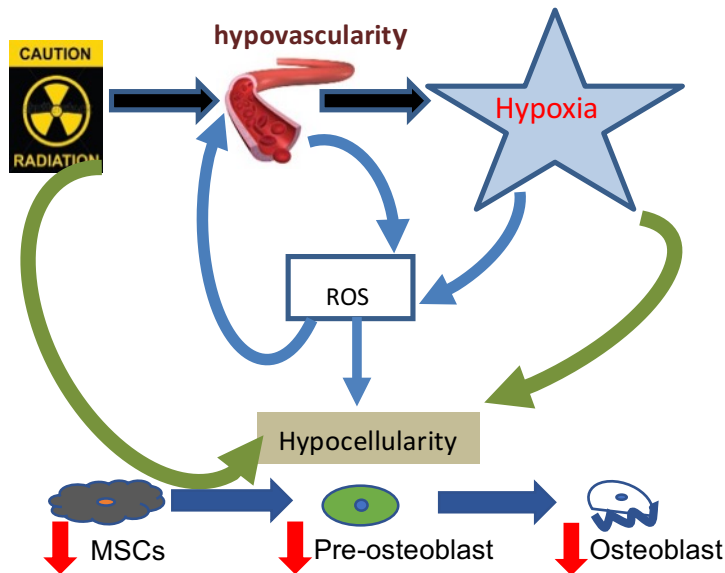
Figure 2. Relationship between radiation, hypovascularity, hypoxia and hypocellularity

1.5 DIAGNOSIS AND STAGING OF JAW OSTEORADIONECROSIS

Jaw osteoradionecrosis is diagnosed based on clinical and radiographic presentation in a patient who presents with a previous history of head and neck radiation therapy. Patient

may be asymptomatic with only findings on radiographic examination or other imaging studies. Patients can also present with pain, infections, orocutaneous fistula, bony sequestrum, compromised soft tissue, trismus and pathologic fractures.

Stage	Length of affected bone/ Associated structures (damaged/exposed)	Presence/absence of symptoms	Treatment
1	<2.5 cm	Asymptomatic	Improve oral hygiene and medication only.
2	>2.5 cm	Asymptomatic with pathologic fracture and/or inferior alveolar nerve (IAN) involvement	Medication treatment only; except for presence of dental sepsis and loose/necrotic bone.
3	>2.5 cm	Symptomatic. No other features of bone necrosis, but presence of persistent symptoms despite medication treatment	Debridement of loose/necrotic bone. Local pedicle flap
4	> 2.5cm	Symptomatic. Pathologic fracture with IAN involvement and/or orocutaneous fistula	Reconstruction with free flap, if patient's overall health allows

Table 1. Adapted from Omolehinwa,T.T. 2016; Lyons, Andrew 2014

Different attempts have been made to classify osteoradionecrosis. One of the more recent classifications is based on the extent of the necrotic bone damage, presence or absent of symptoms and the treatment modality (Table 1) (Lyons, Andrew 2014; Omolehinwa,Temitope T. 2016). Stage 1 of this classification can be interpreted to include intact soft tissue clinically, but with radiographic findings of damaged bone.

1.6 MANAGEMENT OF OSTEORADIONECROSIS

Management range from improving oral hygiene, symptom control with use of analgesics and antibiotics, debriding, sequestrectomy, radical resection, hyperbaric oxygen and pharmacologic agents e.g. Pentoxifylline, alpha tocopherol and clodronate.

1.7 STEM CELLS

Stem cells are cells that have the potential to proliferate, and ability to self-renew and differentiate into multiple cell types. Stem cells can be embryonic, fetal or adult/post-natal in origin (Table 2) (Shanti, Rabie M. 2007).

Types of stem cells

Embryonic stem cells are pluripotent in nature i.e. they are capable of infinitely proliferating. This includes proliferation to form trophoblasts, ectoderm, mesoderm and endoderm of epithelial origin. They can also remain undifferentiated by not committing to any specific lineage (Ulrich, Henning 2017; Shanti, Rabie M. 2007; Thomson,J A. 1998). Embryonic stem cells arise from the inner cell mass of a developing blastocyst. Although embryonic stem cells are ideal for tissue regeneration, their use has been limited by ethical issues (Lo, B. 2009).

Fetal stem cells are capable of extensive proliferation and differentiation and can be isolated from developing fetal organs (Olivier, Valerie 2004). They are also pluripotent in nature (Lo, B. 2009).

Adult stem cells on the other hand are multipotent. They can differentiate into many but still limited cell types. Post-natal stem cells include hematopoietic (from endoderm), mesenchymal (from mesoderm) and epithelial (from ectoderm) stem cells (Table 3) (Shanti, Rabie M. 2007). Bone marrow MSCs are post-natal stem cells isolated from the bone marrow. They have the multilineage properties of adult MSCs and can readily commit to the osteogenic lineage (Yosupov, N. 2017).

Stem Cell	Source	Characteristic
Embryonic	Inner mass blastocyst	Pluripotent, unlimited proliferation
Fetal	Developing organ (e.g. aborted fetus)	Pluripotent, unlimited proliferation and differentiation
Adult/Post- natal	From mesoderm, endodermal and ectodermal origins	Multipotent
Induced Pluripotent stem cell	Re-engineered Adult MSC	Pluripotent

 Table 2. Types of stem cells Omolehinwa 2017

Induced pluripotent stem cells are reengineered somatic postnatal stem cells. They have been reprogrammed with genetic materials from viruses or plasmids vectors to regain the characteristics of embryonic stem cells (Ulrich, Henning 2017; Lo, B. 2009).

Stem Cell	Source Tissue (Location)	Differentiation Potential
Hematopoietic	Bone marrow	Blood, endothelial, myoblast, and hepatic (oval) cells
Epithelial	Epidermis, gut	All cells of epithelium crypts, and all cells of epidermal cells
Mesenchymal	Bone marrow, muscle, trabecular bone, adipose tissue, dermis, periosteum, blood, synovial membrane, periodontal ligament, and deciduous teeth	Adipocyte, chondrocyte, myoblast, osteoblast, cardiomyocyte, hepatocytes, neuron, astrocyte, endothelial, fibroblast, and stromal cells

Table 3. Adult stem cells adapted from Shanti, Rabie M. 2007

Another unique group of stem cells are cancer stem cells. They are stem cells with genetic aberrations, leading to continuous cell proliferation with no signals to turn off the growth of the cells. This leads to tumorigenesis.

This literature review focuses on mesenchymal stem cells (MSCs). This is because MSCs are progenitors of osteoblasts and are implicated in osteoradionecrosis.

1.8   MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are unique population of postnatal (adult) stem cells. They have the potential to self-renew and differentiate into various cell lineages (multipotency) and tissues that include bone, cartilage, muscle and fat (Shanti, Rabie M. 2007; Oh,M. 2015; Sacchetti, Benedetto 2007). MSCs were first isolated from the bone marrow and described by Friendenstein (Friedenstein, A.J. 1976). Apart from the bone marrow, MSCs have also been successfully isolated from the brain, liver, umbilical cord, chorionic villi, Wharton's jelly, fetal tissue, placenta, menstrual blood, dental pulp tissue, skin, muscle, heart, gingival tissue and bone marrow. (Zhang, W.X. October 2006; Gronthos, M. 2000; Ejtehadifar, M. 2015; Madrigal, M. 2014).

Since bone marrow is the most common donor site for isolation of MSCs, (Minguell, J.J. 2001; Levy, Débora), this project will focus on bone marrow MSCs and potential application for bone repair/regeneration in osteoradionecrosis of the jaw.

Properties of MSCs

Based on the position statement the International Society for Cellular therapy in 2006 (Dominici, M. 2006), the properties of MSCs include:

1. An ability to express specific surface antigens. These surface antigens include CD105, CD73, CD44, CD13 CD90. It is however important to note that MSCs do not express the following markers CD45, CD 79α, CD19, CD14, CD34, CD 11b and HLA-DR surface molecules (Table 4) (Ejtehadifar, M. 2015; Dominici,M. 2006).

2. Ability to adhere to plastic surfaces when expanded *in vitro*, while ensuring adherence to standard culture protocols.

3. Must show multipotent differentiation ability when expanded in vitro

4. Should be capable of *ex vivo* expansion (Dominici, M. 2006).

MSCs are also known to have immunomodulatory properties. They exert this effect by inhibiting the proliferation of T-cell (Lotfinegad, P. 2014). This makes MSCs useful in management of autoimmune diseases that require modification of the immune system. An example is Graft-versus host disease (GVHD) especially acute GVHD (Le Blanc, Katarina 2005).

Characteristics of Mesenchymal Stem Cells			
1	Adherence to plastic in standard culture conditions		
2	Phenotype	Positive (≥95% +)	Negative (≤2% +)
		CD105	CD45
		CD73	CD34
		CD90	CD14 or CD11b
			CD79α or CD19
			HLA-DR
3	*In vitro* differentiation: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of *in vitro* cell culture)		

Table 4. Characteristics of MSCs based of surface markers (Dominici,M. 2006)

The unique site-specific properties of bone marrow mesenchymal stem cells

Previous studies have shown that bone marrow MSCs have unique phenotypic and behavioral properties depending on their site of harvest. This was especially noted in

MSCs of the orofacial region (especially jaw bones). It was discovered that orofacial MSCs have features that differentiate them from MSCs of non-jaw sites. These properties include a higher proliferative capability and delayed senescence (Figure 3), more radiosensitivity and higher capacity for *in vitro* osteogenic and adipogenic differentiation (Akintoye,S.O. 2006; Damek-Poprawa,Monika 2010). These differences have been noted in MSCs of humans, porcine, canine, mice and rat (Akintoye,S.O. 2006; Bugueño, Juan 2017; Yamaza, T. 2011)
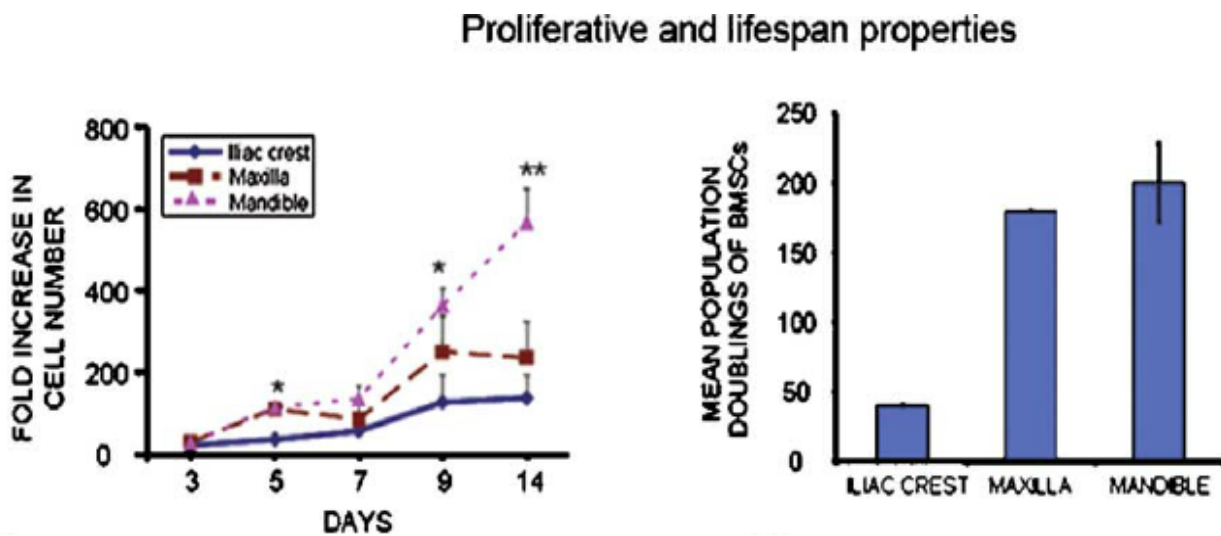
Figure 3. Skeletal site-specific proliferative capacity of MSCs.

OFMSCs are significantly more proliferative than non-oral MSCs based on proliferation and population doubling properties (Akintoye,S.O. 2006).

The site- specific properties of the jaw MSCs are possibly associated with jaw-specific disorders like cherubism, medication related osteonecrosis of the jaw (MRONJ), craniofacial fibrous dysplasia and hyperparathyroid jaw tumor syndrome (Akintoye,S.O.

2006). Additionally, the high proliferative properties of orofacial MSCs can be associated with a disproportionate sensitivity to hypoxia and consequent jaw susceptibility to ORN.

Plasticity of mesenchymal stem cells

The ability to differentiate in a multilineage fashion, regardless of the site/tissue of origin is referred to as plasticity (Zhang, W. October 2006; Brazelton, Timothy R. 2000). This property of the adult stem cell makes it particularly clinically relevant in the treatment of several medical conditions, including but not limited to diseases of the orofacial region such as osteonecrosis of the jaw, radiation induced salivary gland damage and Sjögren's syndrome (Catacchio,I. 2013).

Among the body tissues, the bone marrow MSCs have the highest plasticity, with multiple lineage differentiation potential (Ejtehadifar, M. 2015) and a higher prospect for osteogenic differentiation (Cicione, C. 2013; Muraglia, A. 2000).

The plasticity of the bone marrow MSCs is associated with its oxygen gradient, a hypoxic state being required to keep the cell in a quiescent or inactive state (D'Ippolito,G. 2006).

A lot of studies have been carried out to study the effect of hypoxia on cell plasticity (Jiang et al. 2015; D'Ippolito et al. 2006; Cicione et al. 2013; Grayson et al. 2006). However, the effects of hypoxia on differentiation capabilities of these cells are still unclear (Cicione et al. 2013; Grayson et al. 2006; Holzwarth et al. 2010). The exact knowledge of the mechanisms of action of hypoxia on cell plasticity is still under study.

Mesenchymal Stem cell niche

Adult stem cells reside in a specified area within the tissues/organ. This distinct area is called the stem cell niche/microenvironment. MSC niche are found in perivascular areas, in close proximity to blood vessels. (Farrington-Rock,C. 2004). These cells are spindle shaped or fibroblast like (Friedenstein, A.J. 1976; Marquez-Curtis,Leah A. 2015). Within the niche, MSCs are quiescent until induced to differentiate and commit to a specific lineage by events such as irradiation, bone loss and other types of insults (Figure 4).
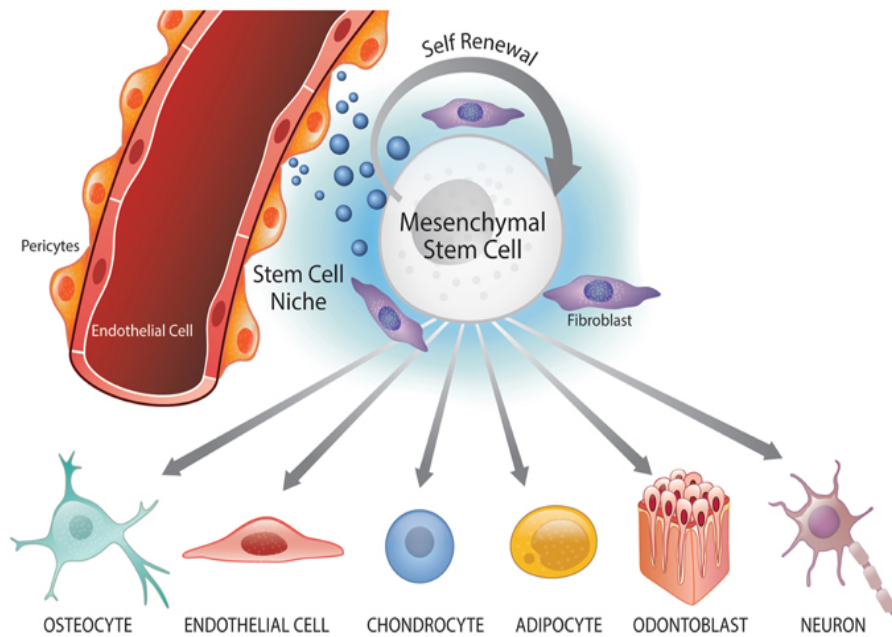
Figure 4. The Stem cell niche (Oh, M. 2015)

Isolation of Bone Marrow Mesenchymal stem cells

Although MSCs are only 0.001 – 0.01% of cells within the bone marrow compartment (Marquez-Curtis,Leah A. 2015), significant amount of MSCs can still be isolated by plastic adherence in a plastic culture dish. It is not practicable to obtain pure MSCs by plastic adherence because of the close proximity of MSC niche with the hematopoietic niche and other stem cell niches. Therefore, MSCs isolated by plastic adherence usually display heterogeneous properties. Each progenitor MSCs proliferates into a clone of 50 or more heterogeneous fibroblast-like cells referred to as colony-forming units fibroblastic (CFU-F). The CFU-F ability of MSC is a mark of stemness and decreases with advancing age of the donor.

The expanded cells must secondly be tested for MSC specific surface antigens as noted in Table 4 above, by cell sorting (usually Fluorescence activated cell sorting –FACS).

Human primary bone marrow mesenchymal stem progenitor cells, in addition to the above stated markers (Table 4), have some specific markers exclusive to this group of cells. They include STRO-1, CD 146 (also known as MUC 18), SUSD2, CD106, FZD9, CD271 and LEPR (Li, Hongzhe 2016).

The media in which the MSCs are cultured is also important. Growth medium supplemented with Fetal Bovine Serum (FBS) are essential components of the culture medium.

Cryopreservation, Storage and revival of mesenchymal stem cells

MSCs have application in tissue engineering. It is important that MSCs can be expanded ex vivo and cryopreserved for long term storage. The use of appropriate freezing medium and long-term storage in liquid nitrogen chambers have been effective in maintaining viability of MSCs. This storage approach slows down MSC metabolic activity while still maintaining viability (Marquez-Curtis,Leah A. 2015).

1.9   CANCER STEM CELLS

Cancer stem cells (CSCs) of the head and neck tumors were first identified in 2007 (Prince,M.E. 2007). They are believed to be unique/rare stem cells that do not commit to any lineage, but continually self-renew in a hap-hazard manner, resulting in tumorigenesis (Reya, T. 2001).

Like all other stem cells, CSCs exist in a niche within the cancer and can be distinguished phenotypically by surface markers such as CD44, CD22 and CD133 (Allegra, E. 2012). CD44 positive CSCs are especially known to promote cancer resistance to therapy, this subset of CSCs is also responsible for the self-renewing property that support the growth of solid tumors (Okamoto,A. 2009)

1.10   HYPOXIA

The fetus develops a respiratory and circulatory system early in-utero because oxygen is vital for survival. The inspired oxygen can be effectively circulated to every tissue in the body via hemoglobin transport of oxygen in the red blood cells (Semenza, Gregg L. 1998). A lack of adequate supply of oxygen is termed hypoxia and can be defined as a shortage in the amount of oxygen reaching the tissues. This results in reduced tissue perfusion.

Hypoxia typically halts the cell cycle in mammalian cells, with resultant cell death (Basciano, L. 2011). The body cells and tissues attempts to counteract changes in oxygen tension by activating survival and cellular stress pathways (Buravkova,L.B. 2014).

Some cells in the body however, have adapted to a hypoxic microenvironment. An example is MSCs that have developed the ability to maintain regular cellular activities in spite of the hypoxic environment (Buravkova, L.B. 2014).

One of the major cellular stress/survival pathways activated in response to hypoxia is the hypoxia inducible factor 1 (HIF-1) pathway. HIF-1 pathway is essential for the maintenance of oxygen homeostasis. This pathway acts by reducing tissue oxygen demand while increasing oxygen delivery via vessel dilatation, angiogenesis and erythropoiesis (Semenza, Gregg L. 1998). The stability of HIF-1 is crucial in its response to tissue hypoxia (Weng and Semenza (2003); Buravkova,L.B. 2014).

It has been shown in HeLa S3 cell lines that HIF-1 has a short half-life of one minute and can be detected in the nucleus within 2 minutes of exposure to 0.02 – 5% oxygen concentration. HIF-1 was also shown to reach a maximum level of expression after 1 hour of continuous exposure to hypoxia (Jewell, U.R. 2001).

HIF isoforms

Existing HIF isoforms include HIF-1α, HIF-2α, HIF-3α and HIF-1β. HIF- 1α and HIF-2 α are closely related and bind to hypoxic responsive elements (HRE) that activate transcriptional target genes. Nonetheless, the target genes of both isoforms are different. HIF-2α for example has been shown downstream to activate erythropoietin (EPO) a gene important for erythropoiesis. This is the case especially in hepatocytes. On the other

hand, HIF- 3α has antagonizing actions against HRE (Ratcliffe, P.J. 2007). HIF-1β is essentially involved under hypoxia with HIF-1α and HIF-2α binding to it, under hypoxic conditions.

Hypoxia and Mesenchymal stem cells

Hypoxia in tissues could be physiologic or pathologic in origin. Whether physiologic or pathologic, hypoxia causes modifications in cellular responses such as angiogenesis, red blood cell formation, as well as cell proliferation and differentiation (Frolova,O. 2012;).

In MSCs, oxygen gradient is maintained between 1% (Xu,L. 2014; Holzwarth et al. 2010) and 12.5% (Heppenstall, Grislis, and Hunt 1975, Ito, A. 2015). MSCs have adapted to this hypoxic state by stabilizing HIF-1α. However, activation of the HIF-1 pathway and translocation of HIF-1α subunit into the nucleus occurs after exposure of MSCs to hypoxia for a duration that can range from 6 – 72 hours (Buravkova,L.B. 2014). Under physiologic hypoxia, MSCs have a higher proliferation capacity and colony forming efficiency. (Buravkova,L.B. 2014; Basciano, L. 2011).

Interestingly, when *ex vivo* expanded MSCs are subjected to physiologic hypoxic conditions (1-12% O_2), they are able to retain their stem cell properties with increased self-renewal ability and delayed senescence compared to those cultured under normoxic conditions. (Ito, A. 2015}

The effect of this severe hypoxic state on the ability of the MSC to differentiate from one lineage to the other has not been fully clarified. A study by Ito et al observed that MSCs are likely to die by apoptosis when exposed to oxygen concentration as low as 0.4% (Ito, A. 2015, Xu,L. 2014, Ceradini,D.J. 2004). However, Jiang C. et al in 2015 reported that

osteogenic differentiation was severely impaired in severe hypoxic state using 0.2% oxygen, while adipogenic differentiation was enhanced. (Jiang,C.M. 2015). Both human MSCs (hMSCs) and rat MSCs have demonstrated increased osteogenesis and suppressed adipogenesis at 2% and 1% oxygen concentrations (Xu,L. 2014; Wagegg, M. 2012; Lennon, D.P. 2001).

Hypoxia at 1% O_2 tension) was reported to diminish chondrogenesis in MSCs obtained from the iliac crest of patients undergoing hip replacement (Cicione,C. 2013) while a lot more studies reported an increase in chondrogenesis under hypoxia (Kanichai, M. 2008). This was supported by elevated expression of SOX9, SOX5, AGC1, COL2A1 and other collagen types, SOX6, choindrointin-4 sulphate, aggrecan and glycosaminoglycan (Buravkova,L.B. 2013; López, Y. 2013; Buravkova,L.B. 2014).

Early progenitor MSCs have the innate property of differentiating in a multilineage fashion (Catacchio et al 2013). Based on preliminary data from our study, severe hypoxia apparently modulates the survival of early MSC progenitors. However, it is unclear if early MSC progenitors can recover from the stress of severe hypoxia and commit to lineage differentiation. Other previous studies suggest that severe hypoxia alters multilineage differentiation pattern of MSCs from non-oral skeletal sites (Cicione et al 2010) but it is unclear if this responsiveness to hypoxia is skeletal site-dependent.

Figure 5. Heterodimeric structure of HIF-1α (Wu, D. 2015)

HIF-1α

HIF-1α is a transcriptional factor that encodes for the HIF-1α gene and is responsible for cellular response to hypoxia (Kanichai et al 2008). It is accepted as the hallmark of hypoxia (Oudina et al 2011;Schipani 2005). It has a heterodimeric structure, containing two alpha-helix structures, which are connected by a loop (Figure 5). HIF-1 was first named in 1992 by Semenza and Wang, when they noted a certain protein that was bound to hypoxia only in Hep3B human hepatoma cells (Semenza, G.L. 1992).

HIF- 1α is present in all tissues (Pal Singh,R. 2012) and transiently present in the cell cytoplasm under normoxic conditions. HIF-1α is unstable under normoxia as it is quickly degraded. This is achieved by hydroxylation of proline residues and ubiquitination by the interaction of the hydroxylation process with von Hippel Lindau tumor suppressor protein (Figure 6).
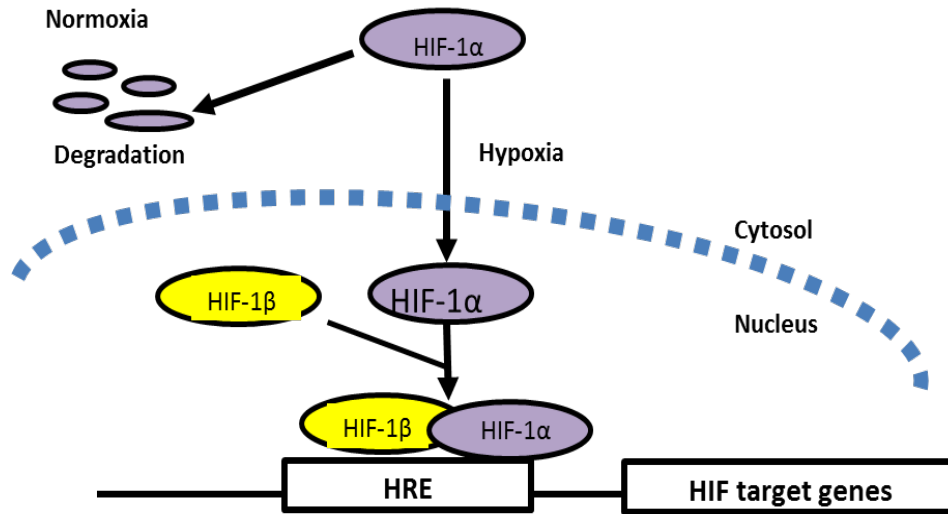
Figure 6. Schematic diagram of HIF-1 pathway (Omolehinwa 2017)

However, under hypoxic conditions, HIF-1α is more stable and translocates into the nucleus. It binds with HIF-1β in the nucleus and the coupling of HIF-1α and HIF-1 β binds to the hypoxic responsive element at the DNA binding site. This results in the activation of downstream target genes like VEGF which is responsible for angiogenesis (Weidemann, A. 2008).

The HIF-1 signaling pathway is activated in regions of physiologic hypoxia (1-1.8%) (Xu, L. 2014) such as during chondrocyte differentiation at the embryonic growth plate (Schipani 2005). HIF-1α plays a role in maintaining MSCs in an undifferentiated quiescent state (Park, I.H. 2013, Holzwarth,C. 2010), while also maintaining MSC plasticity (D'Ippolito,G. 2006). The role of HIF-1α in cancer biology is well defined, especially with hypoxia in tumor cells being responsible for resistance of affected tissues to radiation therapy (Meijer, Tineke W.H. 2012)

However, it is unclear if this mechanism is dysregulated under severe hypoxic conditions beyond physiologic hypoxia.

Endoplasmic reticulum stress response

In addition to the HIF-pathway, the endoplasmic reticulum stress response (ERSR) is also activated under hypoxic conditions. It is unclear whether HIF and ERSR pathways co-regulate MSC responsiveness to hypoxia. The rough endoplasmic reticulum is an intracellular organelle responsible for protein synthesis. Protein folding also occurs in the ER, followed by transport of the folded proteins to the Golgi apparatus. This process of folding and transport of protein occurs in the presence of chaperone proteins, which ensures that the transported proteins are properly folded. An example of this chaperone protein is BiP (Binding immunoglobulin Protein). Under some stressful situations to the endoplasmic reticulum, like the presence of viral infections, disruption of normal redox reactions, glucose and calcium dysregulation or even the presence of hypoxia; there is improper protein folding, which is called unfolded protein response or ER stress response (ERSR). Unfolded protein response is regulated by three signaling pathways: Protein kinase RNA-like ER kinase (PERK), Activator transcription factor 6 (ATF6); which both under physiologic conditions of folded proteins, bind to Bip and keep Bip in an inactive form. Bip however becomes activated when many unfolded proteins are present in the ER, with resultant dissociation of Bip from ATF6, PERK, and IRE1α. Under ER stress, PERK also prevents mRNA translation which prevents the influx of newly produced proteins into the already stressed ER. This is accomplished by the phosphorylation of

eukaryotic translation Initiation factor 2 (eIF2α). eIF2α activates activator transcription factor 4 (ATF4), which downstream results in activation of VEGF.

The third signaling pathway, Inositol-requiring protein 1α (IRE1α), binds directly to unfolded proteins. In a situation where the ER stress response is prolonged with a continuous burden of unfolded proteins, cellular dysfunction occurs with resultant cell death/apoptosis. IRE1 α is believed to be responsible for activation of apoptosis pathway (Sano,Renata 2013)

Endoplasmic Reticulum Stress response and Hypoxia in MSCs

Since VEGF is a downstream effector of both HIF and ERSR pathways, it is still unclear if both pathways co-regulate VEGF-dependent angiogenesis and recovery from hypoxia. A study by Li Z in 2010, showed that hypoxia and serum deprivation of MSCs induced cell death and activated ER stress response (Li, Zongwei 2010). However, there is still paucity of information on the role of HIF-1α and ERSR pathways in recovery of cells from hypoxic insult.

CHAPTER TWO

2.0   RESEARCH AIMS

2.1   PURPOSES

The management, especially prevention of osteoradionecrosis till date has not been figured out in its entirety.

The purpose of this study is to determine whether severe hypoxia suppresses undifferentiated orofacial mesenchymal stem cell (OFMSC) population and their multipotent differentiation capacities.   We will also assess the modulatory roles of hypoxia-inducible factor (HIF)-1α and Endoplasmic Reticulum stress response (ERSR) signaling in the responsiveness of orofacial MSCs subjected to severe hypoxia. The following specific aims will be embarked upon:

2.2   SPECIFIC AIMS

Aim 1: To determine whether severe hypoxia depletes undifferentiated jaw mesenchymal stem cells (OFMSCs)

Hypothesis: Severe hypoxia suppresses OFMSCs

Rationale: MSCs characteristics are phenotypically and functionally skeletal site-specific

Approach: MSCs from mandible/maxilla (OFMSCs) were subjected to severe hypoxia (0.1% O_2) and characterized based on cell surface markers of stemness and survival. Iliac crest (ICMSCs) was used as control.

Aim 2: To determine whether severe hypoxia modulates OFMSC multilineage differentiation

Hypothesis: Severe hypoxia dysregulates OFMSC multi lineage differentiation

Rationale: Post-hypoxic repopulation of early progenitor cells and multilineage differentiation are essential for bone recovery and healing

Approach: Multilineage differentiation capacities of OFMSCs subjected to severe hypoxia was assessed based on osteogenesis and adipogenesis, using immunological and molecular approaches.

Aim 3: To determine whether OFMSC susceptibility to severe hypoxia is dependent on HIF 1 and endoplasmic reticulum stress response (ESRS) signaling pathways

Hypothesis: Altered HIF and ERSR signaling undermines survival of OFMSC following severe hypoxia

Rationale: HIF 1 alpha and ESRS signaling regulate cellular response to hypoxia and cellular stress response respectively.

Approach: The activities of HIF-1α and ERSR via its PERK arm on OFMSCs subjected to hypoxia were assessed. Pharmacological and molecular approaches were used to upregulate or downregulate both HIF 1 alpha and ERSR pathways to assess their downstream effects on VEGF activation.

CHAPTER THREE

3.1 EXPERIMENTAL OUTLINE


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        ┌─────────────────────────────────────┐
        │  Orofacial Mesenchymal stem cells   │
        └─────────────────────────────────────┘
           ↙                            ↘
┌──────────────────────┐      ┌──────────────────────┐
│ Severe hypoxia (0.1% │      │  Normoxia (21% O₂)   │
│         O₂)          │      │                      │
└──────────────────────┘      └──────────────────────┘
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┌──────────────────┐   ┌──────────────────┐   ┌──────────────────────┐
│   Aim 1:   │   │   Aim 2:   │   │   Aim 3:   │
│   │   │   │   │   │
│ Survival post-   │   │ Multilineage   │   │ Modulatory role of   │
│ severe hypoxia   │   │ differentiation │   │ HIF-1α and ERSR in   │
│   │   │ after severe   │   │ response to severe   │
│   │   │ hypoxia   │   │ hypoxia   │
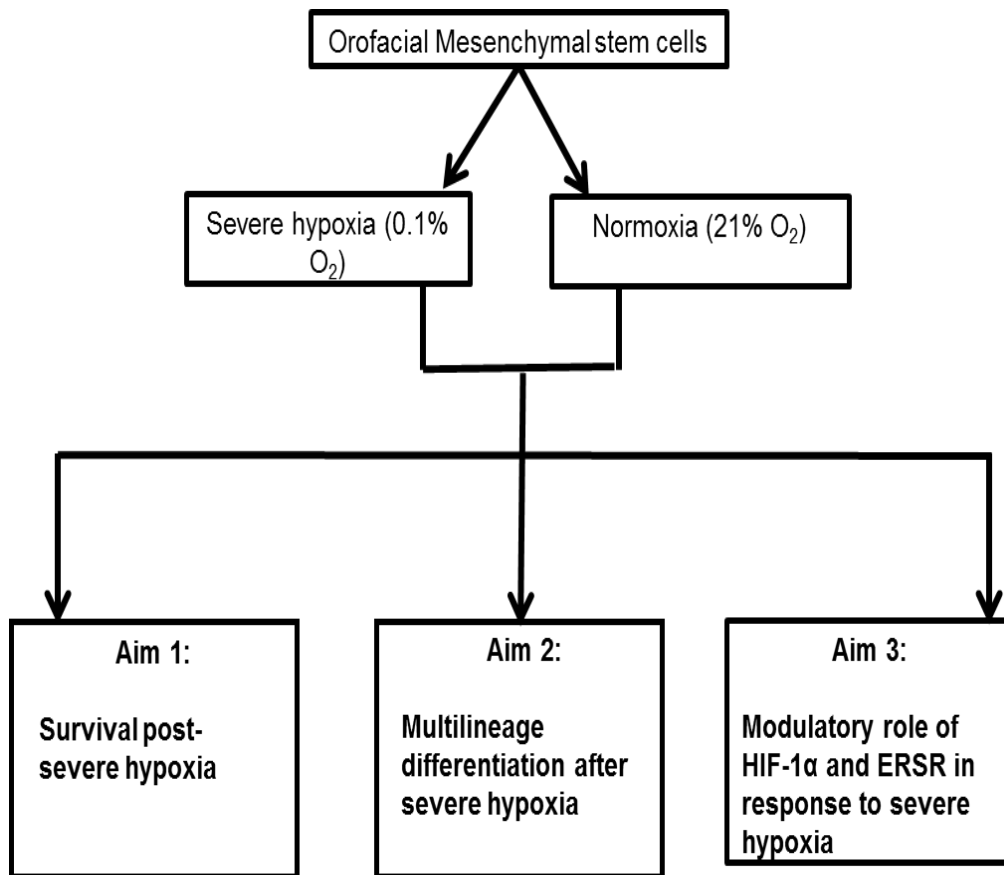└──────────────────┘   └──────────────────┘   └──────────────────────┘

Figure 7. Study outline

3.2 METHODS:

Sample and Cell culture

Human mesenchymal stem cells (MSCs) from the jaw (maxilla or mandible) of three healthy normal volunteers were selected from previously isolated and cryopreserved MSCs in the laboratory of Dr. Akintoye. The maxilla/mandible MSCs termed orofacial MSCs (OFMSCs) were comparatively analyzed with Iliac crest MSCs (ICMSCs) of same individuals. The ICMSCs served as non-oral control MSCs.

The primary hMSCs were further expanded in growth medium containing α-modified Minimum Essential Medium (α-MEM, Life Technologies, Grand Island NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 100 U/ml penicillin, 100 mg/ml streptomycin sulfate and 2 mM glutamine (Gibco, Life technologies, NY). Expanded cells were maintained in a humidified atmosphere containing 5% CO_2 and at a temperature of 37^0C. Twenty-four (24) hours after onset of expansion, MSCs were checked for plastic adherence, while non-adherent cells were removed by suction. After a brief rinse of the MSCs with phosphate- buffered solution (PBS), the growth medium was replenished. This procedure was repeated every 4 days until 80% cell confluence was attained. At this point, cells were detached from flask with 0.5% trypsin (Invitrogen-Life Technologies, Carisberg CA) and re-plated for the rest of the experiments as outlined below. Early cell passages (between P2 to P4) were used for all experiments.

Cryopreservation and thawing of MSCs

Excess MSCs not immediately used for an experiment were saved in freezing medium, made up of growth medium and dimethylsulfoxide (Fisher Scientific, USA) at 1×10^6 cells/ml and cryopreserved in liquid nitrogen for long term storage or $-80^O F$ for short term storage.

Induction and maintenance of severe hypoxic conditions

Two Billups-Rothenberg modular hypoxic chamber (California, USA) were used to maintain severe hypoxia at 2% and 0.1% oxygen respectively for an initial determination of appropriate experimental parameters. The chambers were initially flushed with 2-5 mmHg pressure of either 2% or 0.1% O_2, to eliminate ambient air. This was followed by preconditioning of a serum-free growth medium by closing the chamber for 4 minutes at a pressure of 4mmHg. Thereafter 10% FBS was added to the pre-conditioned medium before being introduced to the MSCs hypoxic treatment group. The hypoxic chambers were again flushed with 25mmHg of the different oxygen (O_2) tensions for 4 minutes, followed by treatment of the cells at a flowrate of 2-5 mmHg for 15 minutes. The hypoxic chambers were transferred to a cell culture incubator and maintained under 5% CO_2 and $37^0 C$ temperature. Early passage OFMSCs (passages 2 to 4) were divided into two groups; one group was cultured under normoxic conditions (21% O_2, 5% CO_2 and temperature of $37.5^0 C$), while the second group was subjected to severe oxygen tensions (0.1% O_2, 5% CO_2).

Selection of optimal hypoxic parameters

OFMSCs seeded at a density of 9.5 X 10^4 cells/ml, were treated with 21%, 2% and 0.1% O_2 as described above. MSC incubation under hypoxia was performed for 1, 3, 6, 12 and 24 hours to assess cell survival using trypan blue staining and a hemocytometer. Results were normalized to cells treated under 21% oxygen and iliac-crest mesenchymal stem cells (ICMSCs) were used as non-jaw site control. The percentage of surviving cells and time point of 50% cell survival was also assessed (lethal dose 50 or LD_{50}. Based on these outcomes, severe hypoxia of 0.1% O_2 and exposure time of 6 hours were selected as optimal parameters simulate a severe hypoxic environment.

Phenotypic identification of mesenchymal stem cell and osteoprogenitor surface markers

Characterization of hMSCs surviving severe hypoxia was carried out by immunophenotyping using immunostaining.

Both OFMSCs and ICMSCs were plated at a density of 8 X 10^3 cells/cm^2 and 1.6 X 10^4 cells/cm^2 in an 8-well chamber slide. At 80% confluence, cells were treated with either 0.1% or 21% O_2 for 6 hours. Immediately after hypoxic treatment, hMSCs were rinsed in 1x PBS and fixed in 4% paraformaldehyde for 10 minutes. Fixed cells were washed three times for 5 minutes each PBS, treated with 0.1% Triton X in PBS for five minutes at room temperature and then blocked with 3% normal goat serum prepared in 1% BSA and 0.05% Triton X in PBS for 30 mins. Both groups of hMSCs treatment, were incubated in humidified tray with mouse monoclonal alpha smooth muscle actin (αSMA) (1:100 dilution) and CD 146 (MUC18) (1:1000 dilution) (Abcam) at 4^0C overnight. A 1:500 dilution

of Alexa Fluor 488-labelled goat anti-mouse (Cell Signaling) was used as secondary antibody and counterstaining was done with 1 μg/mL Hoechst 33342. Both secondary incubation and counterstaining were carried out in the dark. Fluorescence signals was analyzed using a Nikon epiflulorescence microscope.

Post-hypoxic Cell survival

Colony forming efficiency:

To access post-hypoxia clonogenic properties of hMSCs were seeded at a density of 1.9 X10^5 cells/cm^2. At 80% confluence, hypoxic treatment with 0.1% oxygen was initiated for 6 hours, with the control group treated at an oxygen tension of 21%. Cells were trypsinized and counted using a hemocytometer. hMSCs for colony forming efficiency were seeded in T-25 flasks at concentrations of 10^3, 10^2 and 10^1 and kept in culture for 14 days. Cells were fixed in absolute methanol and stained with methyl violet. Colonies containing 50 or more cell aggregates were counted and scored.

Population doubling and lifespan of surviving OFMSCs:

OFMSCs were plated with a cell density of 7.5 X 10^5 cells/flask. Expanded cells were re-plated at a density of 1.9 X10^5 cells/cm^2 and separated into normoxic and hypoxic treatment groups. At the end of the treatment cycle as previously described, hMSCs were trypsinized and counted. Population doubling capacity was determined based on repeated passaging at 1:10 split ratio until cells attained replicative senescence.

Assessment of Apoptotic hypoxic-induced MSC death

<u>Caspase 3/CPP32 Activity</u>

Cells were re-plated at a density of 1.7 x 10^5 cells/cm^2. At 80% confluence, OFMSCs were exposed to either 0.1% or 21% O_2 for 6 hours, trypsinized and counted to ensure approximately 5 x 10^6 cells were used for the cell activity assay. The counted cells were pelleted at 10,000g for 5 minutes at 4^0C. Pellets were re-suspended in lysis buffer (EnzoScientific NY, USA), incubated on ice and re-centrifuged at 10,000g for 1 minute to extract the cytosolic contents. After protein assay to determine protein amount, 50 µg/µl, was added to reaction buffer containing DTT and DEVD-pNA substrate. The mixture was incubated at 37^0C for 2 hours and absorbance read at 405nm in a spectrophotometer. All samples were tested in triplicates.

<u>Western blotting</u>

Using the protein lysates prepared above, immunoblotting was also carried out using equal protein amounts. The blots were probed with rabbit polyclonal caspase 3 antibody (Abcam) at 1:1000. This was followed by incubation in secondary antibody with Anti Rabbit, IgG HRP-linked antibody (Cell Signaling), using a 1:3000 dilution in 5% milk buffer. β-actin (1:1000) served as loading control. Immunoreactive bands exposed with Konica Minolta developer (China) and analyzed using Image J.

Assessment of hypoxia-induced autophagic responsiveness of hMSCs

Autophagic response of undifferentiated hMSCs were determined following exposure of OFMSCs and ICMSCs to severe hypoxia. Following similar platting parameters described above cell lysate was collected and protein assay was performed so equal protein

amounts can be immunoblotted. LC3B a marker of autophagy was assessed by immunoblotting using rabbit polyclonal AntiLC3B (Abcam) and real time PCR was performed using custom made LC3b primer sequences: 5'ATT CGA GAG CAG CAT CCA AC-3' (Forward) and 5'-CTG CCG TTC ACC AAC AG-3' (Reverse). TATA binding protein served as internal control. Primer sequences were 5'-GGA GCT GTG ATG TGA AGT TTC CTA-3' (Forward) and 5'-CCA GGA AAT AAC TCT GGC TCA TAA C -3' (Reverse). Additionally, autophagic activity was further assessed by immunofluorescence. Both groups of hMSCs were re-plated at a density of 8×10^3 cells/cm^2 and 1.6×10^4 cells/cm^2 in an 8-well chamber slide. At 80% confluence, cells were treated with either 0.1% or 21% O$_2$ for 6 hours as previously described. Immediately after hypoxic treatment, hMSCs were fixed in 4% paraformaldehyde, rinsed in PBS-Tween 20 and then blocked with 5% normal goat serum in PBS-Tween 20 for 30 mins. hMSCs were incubated in humidified tray with 3µg/mL and 4 µg/mL of rabbit polyclonal anti- LC3B antibody (Abcam) at 4^0C overnight. 5 µg/mL of Alexafluor 488-labelled goat anti-rabbit (Cell Signaling) was used as secondary antibody followed by counterstaining with 1 µg/mL Hoechst 33342. Fluorescence signals was analyzed using a Nikon epiflulorescence microscope.

Hypoxia-induced reactive oxygen species and oxidative stress

Level of reactive oxygen species (ROS) induced by severe hypoxia was assessed using the dichlorofluorescein diacetate (DCFDA) cellular ROS Detection Assay kit (Abcam). OFMSCs were seeded on duplicate 96-well plates, at a density of 2.5×10^4 cells/ cm^2 per well. At confluence, one plate was kept under normoxia, while the other was treated with 0.1% O$_2$. Cells were then washed and stained with 25µM of DCFDA for 45 minutes at

37^0C. Fluorescence signal was then read at 480nm. Control hMSCs were not treated with DCFDA.

In vitro osteogenic differentiation

Osteogenic differentiation assay was performed as previously described (Akintoye et al 2006; Damek-Poprawa et al 2010) using hMSCs plated at 1 x 10^4 cells/cm^2. OFMSCs from same individuals were each divided into 4 groups: 1). treated with either 21% O_2 (normoxia), 2). 6 hours of 0.1% O_2 (severe hypoxia), 3). 7 days of sustained severe hypoxia (0.1% O_2) followed by 7 days of normoxia, or 4). 14 days of 0.1% O_2 sustained severe hypoxia. Each group of 4 treatment types were osteogenically stimulated for 14 days and all the hypoxic groups were initially treated with unstimulated hypoxic medium for six hours before osteogenic induction. The osteogenic medium consisted of normal growth medium supplemented with 100μM L-ascorbic acid 2-phosphate (10^{-4}M), 2mM dexamethasone sodium phosphate (American Regent Laboratories, Shirley, NY, USA). To assess in vitro mineralization (calcium deposition), similar experimental plates as above were set up but the osteogenic medium was fortified with 50mM β-glycerophosphate. After 14 days, hMSCs in all the treatment groups were fixed in 10% formalin for one minute and rinsed with deionized water. Calcium accumulation based on mineralization assay was determined by staining with1% alizarin red dissolved in 2% ethanol. Unattached stain was rinsed copiously with deionized water. After air-drying the plates, attached alizarin red was quenched with 0.5N HCL and 5% sodium dodecyl sulphate (SDS) at room temperature for quantitative analysis. Absorbance was read in 96-well plates at 405nm, using a microplate reader (Molecular Devices, USA).

Immunoblotting and real time PCR were also used to assess protein and RNA levels of the following osteogenic markers: Alkaline phosphatase, osteopontin osteocalcin and bone sialoprotein. Rabbit polyclonal alkaline phosphatase placental (ALPP) (Novus Biologicals) and anti Osteopontin (Rockland Inc., USA) were used as primary antibodies while real time PCR was performed with these primers: Osteopontin (OPN) (forward 5'-TGGAAAGCGAGGAGTTGAATG-3' and reverse CATCCAGCTGACTCGTTTCATAA-3')

Alkaline phosphatase (ALP): 5'-CCGTGGCAACTCTATCTTTGG-3' (Forward) and 5'-GATGGCAGTGAAGGGCTTCTT-3' (Reverse)

Bone Sialoprotein (BSP): 5'-AAC GAA GAA AGC GAA GCA GAA-3' (Forward) and 5'-TCT GCC TCT GTG CTG TTG GT-3' (Reverse)

Osteocalcin (OCN): 5'- AAG AGA CCC AGG GCG GCT ACC T-3' (Forward) and 5'- AAC TCG TCA CAAG TCC GGA TTG-3' (Reverse).

In vivo osteogenic differentiation

Bone regenerative capacity of hypoxia-treated ICMSCs and OFMSCs was assessed by transplantation of 2×10^6 cells attached to 40mg spheroidal hydroxyapatite-tricalcium phosphate (particle size 0.5-1.0 mm, Zimmer, Warsaw, IN) into the subcutis of 8-week old immunocompromised female nude mice (NIH-III NU/NU, Charles Rivers Laboratories, Wilmington, MA) as previously established in Dr. Akintoye's lab (The IACUC protocol was approved by the University of Pennsylvania office of Regulatory Affairs). The hMSCs were divided into the treatment types described above under *in vitro* differentiation. Non-induced and osteogenically induced OFMSCs and ICMSCs were transplanted into 4 separate subcutis pocket of 5 different animals. At 6, 8 and 12 weeks, transplants were

harvested, fixed in 4% paraformaldehyde for 48 hours, decalcified in 10% EDTA (pH 8.0) and paraffin-embedded. Five micrometer sections were stained with hematoxylin/eosin for histological analysis. Digital images were captured with Nikon Eclipse80i fluorescent microscope (Nikon Instruments, Melville, NJ). Bone regeneration within the transplants was assessed using an established semi-quantitative bone scoring system (Akintoye,S.O. 2006): Bone scores ranged from 0 (no bone formation observed in transplant); 1(minimal bone formed in transplant); 2 (weak bone formation in transplant, occupying a significant portion, but less than 50%);3 (moderate bone formation occupying a significant portion but less than 50% of the transplant) and 4 (abundant bone formation, occupying more than 50% of transplant).

Adipogenic differentiation

ICMSCs and OFMSCs were cultured at 1.8 x 10^3 cells/cm^2. At 90% sub confluence, adipogenic differentiation was induced using established adipogenic parameters for 21 days (Akintoye et al 2006; Osyczka et al 2002). Adipogenic medium contained 10^{-8}M dexamethasone, insulin (1µg/ml), 5 X 10^{-8} M 1-methyl-3-isobutylxanthine (IBMX) ,10^{-4} M indomethacin and ten percent (10%) fetal bovine serum (FBS), penicillin, streptomycin, glutamate and alpha MEM. Adipogenically induced ICMSCs and OFMSCs were subjected to the following treatments: normoxia treated for 21 days, 7 days of sustained hypoxia followed by 14 days of normoxia, 14 days of sustained hypoxia followed by 7 days of normoxia and 21 days of sustained hypoxia. The control group hMSCs were not induced adipogenically but retained in normal growth medium. At 21days, hMSCs were washed in phosphate buffered saline, fixed in 4% paraformaldehyde for 10 mins at room

temperature, stained with Oil Red O for 1 hour and counterstained with 1% fast green dye for another 10 minutes. Lipid laden cells were evaluated and quantified microscopically to determine the number of adipocytes. In parallel experiments, protein and RNA were collected to quantify markers of by western blotting and real time PCR respectively. Primary antibodies to peroxisome- proliferator-activated receptor gamma (PPAR gamma), an early marker of adipogenesis and cytoplasmic phospholipase A2 (PLA2) (late marker of adipogenesis) were used for immunoblotting. The following primers were used for real time PCR: peroxisome- proliferator-activated receptor gamma (PPAR gamma) (primers: forward 5'TGAATGTGAAGCCCATTGAA-3' and reverse 5'-AGCGGGTGAAGACTCATGTC-3'); phospholipase A (cPLA2) (primers: forward 5'-GGGGGCCTTTGGTGACATGCT-3' and reverse 5'-ACCACAGGCACATCACGTGCA-3') and LPL (primers:5'- ACG GCA TGT GAA TTC TGT GA -3'(forward) and 5'- GGA TGT GCT ATT TGG CCA CT -3' (reverse), which are early and late markers of adipogenesis.

Assessment of HIF-dependent response of OFMSC to severe hypoxia

HIF-1 alpha expression of OFMSCs under normoxia and severe hypoxia were assessed by immunofluorescence, immunoblotting and real time PCR. Primary antibody of mouse anti-HIF-1α (Abcam) at 1: 1000 dilution was used for both immunoblotting and immunofluorescence. Secondary antibodies were Alexafluor 488 (1:500 dilution) and Alexaflour 555 (1:500 dilution) for immunofluorescence and anti-mouse (1:3000) for immunoblotting. Counterstaining was carried out with 1µg/ml of Hoechst 33342. HIF-1α primer sequences for real time PCR were: 5'-ACG TTC CTT CGA TCA GTT GTC A-3' (forward) and 5'-TTT GAG GAC TTG CGC TTT CA-3' (reverse).

Assessment of OFMSCs endoplasmic reticulum stress response post severe hypoxia

OFMSCs protein lysate and RNA samples were collected post severe hypoxia. Immunoblotting and real time PCR were used to assess the activation of endoplasmic reticulum stress response (ERSR) in OFMSCs following treatment with 21% and 0.1% oxygen tensions as described above. The following ERSR markers were assessed: eukaryotic initiation factor 2α (eIF2α), phosphorylated eukaryotic initiation factor 2α (peIF2α), activating transcription factor 4 (ATF4), and binding protein (Bip).

For immunoblotting, equal protein amounts were used to determine the expression levels of Bip and eIF2α. The blots were probed with rabbit polyclonal anti-Bip (1:1000) (BD Biosciences) mouse anti-eIF2α (1:1000) (Cell signaling Technology); rabbit anti-peIF2α (1:500) (Cell signaling Technology); and rabbit anti-ATF4 (1:500). Secondary antibodies were anti Rabbit and anti-mouse, IgG HRP-linked antibodies (Cell Signaling) respectively at 1:3000 dilution in 5% milk buffer. Anti-β-actin (1:1000) served as loading control. Immunoreactive bands were developed with a Konica Minolta developer (China) and analyzed using Image J software.

Real time PCR was carried out with 7300 Fast Real Time PCR system (Applied Biosystems, Foster city, CA) as previously described above. The following custom designed primers were used: Bip: 5'- GGA GGT GTC ATG ACC AAA CTG A -3' (forward) 5'TCT TTC ACC TTC ATA GAC CTT GAT TG-3' (reverse); ATF4: 5'-CAG ACC GTG AAC CCA ATT GG -3' (forward) and 5'- CAA CCT GGT CGG GTT TTG TT-3' (Reverse).

Assessment of activation of vascular endothelial growth factor (VEGF)

VEGF expression was also assessed by western blotting, real-time PCR and immunofluorescence to evaluate the role of the two independent pathways in the recovery of OFMSCS after severe hypoxia. Mouse anti-human VEGF antibody (Abcam) was used for both immunoblotting (6µg/ml) and immunofluorescence (1:2000 dilution).

VEGF primer used for real time PCR were: 5' GCA CCC ATG GCA GAA GGA GG-3' (forward) and 5'CCT TGG TGA GGT TTG ATC CGC ATA -3' (Reverse)

The physiologic effect on blood vessels *in vivo* was also assessed by immunohistochemistry.

Pharmacologic perturbation of HIF-1α and ERSR and effects on VEGF activation

OFMSCs were divided into the two treatment groups as previously described above. Cells were treated for 18 hours with Thapsigargin (1µM), Integrated stress response inhibitor (ISRIB) 0.2mM (ER stress response upregulator and downregulator respectively) as well as quinomycin (80nM) and 2,4 diethylpyridine dicarboxylate (50 µM) (HIF-1α downregulator and upregulator respectively). While a set of cells were kept under normoxic conditions, another set was switched after 12 hours and exposed to 0.1% oxygen for an additional 6 hours. Effect of upregulation and downregulation of HIF-1 alpha and ERSR on VEGF levels were assessed by real time PCR.

Statistical analysis

Experiments were analyzed using Prism 6 (GraphPad Software Inc. La Jolla CA). Each set of experiments was performed at least three times and cells were set up in triplicates plates. All data were subjected to descriptive analysis and expressed as mean ± standard deviation. Relationship between cells treated at 21% and 0.1% oxygen was assessed by paired t-test and differences among subjects were assessed by one-way analysis of variance (ANOVA) followed by post-hoc analysis with Bonferroni correction. Statistical significance was set at $p<0.05$.
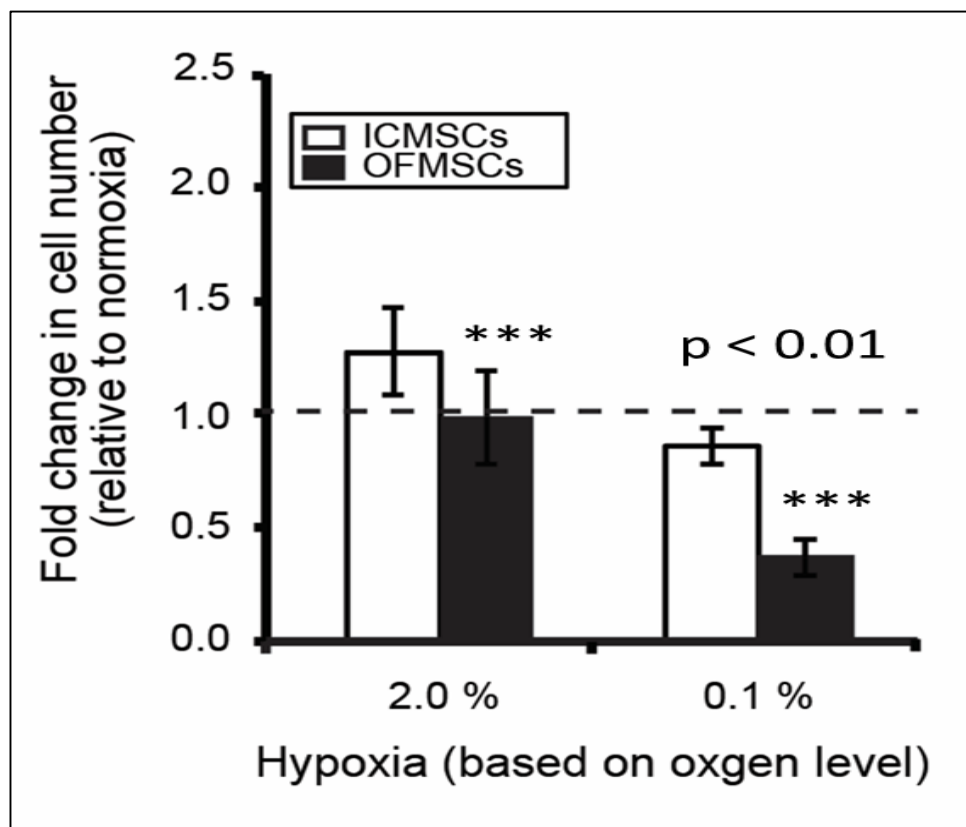
4.0   RESULTS

hMSCs succumb to severe hypoxia

Following exposure of hMSCs from 3 different healthy patients to varying oxygen concentrations of 21% (normoxia), 2% and 0.1%, both ICMSCs and OFMSCs were noted to succumb to severe hypoxia of 0.1% O_2 (Figure 8).


Figure 8. Response of hMSCs (both ICMSCs and OFMSCs) to different oxygen concentrations

MSCs were treated in 21%, 2% and 0.1% oxygen tensions. OFMSCs at 0.1% O2 was noted to succumb more to severe hypoxic insult, with a 0.5-fold decrease ($p < 0.01$) when compared to ICMSCs treated under the same conditions. ICMSCs in the hypoxic group showed a 0.1-fold decrease compared with normoxia. Fold change in cell number was relative to MSCs treated under 21% O2 tension

However, over 50% of OFMSCs were noted to succumb to severe hypoxia, compared to 10% of ICMSCs ($p < 0.01$). To recapitulate severe hypoxia in subsequent experiments, an oxygen concentration of 0.1% was selected. Most of the subsequent experiments focused on OFMSCs while ICMSCs were used as non-orofacial controls.

Selection of optimal time point for hypoxic treatment

OFMSCs were further exposed to 0.1% oxygen for 1, 3 and 6 hours to assess time-dependent effect of severe hypoxia on hMSCs. Comparison of percentage of surviving and dead cells demonstrated that 6 hours was an optimum time point of maximum cell survival (Figure 9). Six-hour time point was designated as t_{50}, at which 50% of hMSCs were still viable. Exposure of hMSCs to severe hypoxia was conducted for 6 hours. Any exposure beyond 6 hours was regarded as sustained severe hypoxia
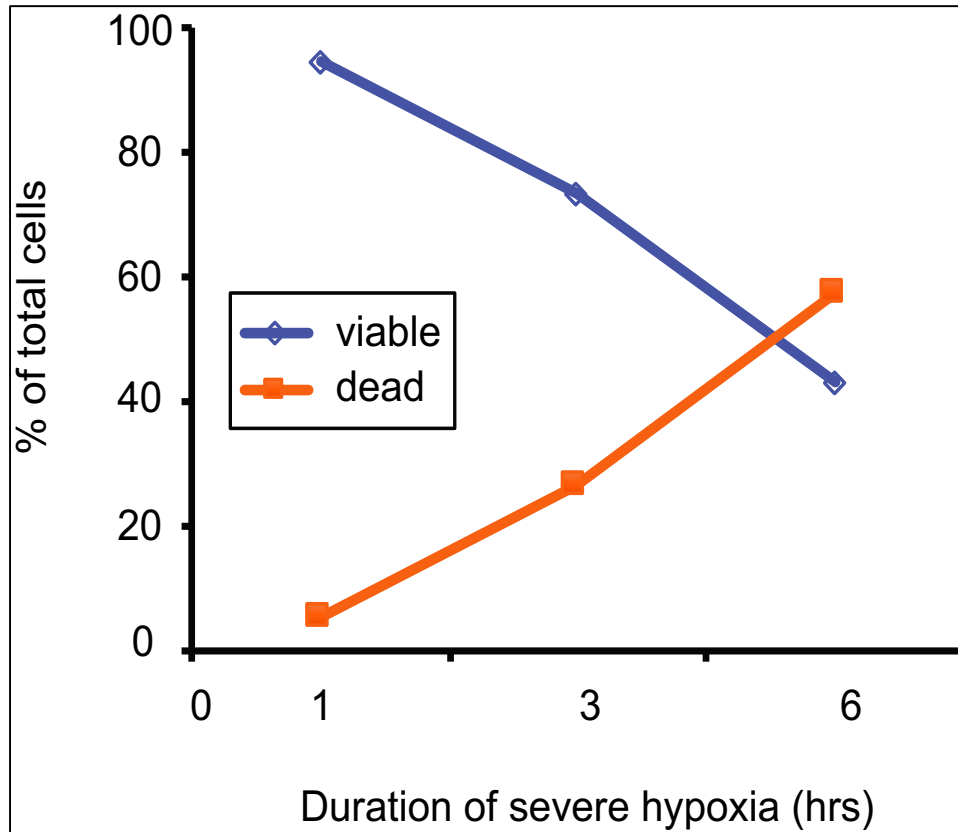
Figure 9. Post-hypoxic percentage of surviving cells relative to dead cells.

Time-dependent changes in ratio of live to dead cells after exposure of OFMSCs to severe hypoxia

Severe hypoxia activated oxidative stress and release of reactive oxygen species by OFMSCs.

In addition, post-hypoxic production of reactive oxygen species was assessed in the OFMSC treatment groups. Under severe hypoxia, there was a significant 8-fold increase in the detection of reactive oxygen species (ROS) activity by OFMSCs (p< 0.01) (Figure 10).
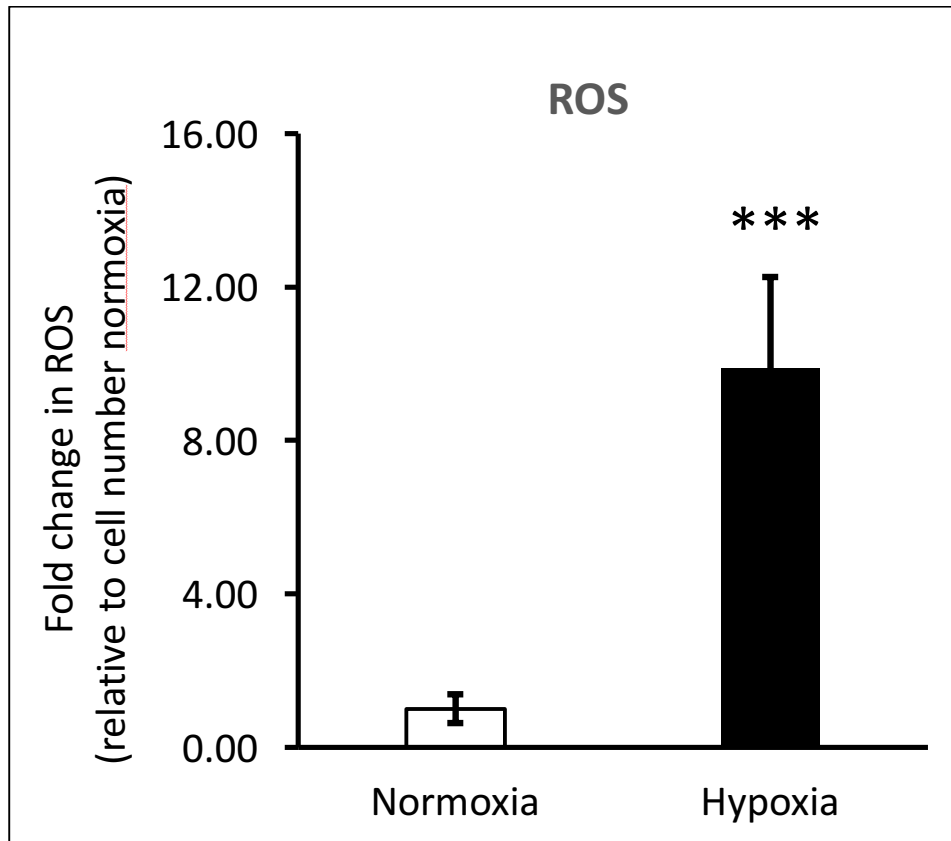
Figure 10. Post-hypoxic oxidative stress based on levels of reactive oxygen species.

OFMSC oxidative stress based on ROS production was significantly higher under hypoxic stress relative to normoxia (n =3, p < 0.01).

**Effect of severe hypoxia on expression levels of OFMSC markers of 'stemness'.**

After exposure of OFMSCs and ICMSCs to severe hypoxia, the expression levels of two major markers of MSC 'stemness', α-SMA and MUC18 (CD146) were assessed by immunofluorescent staining. Results showed that both α-SMA and MUC18 immunofluorescence signal intensities were relatively reduced in OFMSCs compared to ICMSCs. Approximately 250-fold decrease in signal intensity of α-SMA was noted in OFMSCs (Figure 11) treated under severe hypoxia compared with ICMSCs (p < 0.001)

and a 2-fold decrease was noted in fold intensity of MUC18 in the OFMSC severe hypoxia treatment group ($p < 0.01$) (Figure 12).
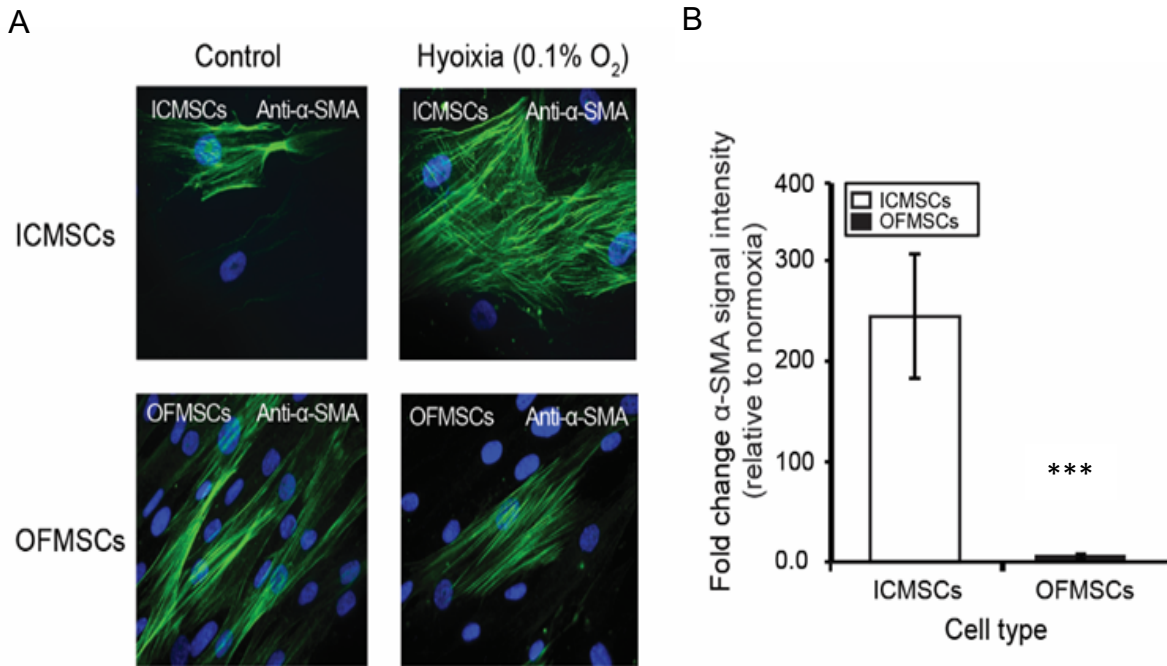
Figure 11. Comparative difference in OFMSC and ICMSC expression levels of αSMA.

ICMSCs (upper panel) after exposure to 0.1% oxygen showed higher immunofluorescent signal intensity compared to OFMSCs treated under similar conditions (lower panel right- 11A). Normoxia (control) MSCs were exposed to 21% oxygen tension) (left upper and lower panel (11A). Quantitative analysis of signal intensities (11B) also demonstrated the significant differences in αSMA expressions between OFMSCs and ICMSCs (*** $P < 0.0001$).
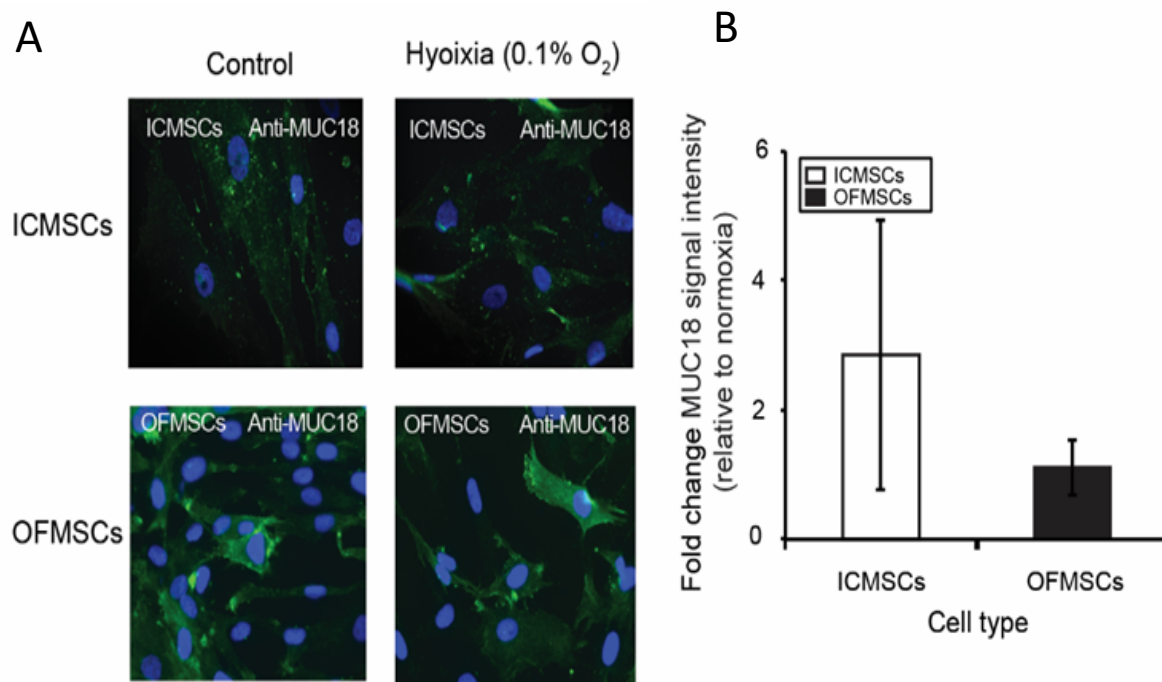
Figure 12. Comparative difference in OFMSC and ICMSC expression levels of MUC18.

ICMSCs (upper panel) after exposure to 0.1% oxygen showed stronger immunofluorescence signal intensity compared to OFMSCs treated under the same conditions (lower panel right- 12A). Normoxia (control) MSCs were exposed to 21% oxygen tension) (left upper and lower panel- 12A). Quantitative analysis of signal intensities (12B) also demonstrate a difference in MUC18 expressions between OFMSCs and ICMSCs
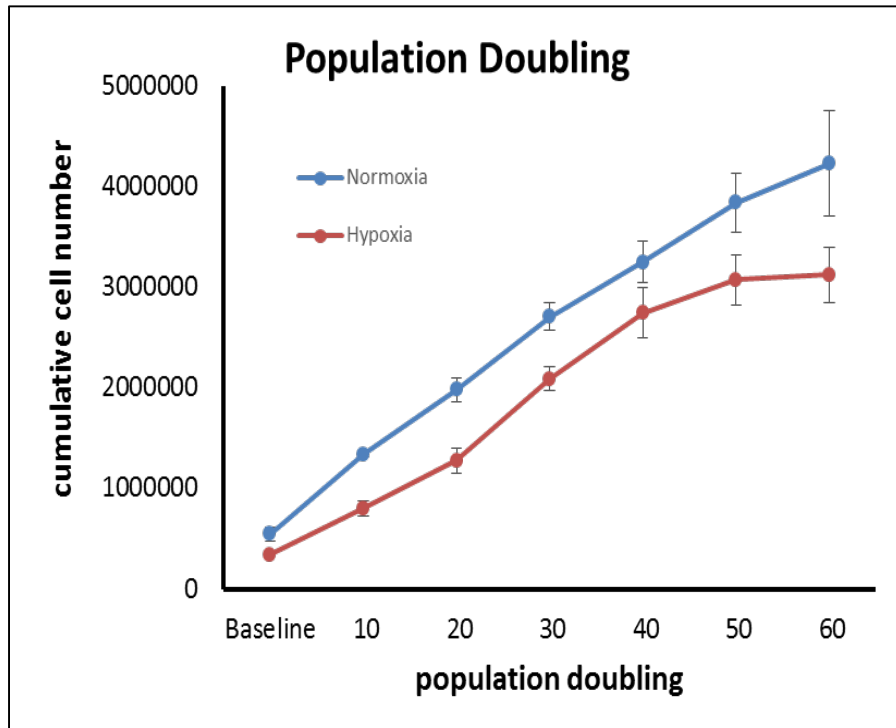
Post-hypoxic OFMSC survival

<u>Cell proliferation/Population Doubling</u>

Furthermore, post-hypoxic recovery capacity of OFMSCs was assessed directly and

indirectly following evident presence of fewer surviving OFMSCs after hypoxic treatment.

Assessment of population doubling properties of the hMSCs demonstrated survival up to

50 population doublings in the hypoxia-treated OFMSCs. The normoxic-treated cells however, survived up to 60 population doublings (Figure 13).

Figure 13. Post hypoxic population doubling capacity of OFMSCs

Similar proliferation patterns between the normoxic and hypoxic groups of OFMSCs. OFMSCs treated under hypoxic conditions were noted to survive up to 50 population doublings (PD), while the normoxic treatment group survived up to 60 PD. [Representative graph of one of the patient samples; n=3 samples. (p = 0.356).

Indirect evaluation of OFMSC recovery capacity by assessing apoptosis showed a 0.2-fold decrease (p= 0.076) (Figure 14A & 14B) in expression of Caspase 3 (apoptotic marker) in the hypoxic group (by immunoblotting). This was further confirmed by a 0.1-fold decrease in Caspase 3/CPP activity in the hypoxic group (p = 0.49) (Figure 14C).
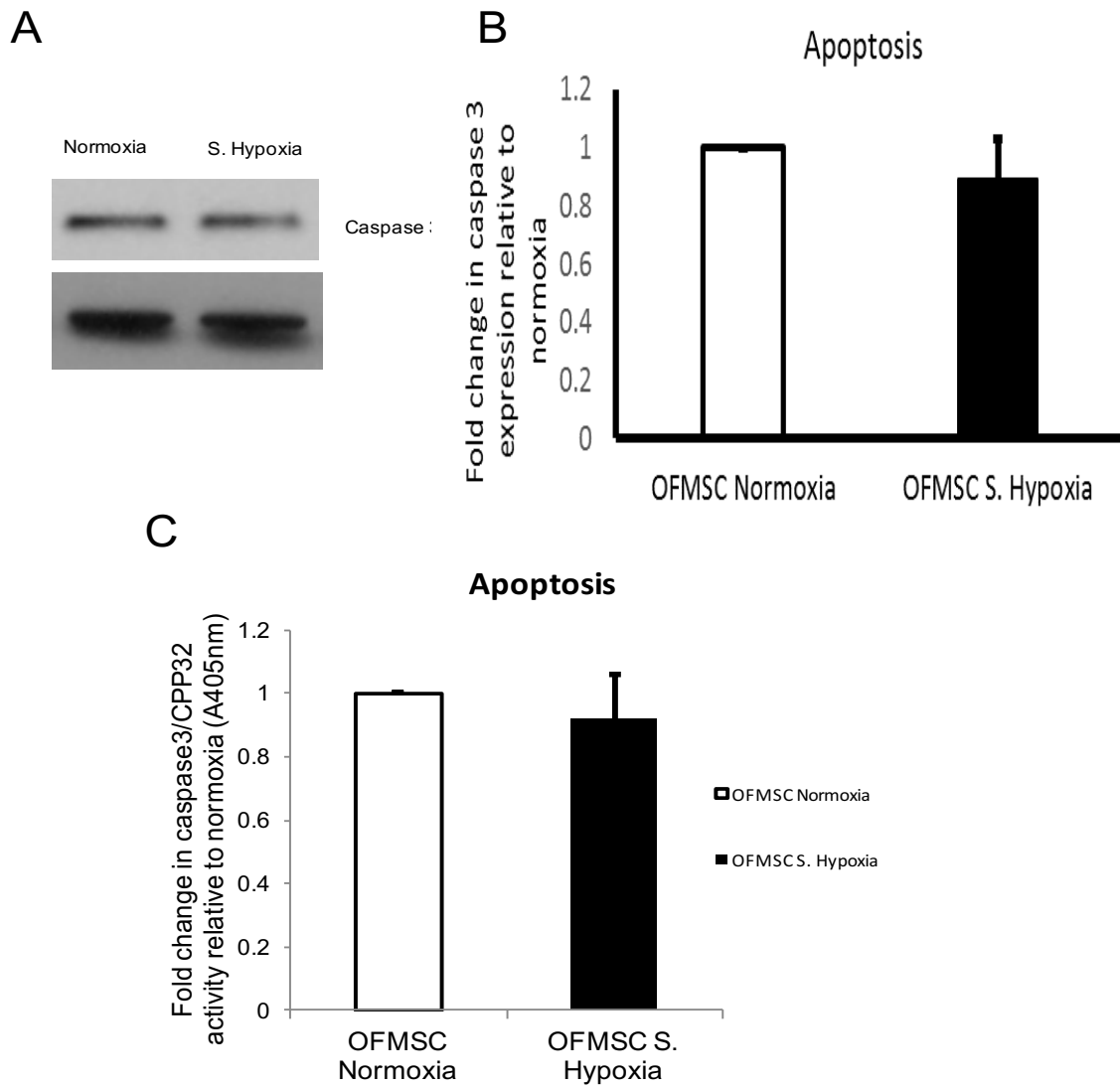
Figure14. Apoptosis in hypoxic-treated OFMSCs.

A 0.2-fold decrease in caspase-3 protein expression noted in the hypoxia-treated group (p = 0.076) (14A & 14B). Caspase 3/CPP activity by colorimetric assay further showed a 0.1-fold decrease in the hypoxic treatment group when compared to normoxia. (14c) (p = 0.49).

The expression level of LC3, a marker of autophagic activity was further assessed by

immunoblotting, real time PCR and immunofluorescence. Hypoxia-treated OFMSCs

displayed a 0.3-fold decrease in protein expression of LC3 (p= 0.05) (Figure 15A &

15B). A more significant difference was noted in LC3 gene expression, with a 0.7-fold

decrease in the hypoxic group (p= 0.011) (Figures 15C). This was further supported by

the fewer immunoreactive cells in the hypoxic group based on immunofluorescence

staining (Figure 15D).
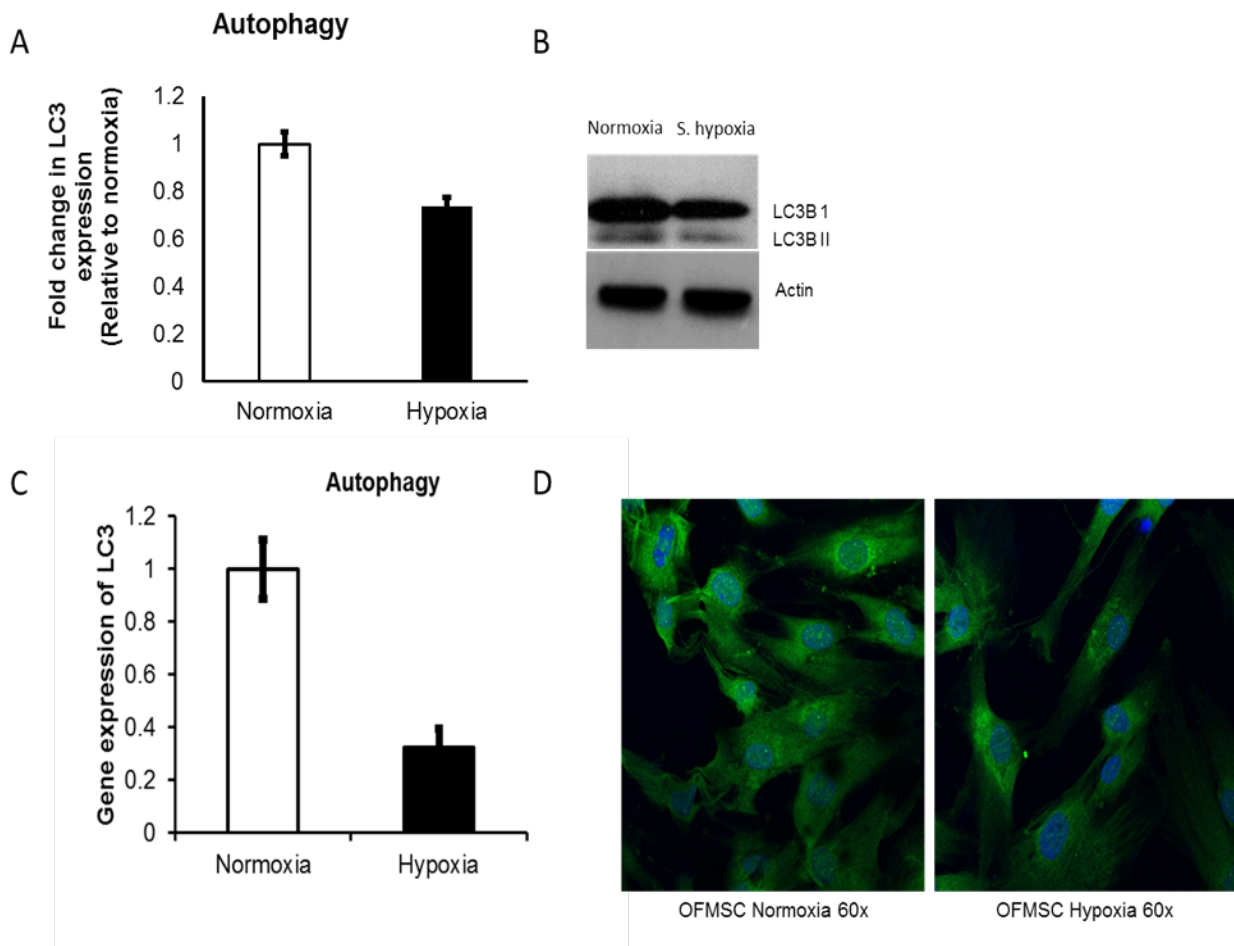
Figure 15. Comparative differences in LC3 expression

LC3 a marker of autophagic activity was assessed by immunoblotting (15A, 15B) and real time PCR (15C). A 0.2-fold and 0.6-fold decrease was noted in the hypoxic treatment group following immunoblotting and real-time PCR (p = 0.05) respectively. Additional immunofluorescent staining also confirmed decreased immunoreactive hypoxic treated OFMSCs (15D).

Finally, a comparable colony forming efficiency pattern was observed in both the normoxic and hypoxic treatment groups with an average of 5 x 10^5 colony forming units (CFUs) in each group (Figure 16).

Normoxia   Hypoxia

OFMSCs Colony forming units
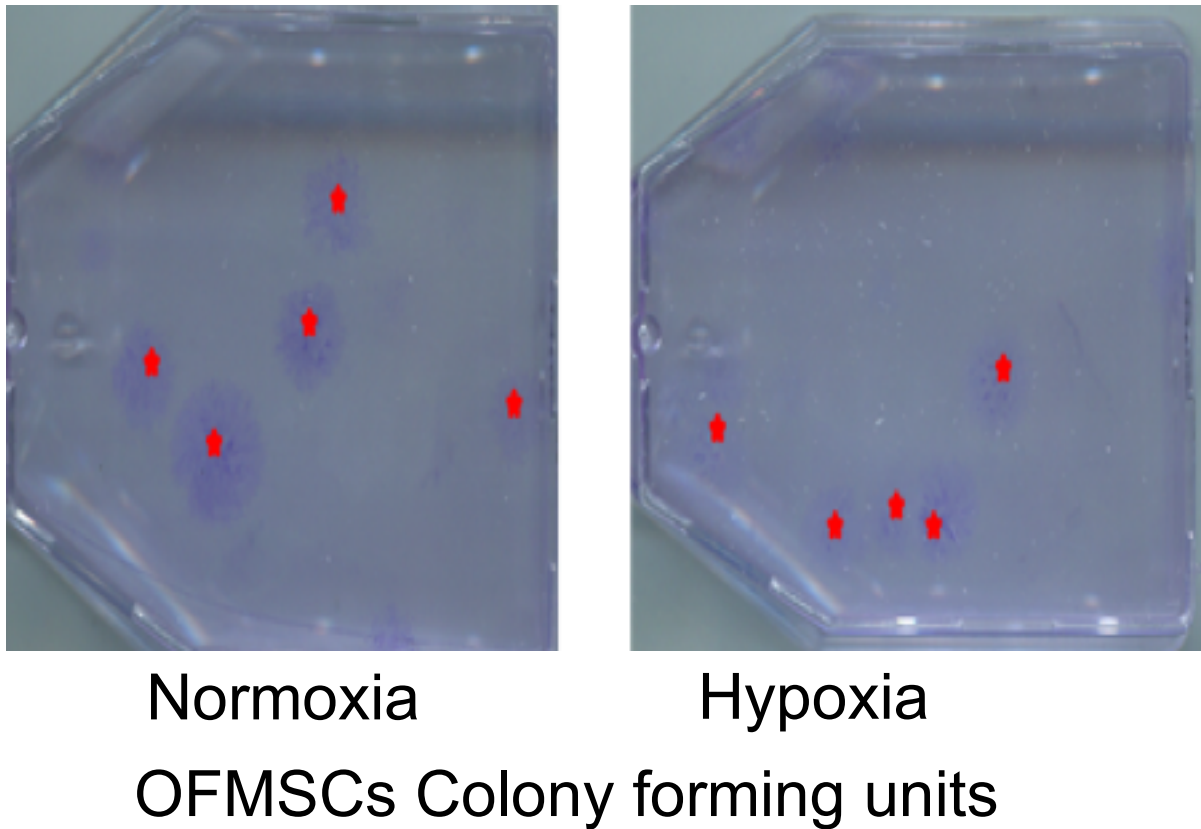
Figure 16. Colony forming efficiency in response to severe hypoxia.

Both normoxic and hypoxic cells displayed similar clonogenic capacity [red star identifies some representative clones

Post hypoxic OFMSC differentiation:

Effect of severe hypoxia on *in vitro* osteogenic differentiation

Osteogenic differentiation capability of surviving OFMSCs post hypoxia was also assessed, with comparable osteogenic differentiation noted in both hypoxic-treated and normoxic-treated groups. This was noted with Alizarin red dye stain (Figure 17). Quantitative analysis however showed a 0.4-fold increase in mineralized OFMSCs in the severe hypoxia treatment group when compared with those treated with normoxia (p = 0.51) (Figure 18).
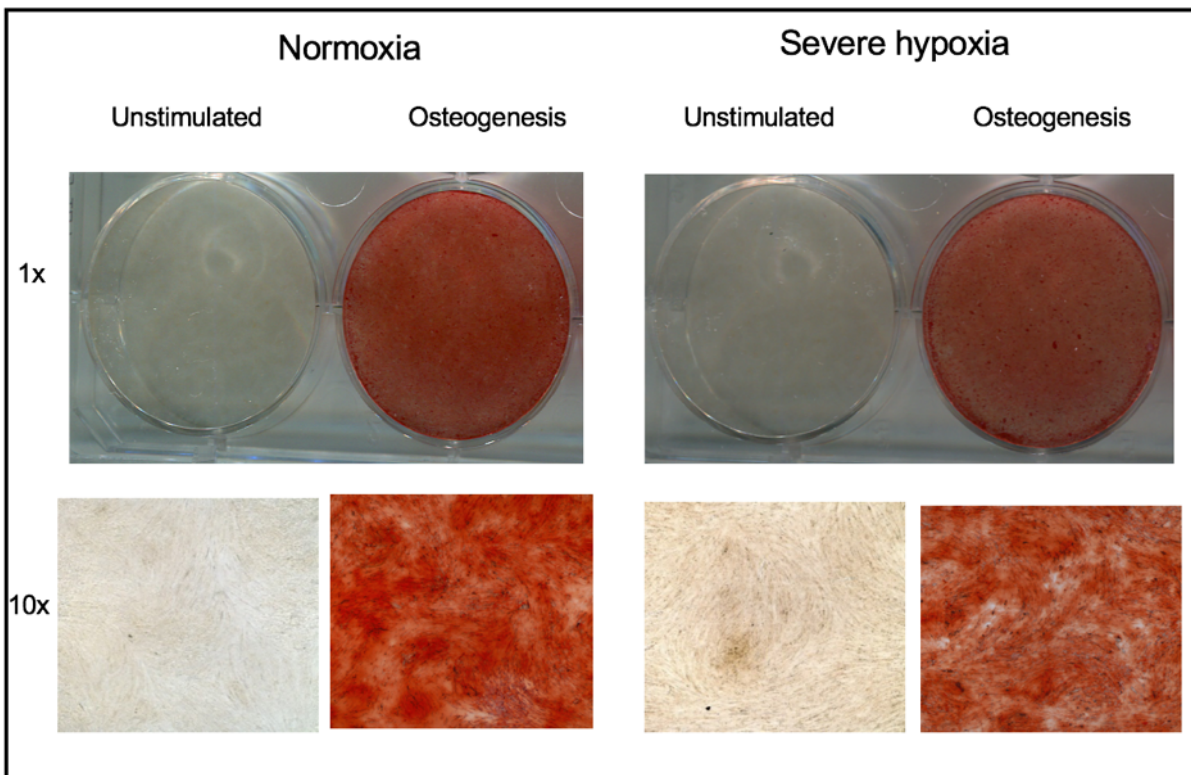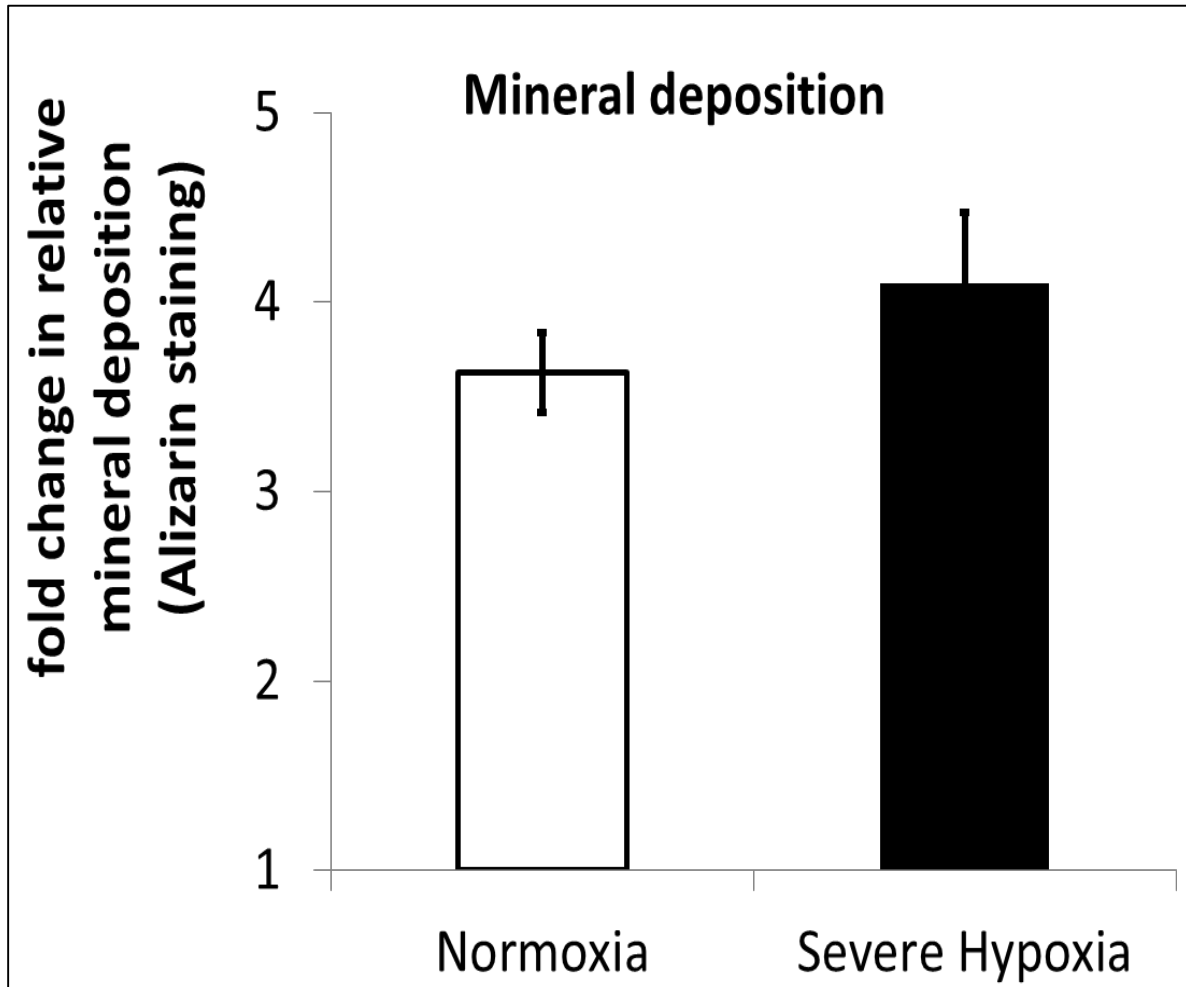

Figure 17. Post-hypoxic osteogenesis based on in vitro mineralization

In vitro mineralization of OFMSCs based on alizarin red staining were not affected by hypoxia as both normoxic and hypoxic cells displayed similar staining patterns. The non-osteogenically stimulated cells served as control.

When gene expression of bone markers was evaluated by real time PCR, the hypoxic treatment group showed non-significant decreased ALP, OCN and BSP activity (Figures

19, 20, 22). OPN however had a comparable expression between the normoxic and hypoxic treatment groups (Figure 21).

Figure 18. Post-hypoxic osteogenic differentiation of OFMSCs based on in vitro mineralization

Quantitative analysis of mineralization shows a non-significant increase in mineral deposition in the severe hypoxic treatment group. [n =3] [p = 0.51].

Further assessment of osteogenic markers by real time PCR showed a non-significant decrease in gene expression of ALP, BSP and OCN (0.3-fold decrease) in the OFMSC hypoxic treatment group (Figure 19-22). OPN however showed similar expression between the two treatment groups (Figure 21).
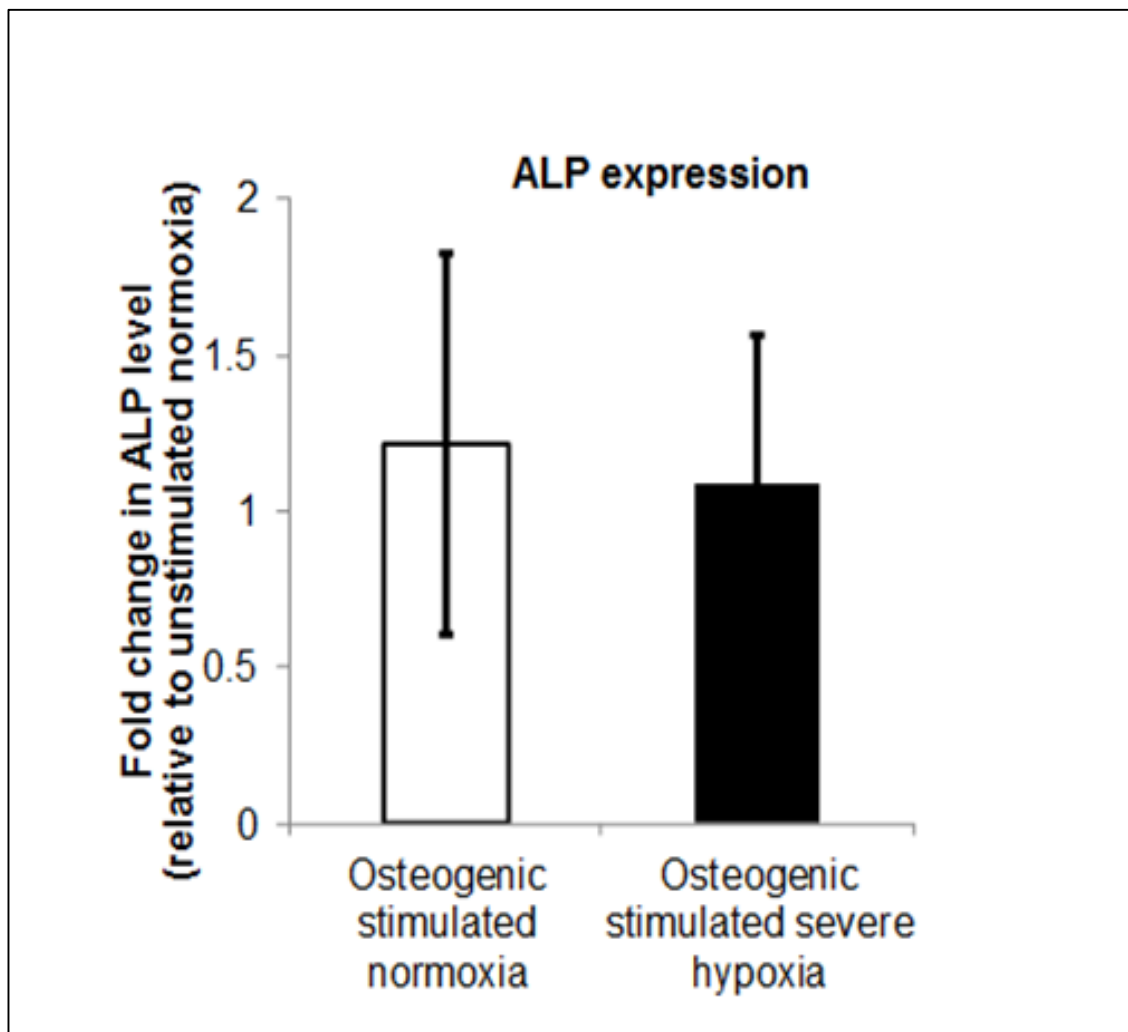
Figure 19. Post-hypoxic osteogenic differentiation based on alkaline phosphatase (ALP) gene expression

Gene expression of ALP in OFMSCs treated under normoxic and hypoxic treatment conditions. The reduced level of ALP in hypoxic treated OFMSCs was minimal and non-significant.
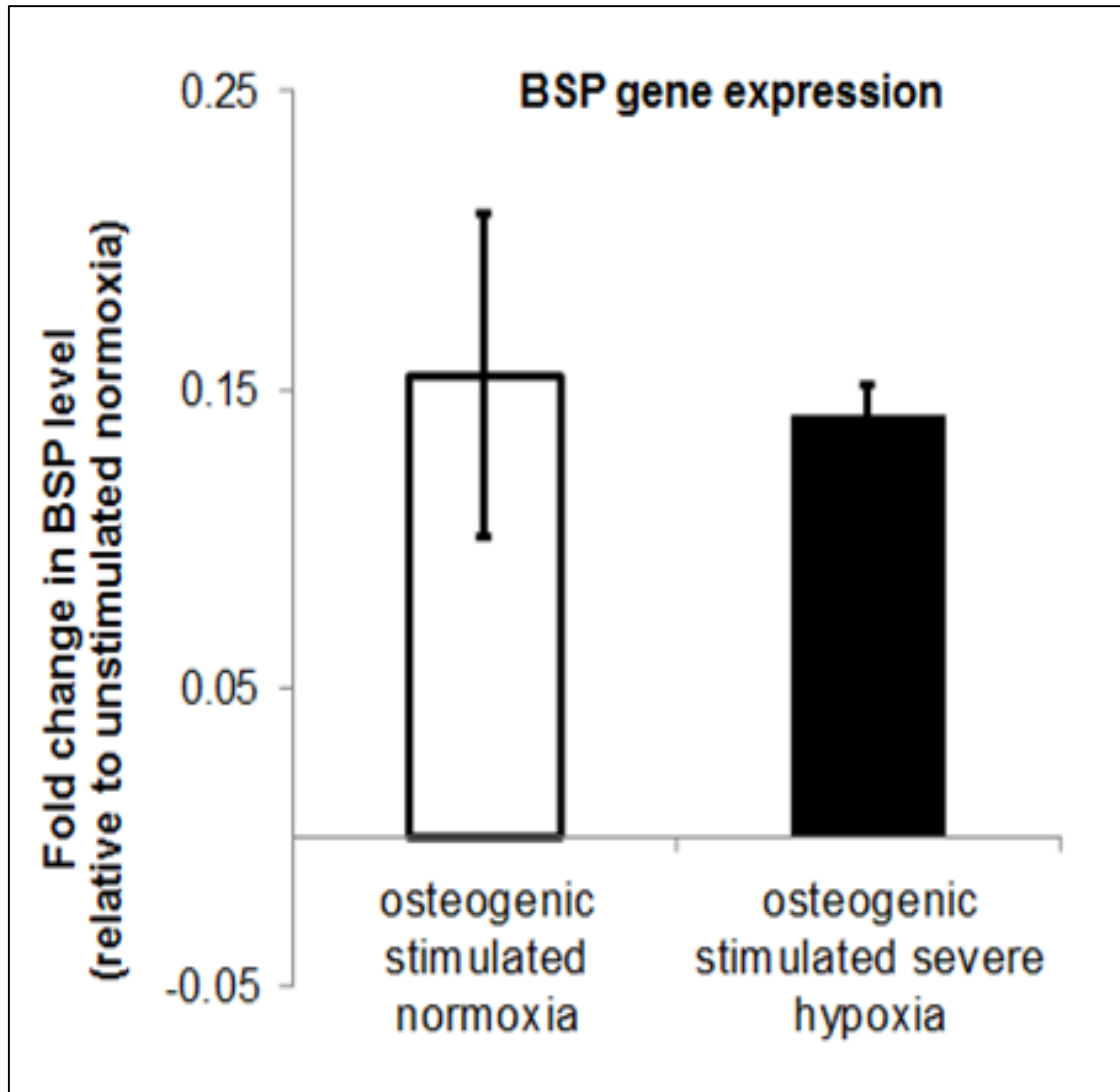
Figure 20. Post-hypoxic osteogenic differentiation based on bonesialoprotein (BSP) gene expression

Gene expression of BSP in OFMSCs treated under normoxic and hypoxic treatment conditions was assessed by real time-PCR. BSP showed decreased expression in the hypoxic treatment group but was not significant.
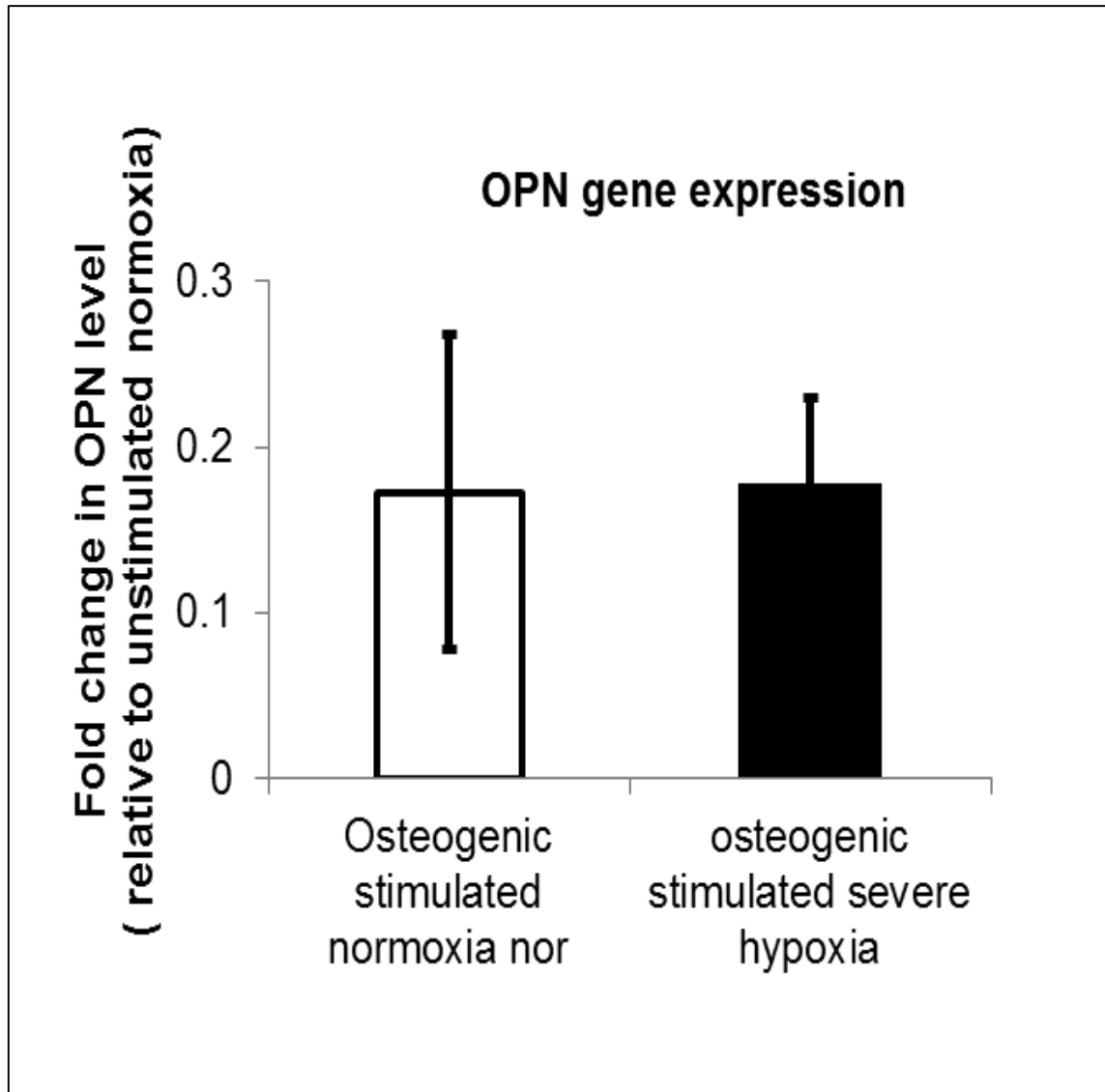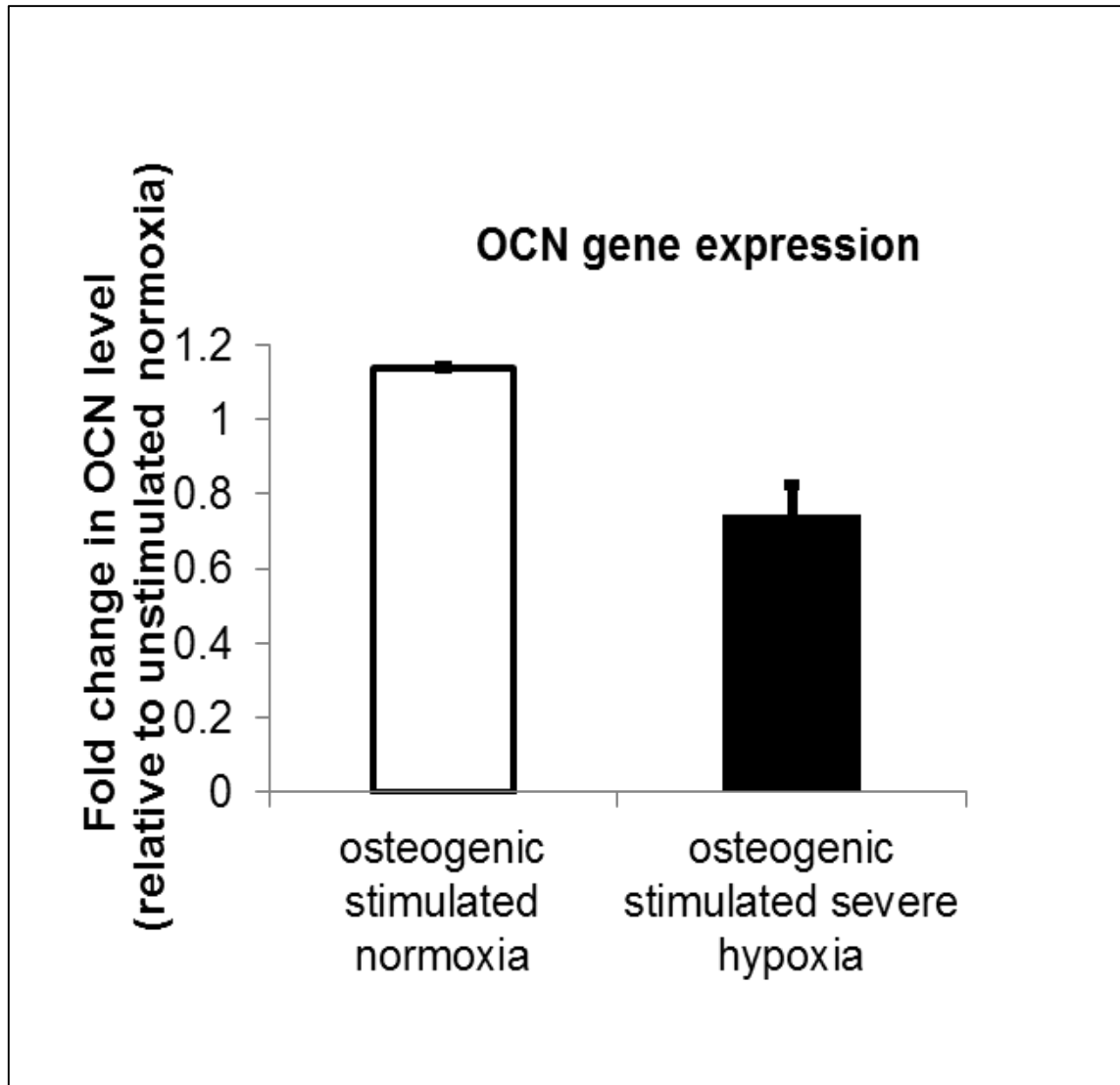
Figure 21. Post-hypoxic osteogenic differentiation based on osteopontin (OPN) gene expression.

Gene expression of OPN in OFMSCs treated under normoxic and hypoxic treatment conditions assessed by real time-PCR showed comparable expression levels in both treatment groups.

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Figure 22. Post-hypoxic osteogenic differentiation based on osteocalcin (OCN) gene expression

OFMSCs treated under normoxic and hypoxic treatment conditions assessed by real time PCR showed a 0.2-fold decreased expression level in the hypoxic treatment group.

Effect of severe hypoxia on i*n vivo* osteogenic differentiation

In addition to *in vitro* osteogenesis, the ability of OFMSCs to recover from hypoxic insult and form bone was also assessed *in vivo*. H&E staining of normoxic and hypoxic treated OFMSCs harvested from the subcutis of immunocompromised mice after 8 weeks of transplant (Figure 23 & 24), showed similar bone formation pattern within the hydroxyapatite in both groups. An average bone score of 4 was assessed in each group.
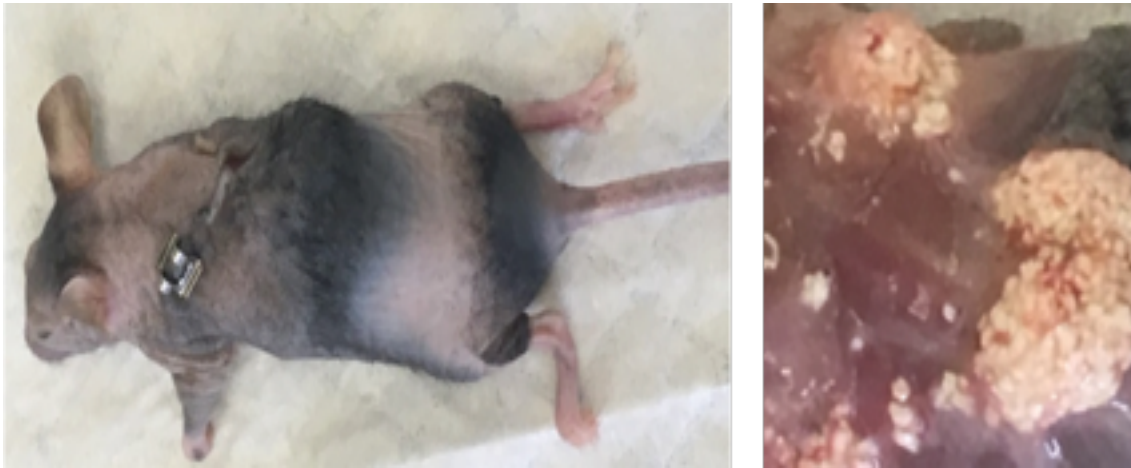
Figure 23. Transplantation of OFMSCs post-hypoxia.

A representative immunocompromised nude mouse showing transplanted OFMSCs with carrier before and after surgical exposure and harvesting.
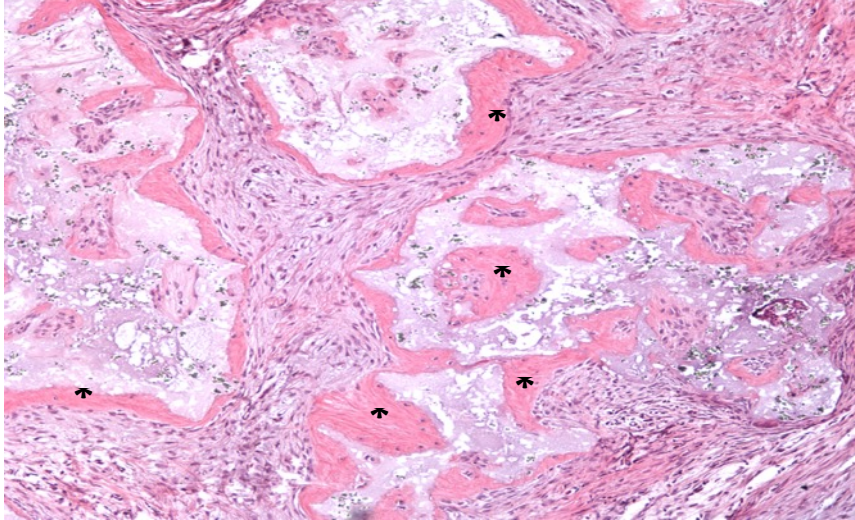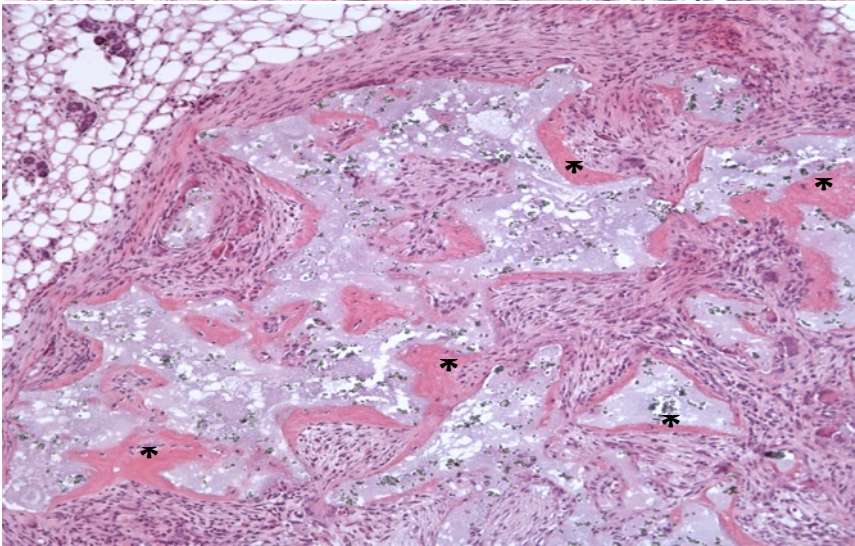
Figure 24. Photomicrograph of H& E stained sections of tissue formed from transplanted OFMSCs

Harvested transplants of OFMSC treated under normoxic conditions (24A above) showing bone formation (black arrow) within the hydroxyapatite after 8 weeks of transplant.Similar bone formation pattern noted in OFMSCs treated under hypoxic conditions (24B below). [Magnification 20x]

Adipogenesis

In further assessing the effect of severe hypoxia on differentiation, OFMSCS were induced adipogenically for 21 days post hypoxia. Oil red-O staining following fixation with 4% formaldehyde showed the presence of adipocytes in both treatment groups. This was evidenced by bead-like red stains in the perinuclear area of the cells as shown in Figure 25.

Additionally, assessment of adipogenic markers post-hypoxia showed a 0-2-fold (p= 0.4) and 0.5-fold (p =0.01) in PPAR gamma and LPL levels respectively (Figure 26, 27), when assessed relative to hypoxia by real time real time PCR. Protein expression of cytoplasmic phospholipase A2, showed a 0.1-fold decrease (p =0.8) in the hypoxic treatment group (Figure 28).
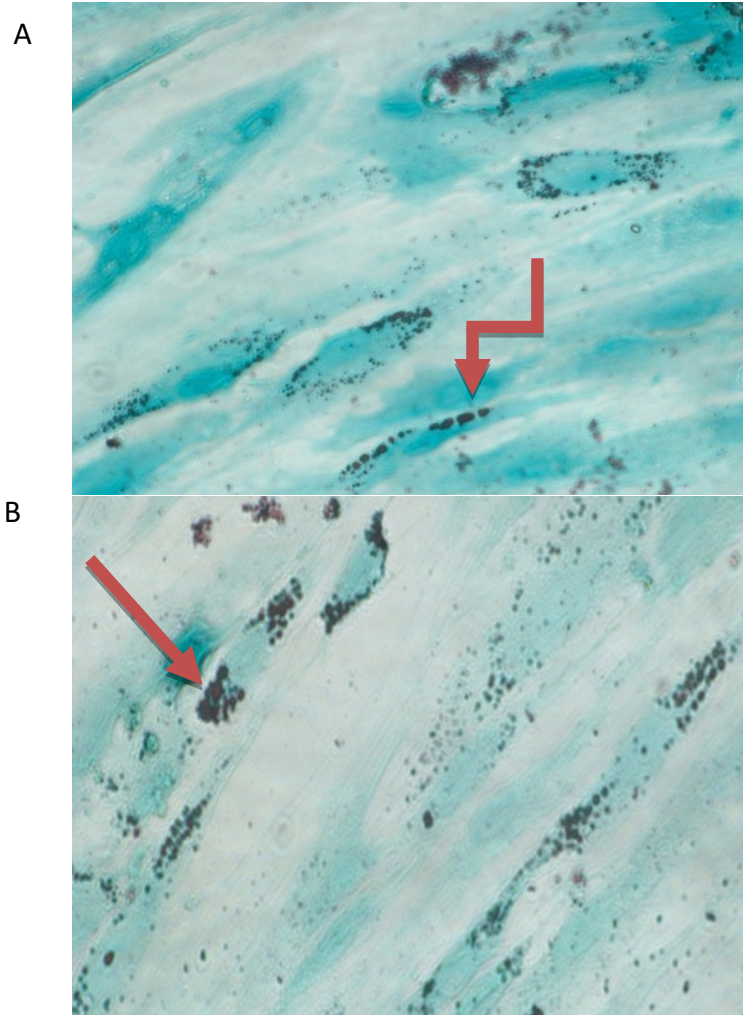
Figure 25. Post-hypoxic adipogenic differentiation based on Oil Red O staining.

The red staining sections show adipocytes surrounding nuclei in normoxic treated OFMSCs (25A, above) and severe hypoxic treated OFMSCs (25B, below). Counterstaining carried out with fast green dye.
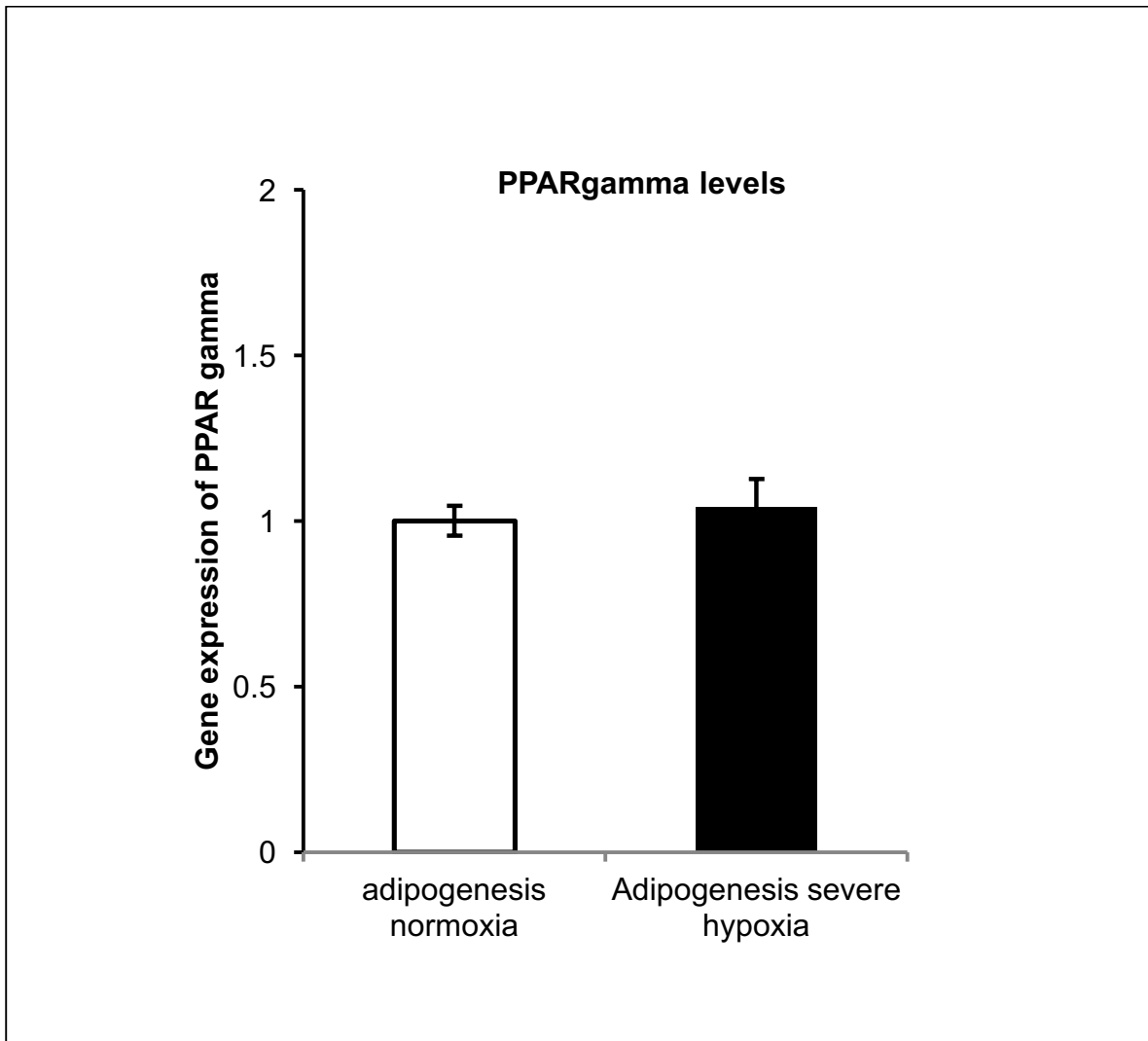
Figure 26. Post hypoxic adipogenic differentiation in OFMSCs based on PPAR gamma expression.

PPARgamma, a marker of adipogenesis was expressed similarly by both normoxic and hypoxic treated OFMSCs (p = 0.4).
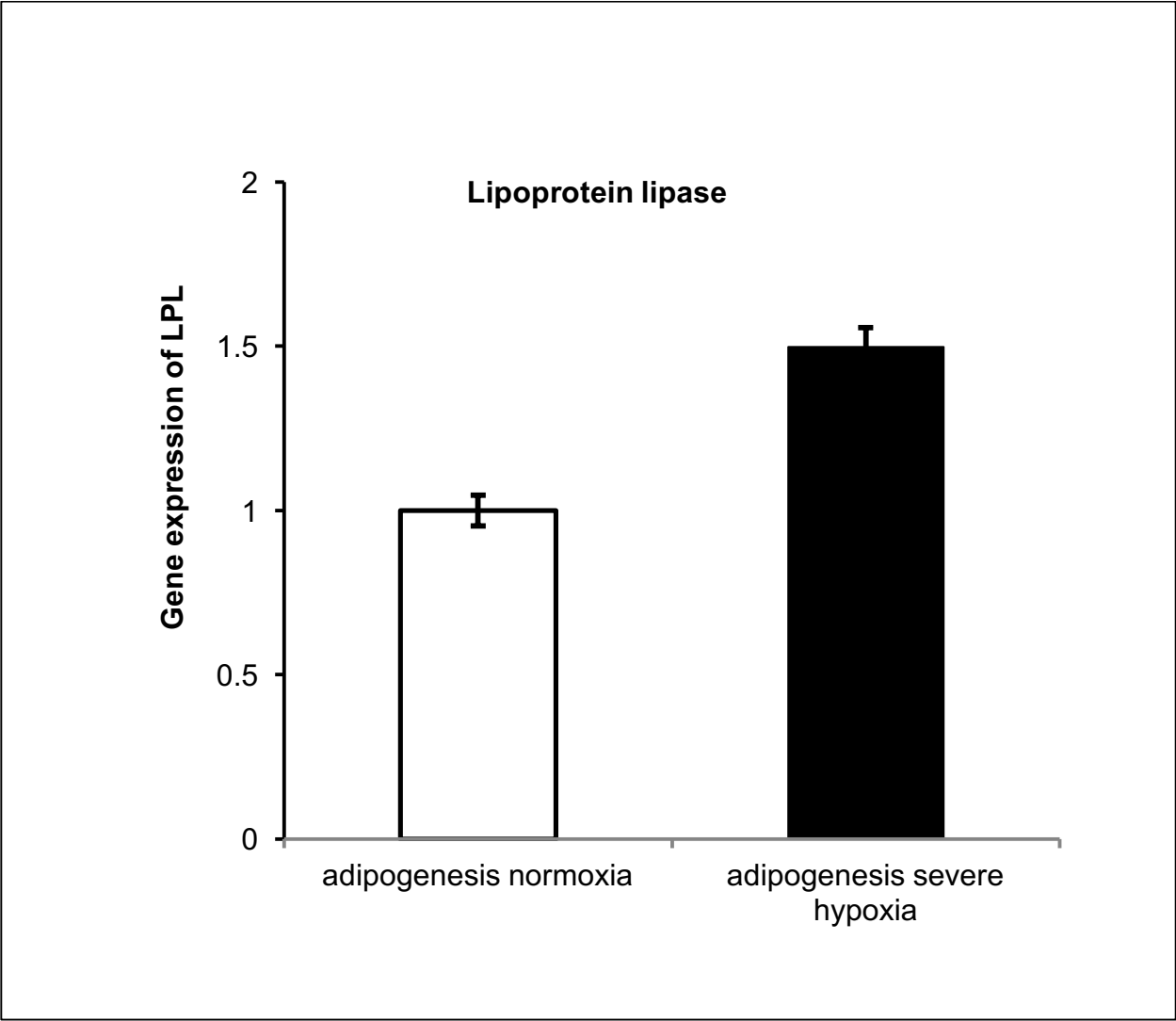
Figure 27. Post hypoxic adipogenic differentiation in OFMSCs (Lipoprotein lipase)

Lipoprotein lipase, a marker of adipogenesis showed a 0.5-fold increase in gene expression in the hypoxic group (p = 0.01).
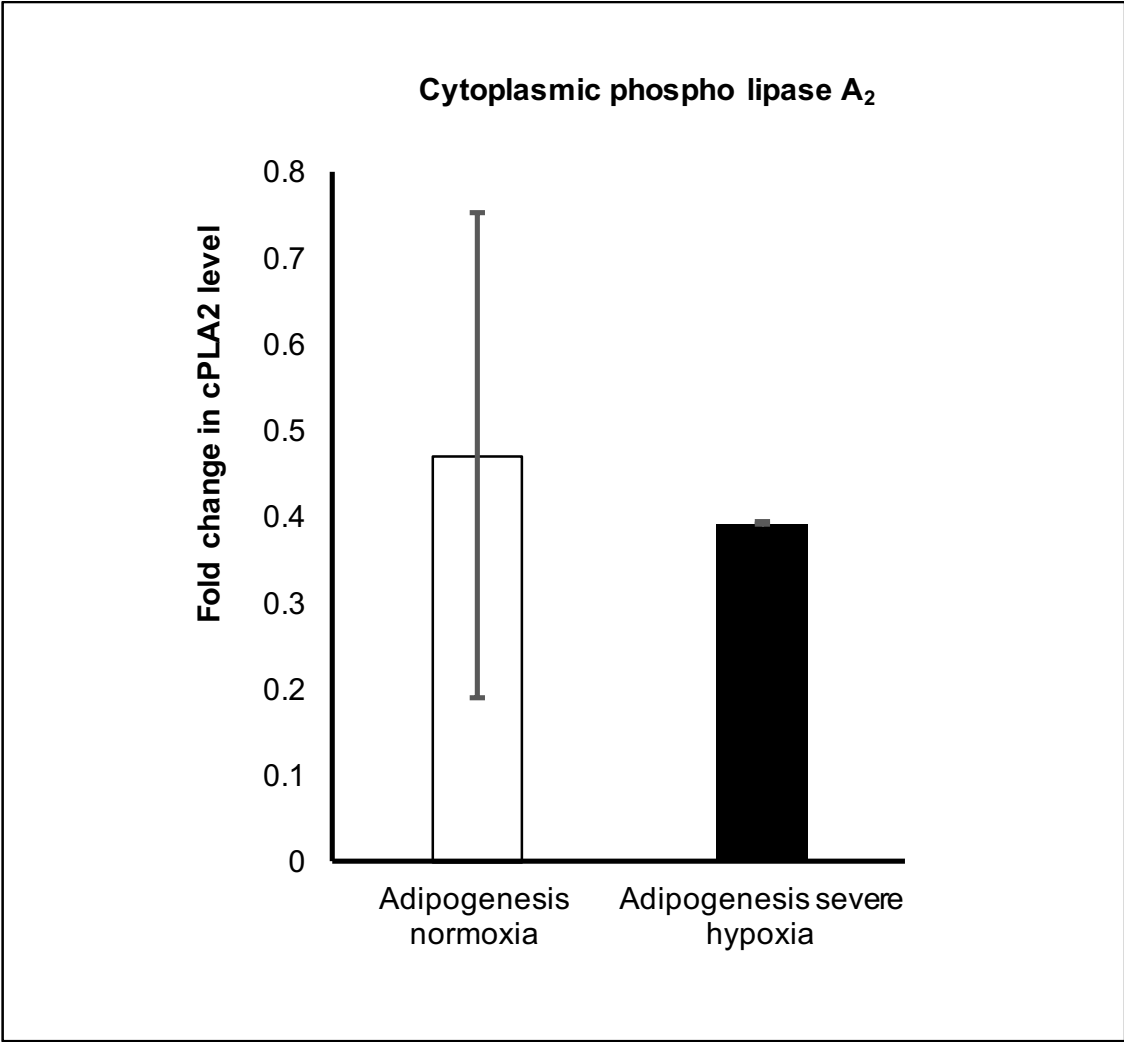
Figure 28. Post hypoxic adipogenic differentiation in OFMSCs based on expression levels of cytoplasmic phospholipase A2.

There was minimal difference in expression levels of cytoplsmic phospholipase A2 between the normoxic and hypoxic groups ($p = 0.8$).

Role of HIF1 in responsiveness of OFMSCs to hypoxia

Mechanistically, HIF-1α signaling pathway, a pathway that regulates cellular response to hypoxic stress when assessed by immunofluorescence, immunoblotting real time PCR in OFMSCs showed the presence of more positive cells (Figure 29) and increased expression levels in the hypoxic treatment group. While protein expression showed a 0.4-fold increase (p =0.015) (Figure 30B & 30B), gene expression demonstrated a 0.1-fold increase (p=0.77) (Figure 30C).
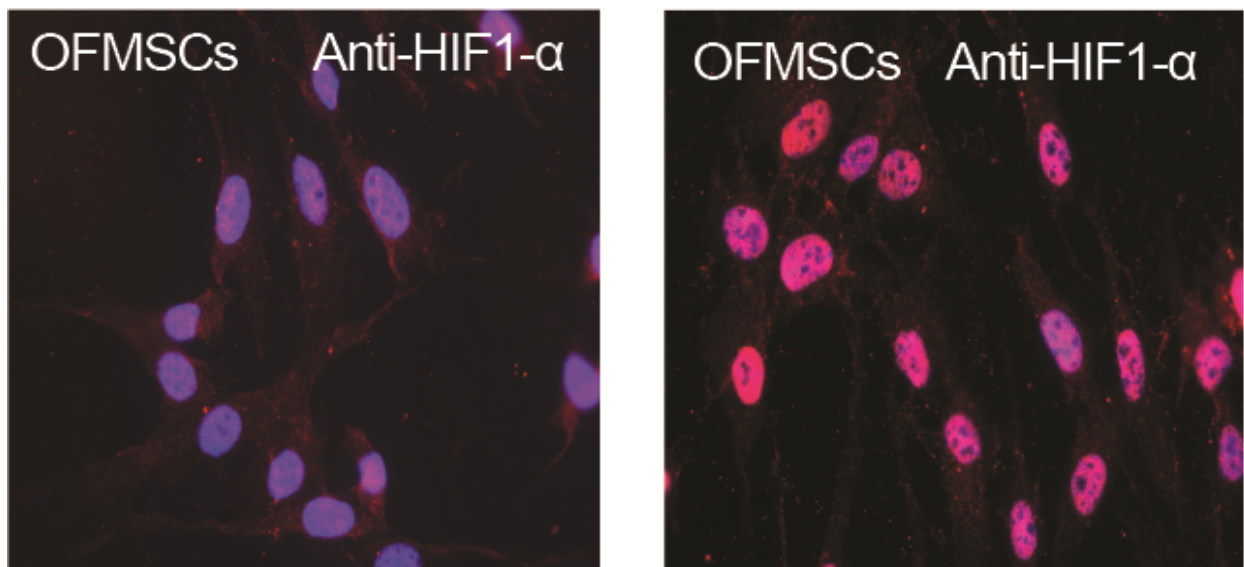
Figure 29. HIF-1α activation in hypoxic OFMSCs

There was a higher number of HIF-1α positive OFMSCs activated by severe hypoxia based immunofluorescent staining with anti-HIF-1α
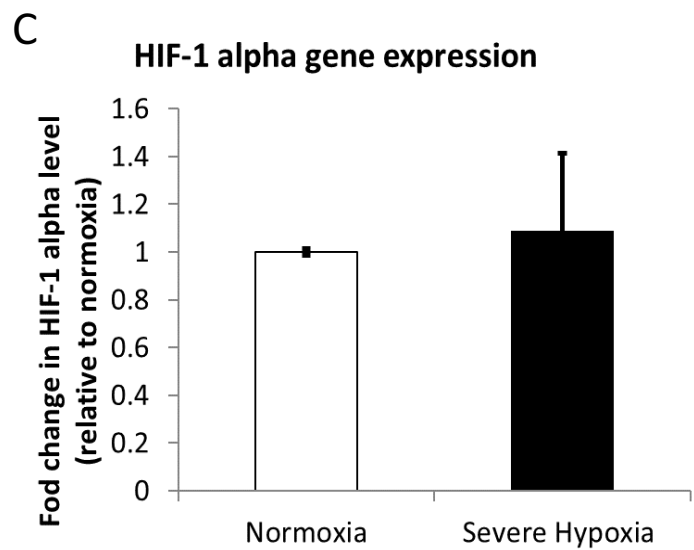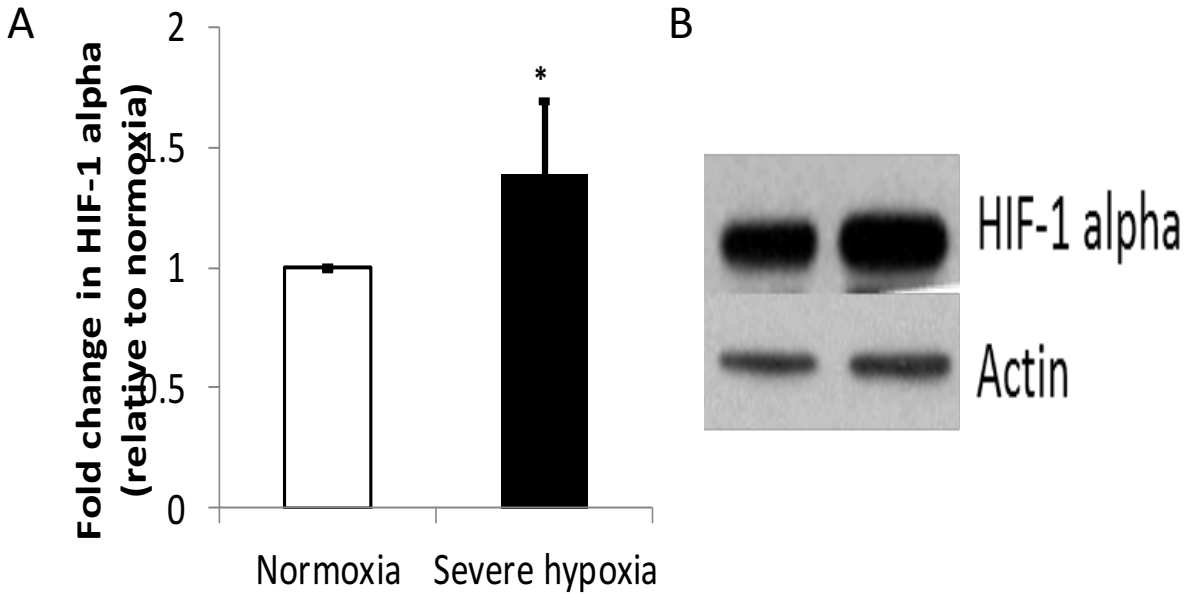
Figure 30. Post-hypoxic assessment of HIF-1α levels

Activation of HIF-1α is demonstrated by 0.4-fold increase by immunoblotting (p =0.015) (30A & 30B) and minimal 0.1-fold increase based on real time PCR (p=0.77) (30C)

Post-hypoxic assessment of ER stress response pathway:

In addition to the mechanistic assessment of HIF-1α, markers of ER stress response signaling pathway, a pathway that responds to cellular stress was evaluated. A 0.5-fold (p =0.28) (Figure 31A & 31B) and 5-fold (p =0.036) (Figure 32) increase in BiP and phosphorylated eIF2α protein expression levels respectively was noted in the hypoxic treatment group.

Furthermore, gene expression of ATF6, which is upstream of phosphorylated eIF2α in ER stress response pathway showed a 0.5-fold (p = 0.28) increase in the hypoxic treatment group (Figure 33).
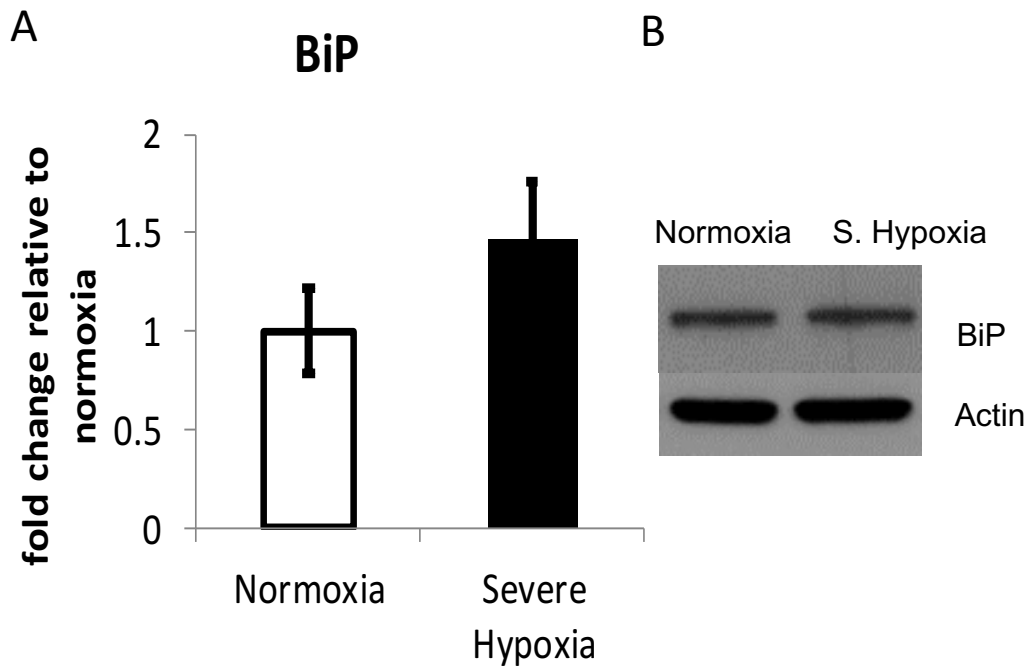
Figure 31. Assessment of post-hypoxic ER stress responsiveness of OFMSCs based on Bip level

Activation of Bip a responsive element in the ERSR pathway is minimally higher based on immunoblotting.
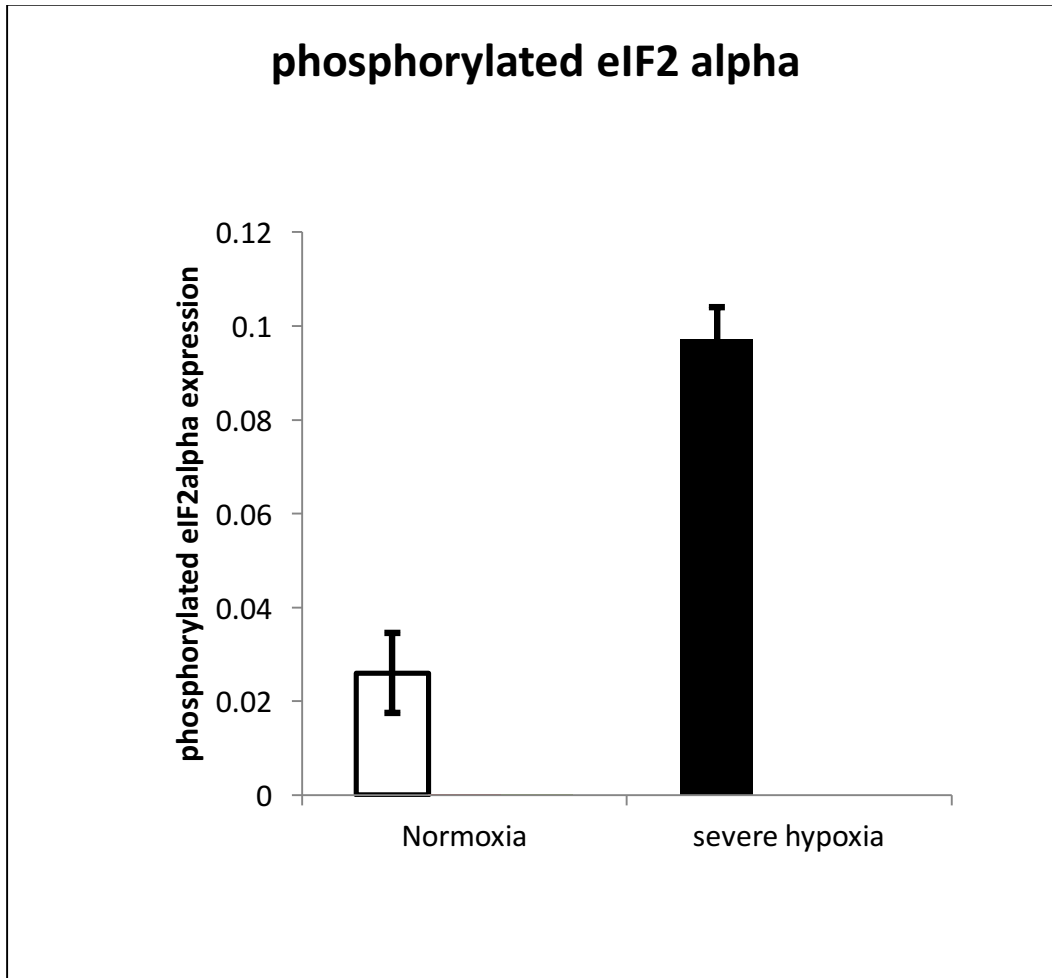
Figure 32. Assessment of post-hypoxic ER stress responsiveness of OFMSCs based on activation of the PERK arm of ERSR

The level of phosphorylated eIF2α based on immunoblotting shows a significant 5-fold (p =0.036) increased in the hypoxic treatment group of OFMSCs.
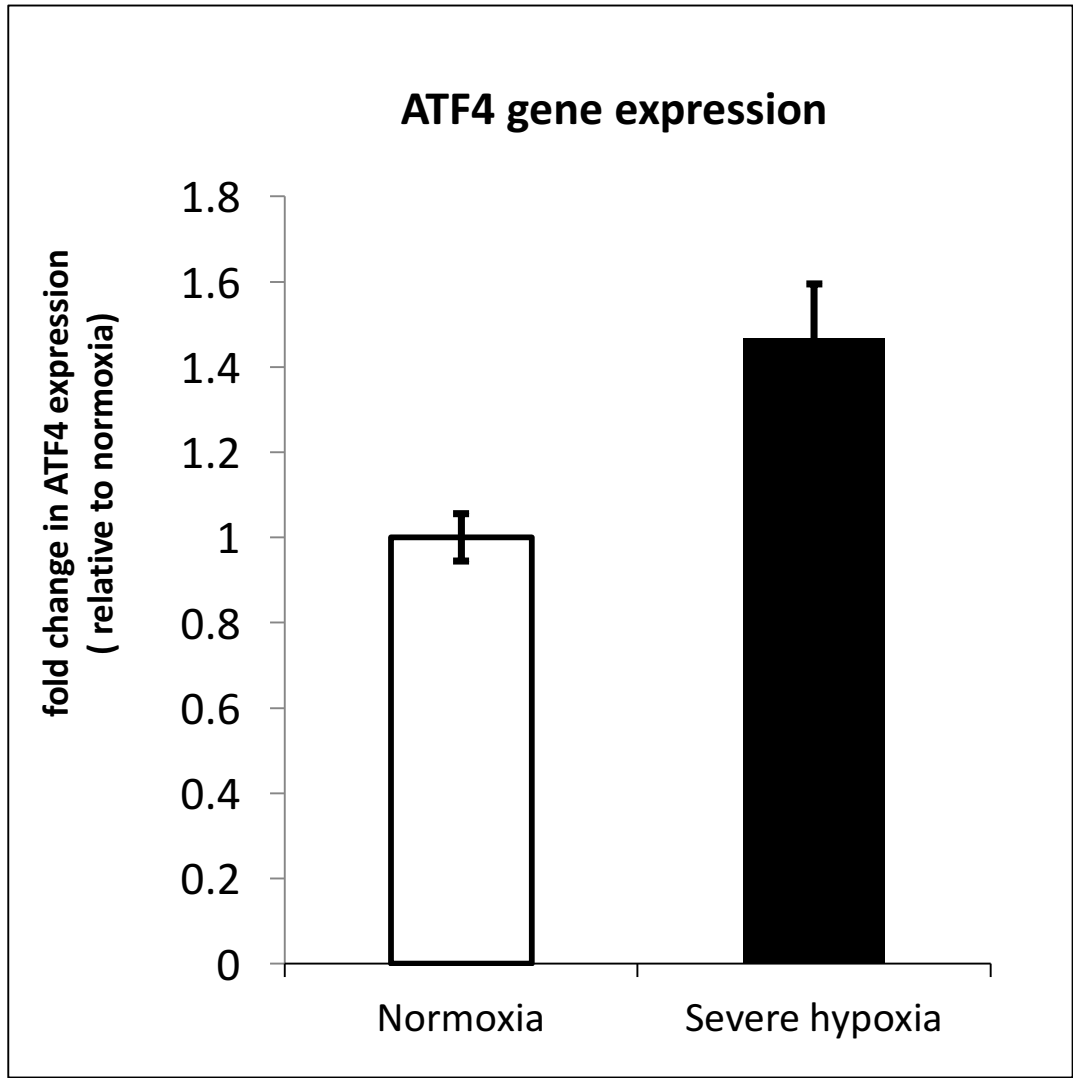
Figure 33: Post hypoxic ER stress response pathway assessment (PERK signals)

The level of ATF4 downstream of phosphorylated eIF2α based on immunoblotting shows a minimal 0.5-fold (p =0.036) increased in the hypoxic treatment group of OFMSCs.

Interplay between HIF-1α and ERSR pathways

We further assessed the interplay between the two mechanistic pathways (HIF-1α and ERSR) with and without chemical upregulation and downregulation of both pathways. Gene expression of HIF-1α showed a 3.5- fold increase (p= 0.015) in the hypoxic treatment OFMSC group, after 18 hours of chemical upregulation of the ERS response pathway by thapsigargin (Figure 34).
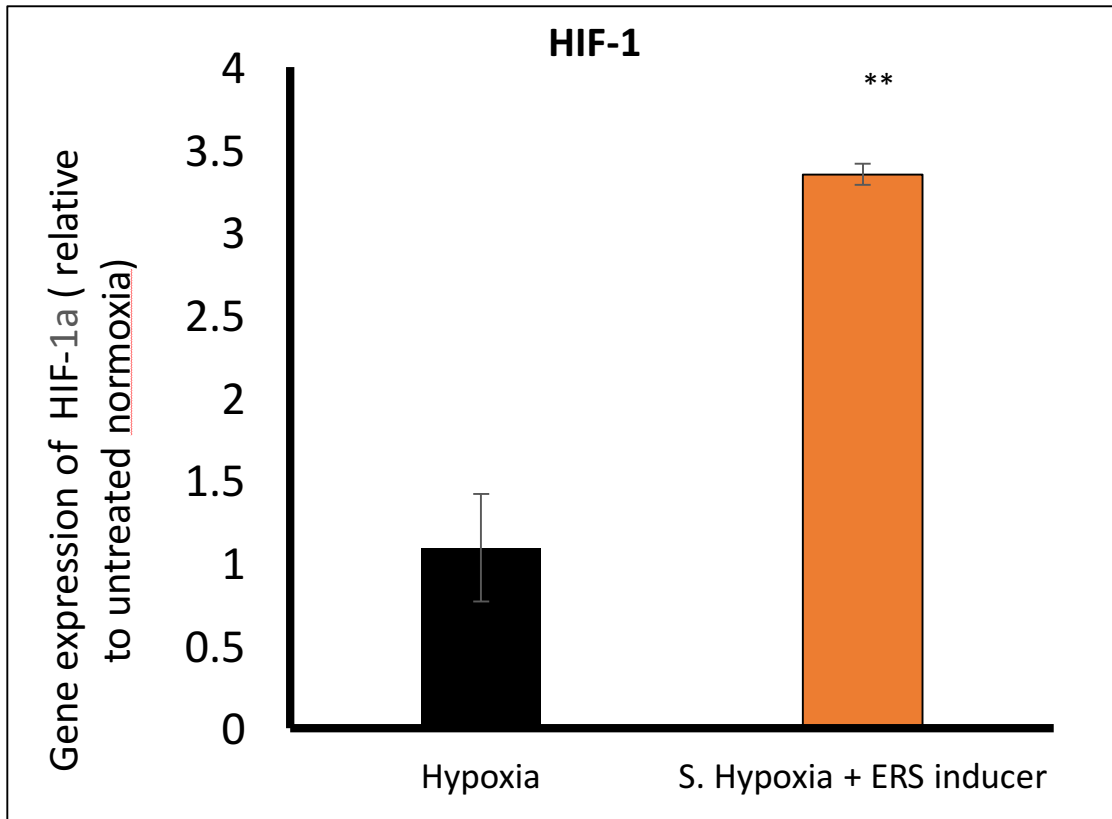
Figure 34. Combined effects of severe hypoxia and ERSR on OFMSCs.

HIF-1α expression in OFMSCs was significantly upregulated by the combined effects of severe hypoxia and ERSR relative to hypoxia only. Fold change is relative to OFMSCs treated with normoxia only. (p = 0.015)

The HIF-1α pathway inducer and downregulator evaluated were 2,4-DPD and Quinomycin respectively.
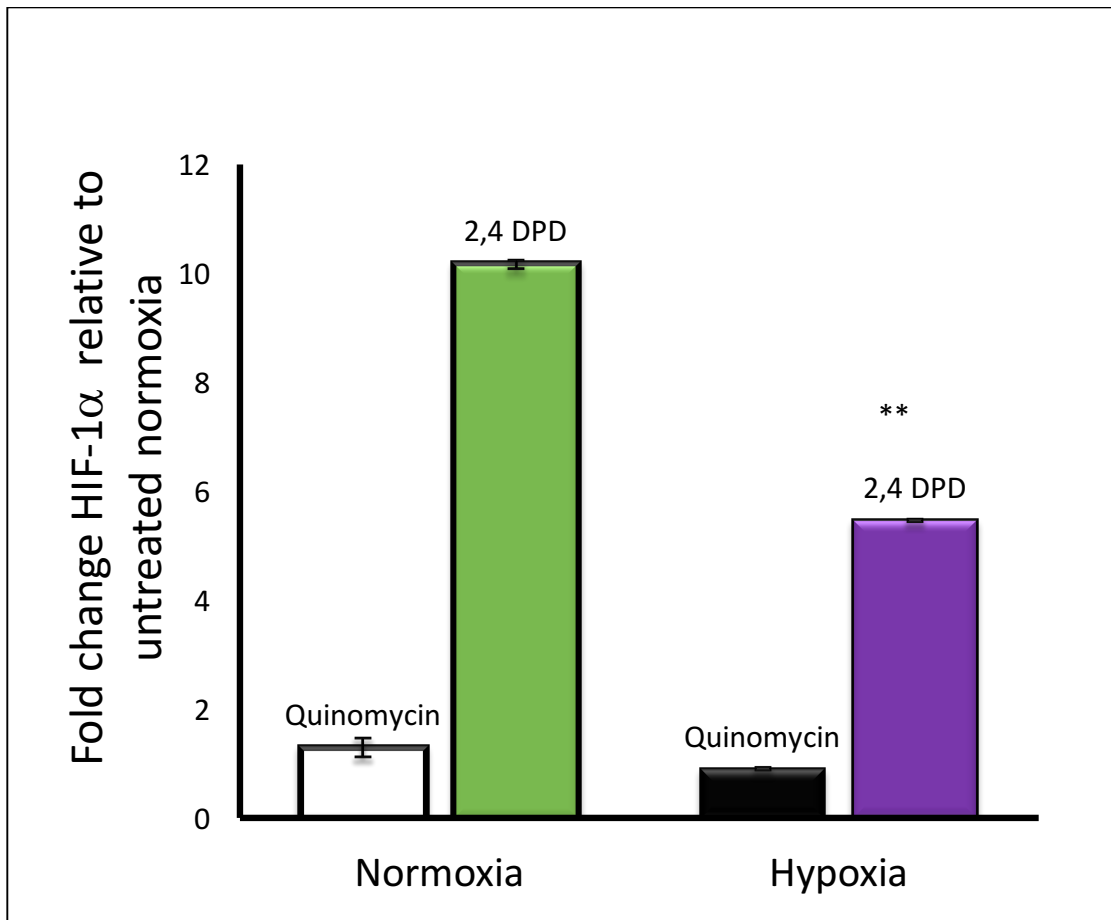
Figure 35: Effects of blockage and upregulation of HIF-1α under severe hypoxia.

While the combination of quionomycin with severe hypoxia showed no effects compared to normoxia, the upregulation of HIF-1α .by 2,4-DPD was attenuated under severe hypoxia (** $p < 0.05$)

Protein expression of Quinomycin when assessed relative to OFMSCS not chemically stimulate, showed an approximately 2-fold increase in the normoxia treatment group and no changes in the hypoxic treatment group. On the other hand, OFMSCs treated with 2,4-

DPD produced a 10-fold and 6-fold increase in HIF-1 α expression under normoxia and

severe hypoxic conditions respectively (Figure 35).
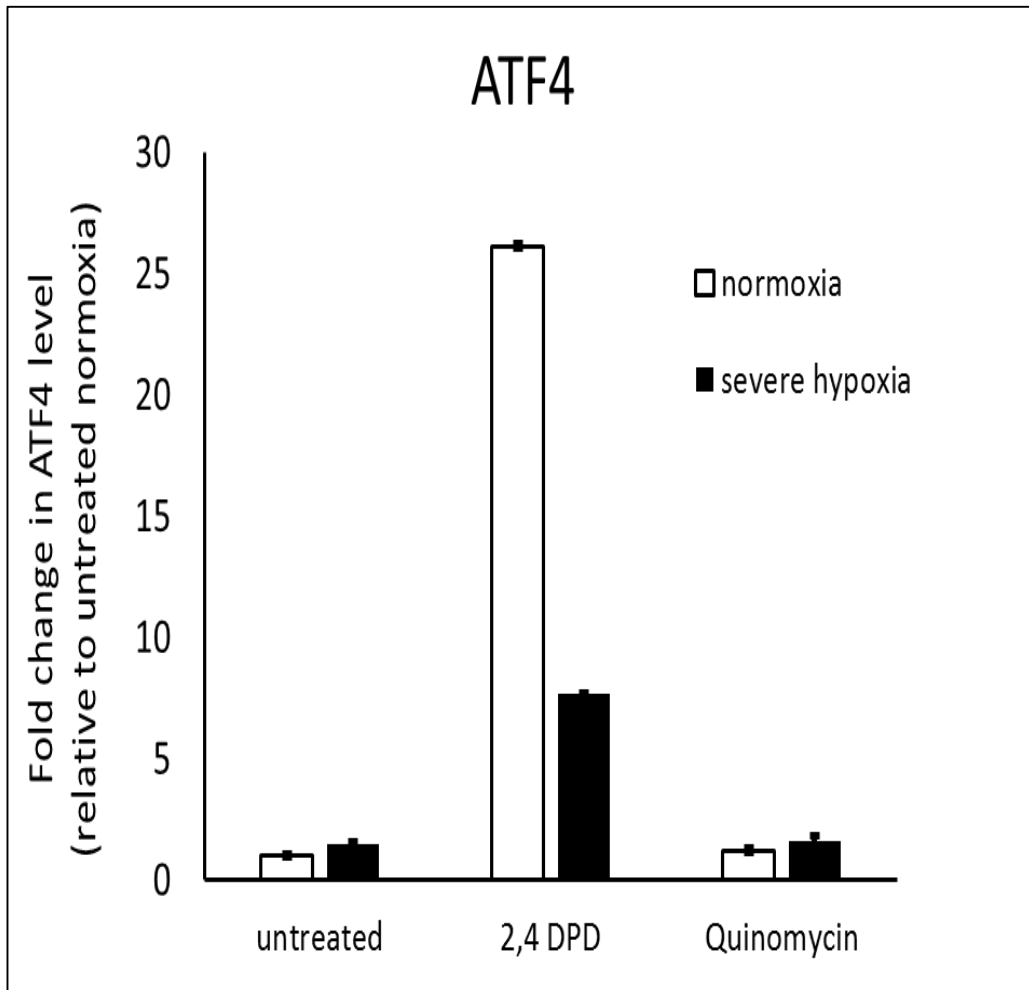
Figure 36: Effects of blockage and upregulation of HIF-1α under severe hypoxia.

While the combination of quionomycin with severe hypoxia showed no effects compared to normoxia, the upregulation of HIF-1α .by 2,4-DPD was attenuated under severe hypoxia (** $p < 0.05$)

Further interactions between both pathways was assessed by evaluating expression level of ATF4 after upregulation and downregulation of HIF-1α in both treatment groups. A 26-fold increase was noted in the expression level of ATF4 following normoxic treatment of OFMSCs with 2, 4-DPD, while an approximate 8-fold increase was noted under severe
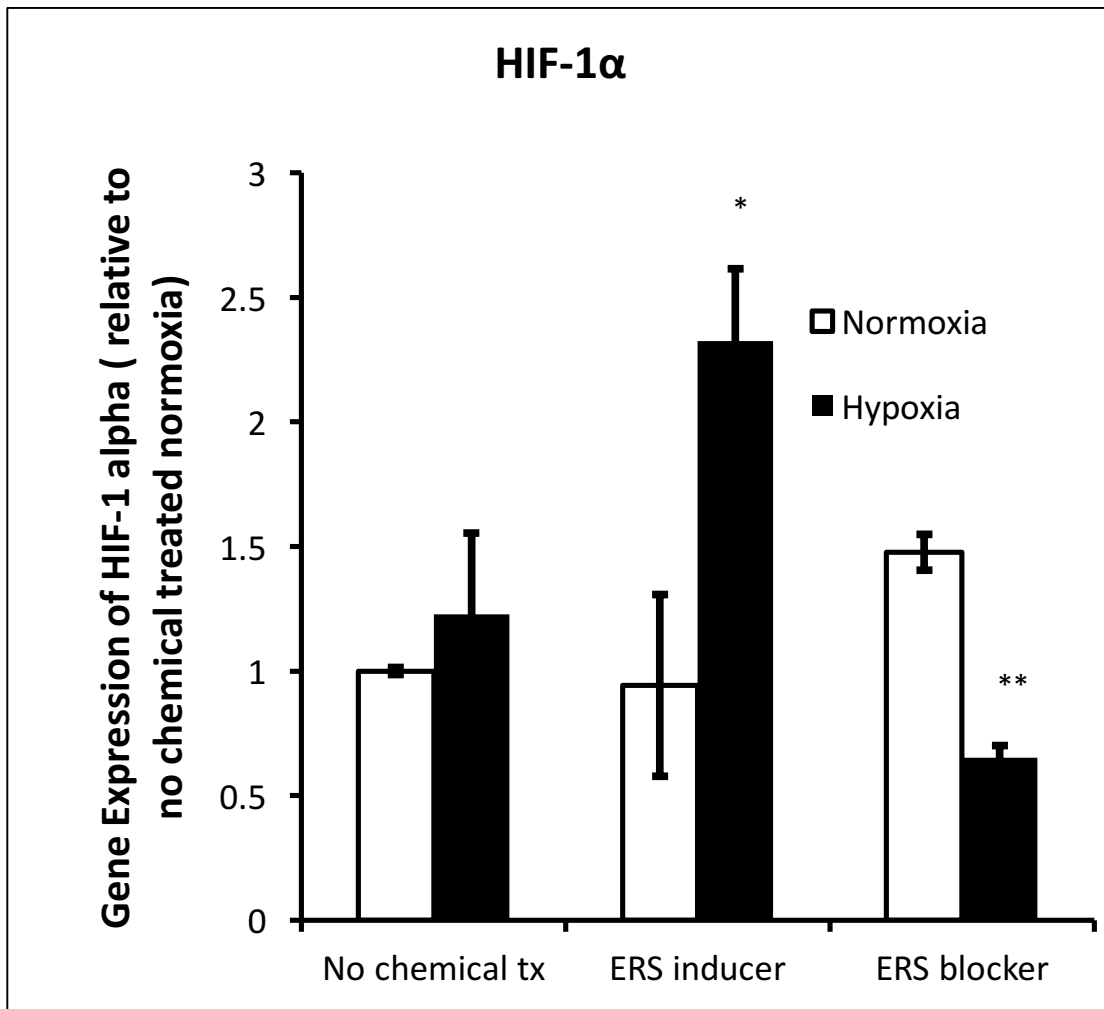
Figure 37: Combination of severe hypoxia and ERSRS activates HIF-1α

hypoxia. On the other hand, expression of ATF4 in quinomycin treated OFMSCs under normoxic and hypoxic conditions, showed similar patterns with the group treated without any inducers or downregulators. (Figure 36).

Finally, gene expression of HIF-1α was also assessed following induction and downregulation of ERS response, using Thapsigargin and ISRIB respectively. A 2.5-fold increase was noted in HIF-1α response of OFMSCs treated under both severe hypoxia and ER stress induction (thapsigargin) (Figure 37).

Post hypoxic assessment of VEGF expression

Since VEGF is downstream of both HIF and ERSR signaling pathways, we assessed the effect of severe hypoxia on both pathways. A 1-fold increase (p = 0.017) in gene expression was noted in the severe hypoxic treated group when compared with normoxia (Figure 38A). This was further confirmed by immunohistochemistry (Figure 39), which showed more reactive VEGF on tissue sections from the harvested transplants in the hypoxic treatment group. Protein expression however showed no significant differences between the two treatment groups (Figure 38B).

The effect of HIF-1α and ERS response pathways on VEGF expression was also assessed by chemical inhibition and enhancement of both pathways. VEGF gene expression was noted to be 16-fold and 8-fold higher in the normoxic and severe hypoxic treatment group pretreated with 2,4 DPD (Figure 40) respectively. Finally, when OFMSCs were induced by thapsigargin, the hypoxic treatment group showed approximately 5-fold increase compared to the normoxic group (Figure 41) (p=0.00085).
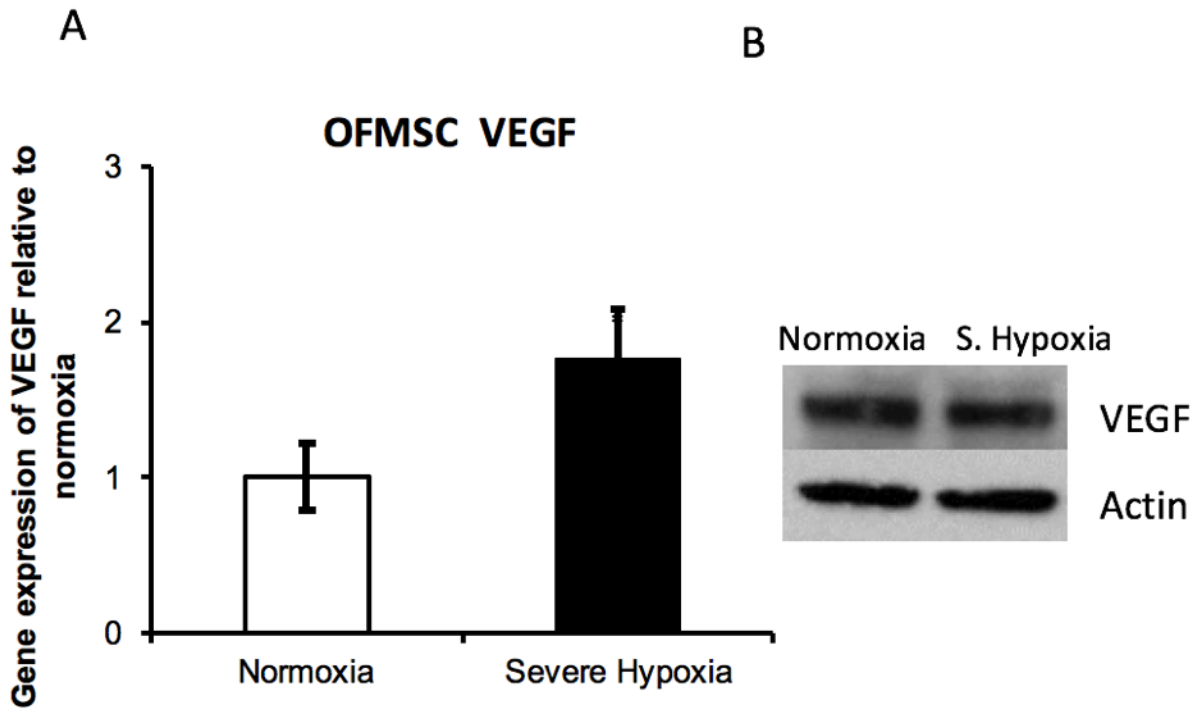
Figure 38: Post hypoxic recovery of OFMSCs (VEGF expression- *in vitro*)

Gene expression of VEGF shows significant upregulation in severe hypoxic treatment group when compared with normoxia, with a 0.8-fold increase in the hypoxic treatment group (p= 0.017). Protein expression however show no difference between the two groups.
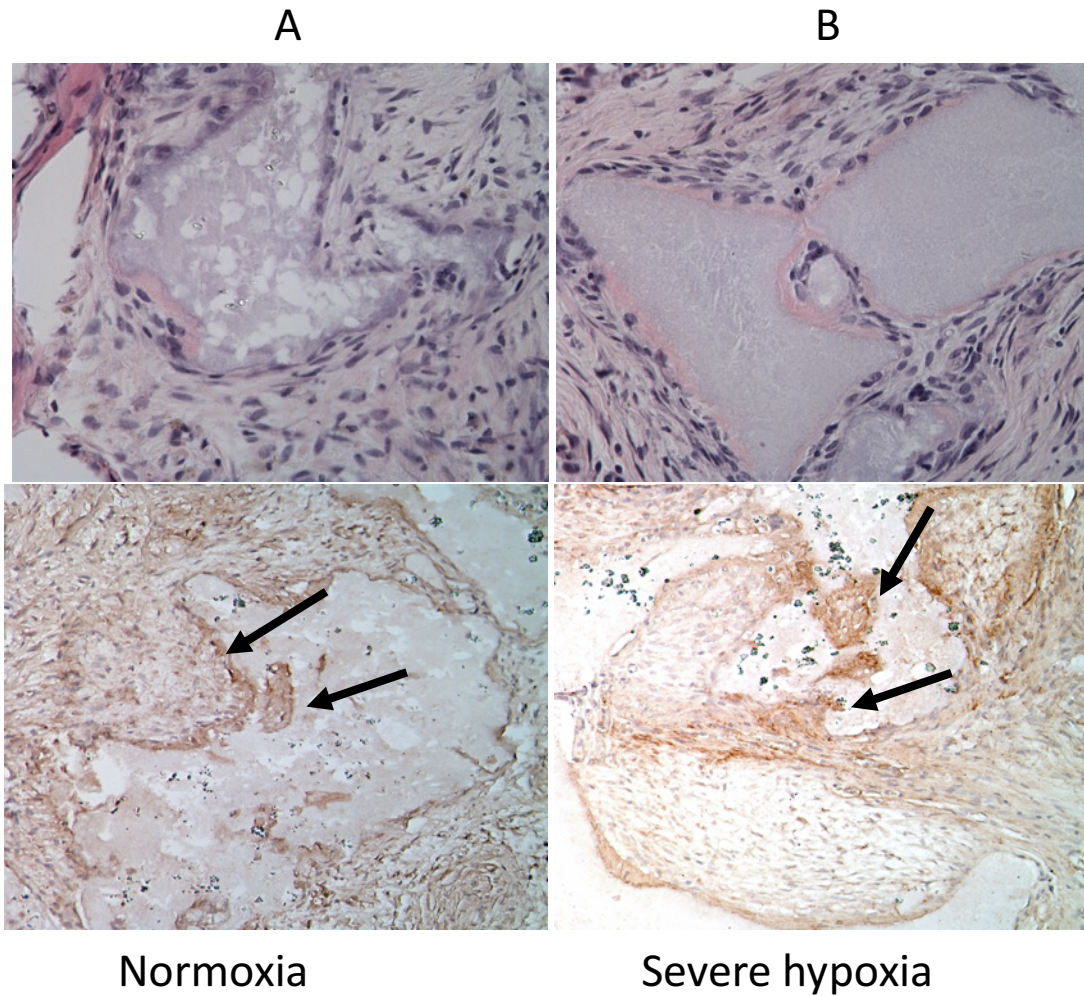
Figure 39: Post hypoxic recovery of OFMSCs based on in vivo bone regeneration and VEGF expression

H&E staining of the transplant tissue. Shows bone formation within the hydroxyappatite. More bone formation noted in the severe hypoxia treatment group. Image below shows VEGF expression within the hard and soft tissues, with more VEGF expresson noted in the transplant pretreated with hypoxia.
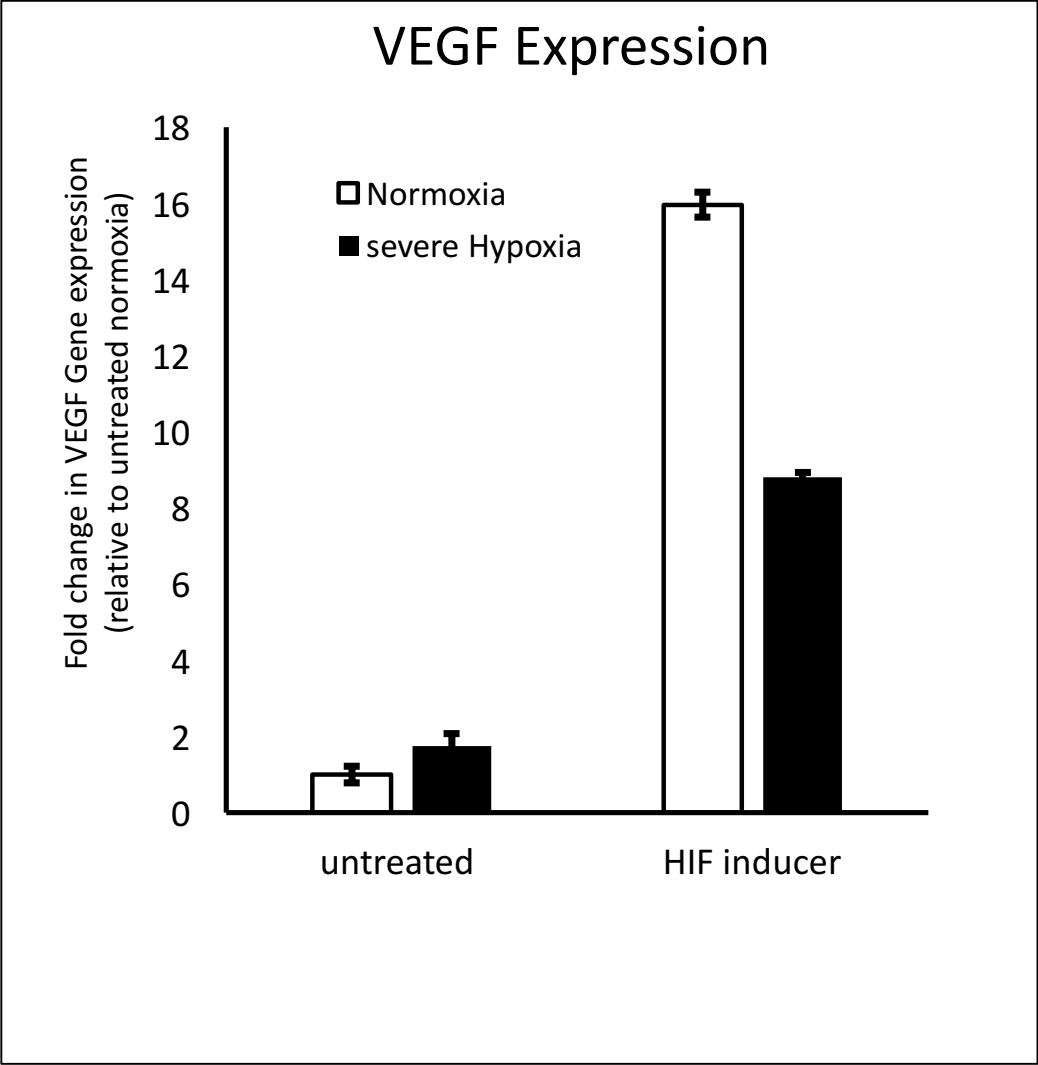
Figure 40: Post hypoxic recovery of OFMSCs based on VEGF expression

Gene expression of VEGF shows significant upregulation in severe hypoxic treatment group when compared with normoxia, with a 0.8-fold increase in the hypoxic treatment group (p= 0.017). Protein expression however show no difference between the two groups (data not shown).
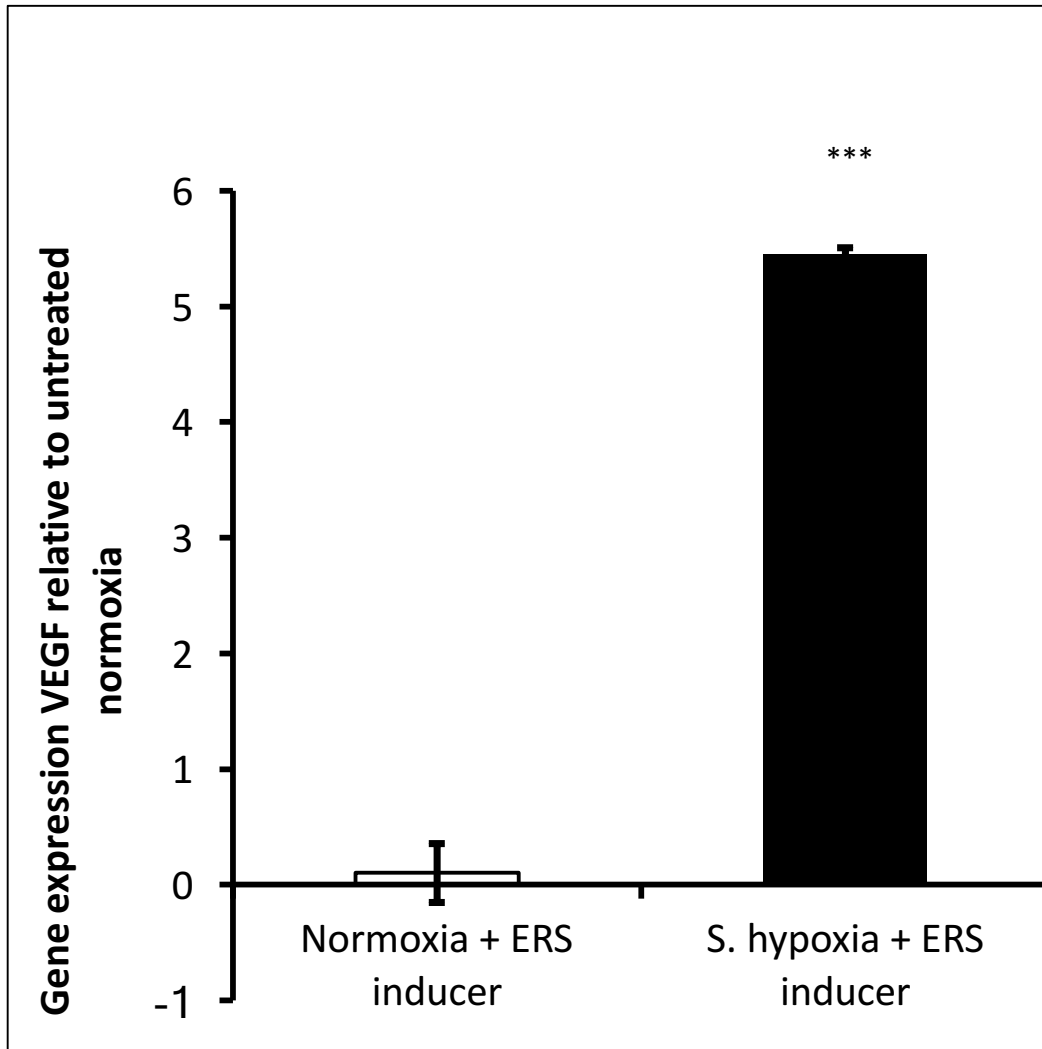
Figure 41: Interplay of ERSR pathway induction and hypoxia on OFMSCs VEGF expression.

Gene expression of VEGF in the two treatment groups pretreated with 1mM of thapsigargin. VEGF expression noted to be higher in the severe hypoxic treatment group. (p=0.00085)

CHAPTER FIVE

5.0   DISCUSSION

Bone MSCs within the bone marrow depend on a physiologic hypoxic niche to effectively maintain their 'stemness, renewal, survival and differentiation properties (Hu X. et al 2014). A combination of stem cell resistance and adaptation to this hypoxic milieu is likely responsible for preserving the phenotypic and functional MSC properties of MSCs (Buravkova, 2014).

There is meager information on responsiveness of orofacial MSCs to non-physiological hypoxia. Most of the previous studies on effects of severe hypoxia on MSCs tested physiological hypoxia rather than severe hypoxia. This is because the oxygen tension used for the studies was compared to atmospheric oxygen *in vitro* rather than the physiologic oxygen conditions that MSCs are familiar with (Hu, X. 2014, Xu 2014, Holzwarth 2010, Ito 2015, Grayson 2007). Furthermore, orofacial MSCs were not included despite the fact that MSCs from many other body sites were tested (Hu, X. 2014; Ito, A. 2015; Holzwarth,C. 2010; Xu,L. 2014; Grayson,W.L. 2006)

Orofacial MSCs were the focus of this project because of its clinical relevance to pathophysiology and management outcomes of jaw osteoradionecrosis, a major cancer complication of head and neck radiation therapy. Hypoxia plays a central role in the hypoxic-hypovascular-hypocellular mechanistic theory of ORN pathogenesis. Additionally, MSCs are phenotypically and functionally unique based on their embryological site of origin.

To simulate radiation-induced hypoxia, we used a hypoxic chamber to induce severe hypoxia at 0.1% oxygen tension. In assessing the effect of severe hypoxia on human OFMSC plasticity, we utilized 0.1% O_2 tension in a hypoxic chamber. Severe hypoxia was tested in this project because stem cells in general are resistant to low oxygen tensions that exist at physiologic levels (around 2% O_2) (Buravkova,L.B. 2014), while extremely low oxygen tension induces pathologic effects (Martin-Rendon,Enca 2007). Our study noted that when compared to OFMSCS treated under normoxic (21% O_2) and physiologic hypoxic (2% O_2) conditions, OFMSCs treated with severe hypoxia (0.1% oxygen) succumbed to the hypoxic insult. A similar finding was reported by Hutton *et al* in 2016. The authors noted that adipose-derived MSCs from female patients succumbed to hypoxic treatment with oxygen tension of 0.2% (Hutton 2016). Our data also suggest that severe hypoxia can change the MSC population because undifferentiated pool of osteoprogenitors were severely depleted based on decrease in STRO1+, CD146 and α-SMA positive cells. The implication of this effect is that when severe hypoxia depletes OFMSCs osteoprogenitors, there are fewer osteogenically active cells that can differentiate to pre-osteoblast. Hence, recovery and bone regeneration post-hypoxia is severely suppressed. This undoubtedly underscore the role of hypoxia in the pathogenesis of jaw ORN.

To further compound the suppression of osteoprogenitors, the increase production of ROS and oxidative stress under severe hypoxia will further reduce the number of surviving OFMSCs(Cicione C. 2013). The interplay of depleted osteoprogenitors, ROS, oxidative stress and decreased OFMSCs survival further enhance jaw susceptibility to ORN.

Since the population of MSCs changed after severe hypoxia, it is imperative to access the recovery capacity of residual MSCs that overcome severe hypoxia. The premise is that if a some MSC populations survive radiotherapy-induced hypoxia, it may be possible to reactivate these cells, promote post-hypoxic healing and reduce susceptibility to ORN. Interestingly, surviving OFMSCs displayed appreciable population doubling capacity and colony forming efficiency and decreased autophagic and apoptotic activities in spite of exposure to severe hypoxia. These outcomes are in line with a previous report that showed increased MSC proliferation at physiologic hypoxic conditions (Pal Singh,R. 2012). A similar pattern was also reported by Buravkova in 2004, where MSCs exposed to short-term hypoxia (up to 72 hours) displayed decreased viability but equivocal response to proliferation and increased reactive oxygen species production (Buravkova 2014). Another study showed increased colony forming efficiency in cord blood MSCs treated exposed to hypoxia (1.5%) (Martin-Rendon,Enca 2007) These reports are consistent with the premise that MSCS and in this case OFMSCs can adapt to environmental stress following severe hypoxia (Greijer, A. E. 2004). Greijer also stated that the severity and length of time of exposure to hypoxia determines if cells undergo apoptosis or environmental adaptation. Our study observed that exposure to acute severe hypoxia resulted in OFMSC adaptation rather than apoptosis. It will be interesting to further evaluate the effect of severe and prolonged hypoxia on OFMSCs as these will further simulated the repeated cycles of radiotherapy associated with management of head and neck cancers.

In addition to maintaining proliferative ability under severe hypoxic conditions, OFMSCs were also noted to maintain osteogenic and adipogenic differentiation capability. This is an interesting finding, considering that hypoxia-induced depletion of osteoprogenitors should negatively affect osteo-differentiation ability. In essence, OFMSCs did not only recover from severe hypoxic insult but were able to retain some degree of differentiation ability. Other studies have also suggested that severe hypoxia alters multilineage differentiation pattern of MSCs from non-oral skeletal sites (Cicione C, et al 2013; Holzwarth,C. 2010). Holzwarth *et al* in 2010, showed comparable adipogenic and osteogenic differentiation between human MSCs cultured at 21% and those cultured at 3%. Most of these studies however, are not comparable to our study as physiologic oxygen tensions were assessed and none of the MSCs studied were from the orofacial region.

Furthermore, HIF-1α was noted to be activated after severe hypoxic treatment, assessed by immunofluorescence, immunoblotting and real time PCR. A similar observation reported by Lynam *et al* in 2015, showed increased in HIF-1α level following severe hypoxic treatment (0.5% O2). (Lynam,E.C. 2015). Similar studies that assessed HIF-1α expression in mice bone marrow MSCs (Ren,Hongying 2006) and human MSCs (Xu. H. 2014) have also reported increased HIF-1 activation under severe hypoxia. Our findings combined with these previous studies underscore the modulatory roles of HIF-1α signaling in MSCs survival post severe hypoxia.

An important downstream effector of HIF-1α signaling is VEGF. HIF-1α has been noted to increase VEGF production under hypoxic conditions (Razban, V. 2012). It was significant to note that VEGF expression was activated in OFMSCs in response to severe

hypoxic treatment. Umbilical cord MSCs have also been shown to expressed increased VEGF levels in response to severe hypoxia (Martin-Rendon,Enca 2007). The protective effect of bone marrow MSCs on cardiomyocytes has been previously demonstrated . Bone marrow MSCs transplanted into an ischemic cardiac environment were able to survive and activated increased HIF-1α-medicated VEGF levels in the hypoxic environment (Razban V. 2012). Based on our data, the ability of hypoxic treated OFMSCs to activate both HIF-1α and VEGF suggest that residual MSCs post-radiotherapy can be activated to improve recovery from jaw ORN. This can be induced by grafting autologous OFMSCs into an osteoradionecrotic area in the jaw. The activity of the donor MSCs have the potential to reactivate residual cells to promote HIF-1α-VEGF associated survival and recovery.

In addition to HIF-1α signaling pathway, the PERK- eIF2α arm of the endoplasmic reticulum stress response pathway was also activated in OFMSCs in response to severe hypoxia. The significant increase in eIF2α indicates that ERSR pathway will slow down protein translation to reduce accumulation of unfolded proteins in the endoplasmic reticulum in response to hypoxic stress (Simon, M.C. 2008). It is interesting to note that both HIF-1α and PERK-eIF2α arm of the ERSR are upstream effectors of VEGF (Figure ??). (Simon, M.C. 2008), (Tamama, K. 2011). Since VEGF level was significantly upregulated in hypoxia-treated OFMSCs, the recovery of OFMSCs and ability of residual cells to retain osteogenesis can be attributed to cooperation of angiogenesis and osteogenesis signaling pathways. Hence angiogenesis-osteogenesis cooperation is major factor essential for post-hypoxia recovery of OFMSCs.

Conclusion

Severe hypoxia possibly plays a huge role in the pathogenesis of jaw osteoradionecrosis. Even though OFMSCs succumb to severe hypoxia, they are able to recover and retain their stem cell properties. This shows a potential usefulness of autologous MSCs as transplants in the management of patients with jaw osteoradionecrosis.

However, before drawing any definite conclusions from this study, it will be advantageous to consider the effects of long-term sustained hypoxia on OFMSCs. An essential future direction is the need for both pharmacologic and genetic disruption of HIF-1α and PERK-eIF2α signaling pathways to further confirm their roles in OFMSC responsiveness to severe hypoxia.

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