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The Role of Oxygen Tension and Insulin-Like Growth Factor Signaling in the Placental Mesenchymal Stem Cell Fate

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The Role of Oxygen Tension and Insulin-Like Growth Factor Signaling in the
Placental Mesenchymal Stem Cell Fate

An Integrated-Article Thesis

by

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Graduate Program in Biochemistry

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ABSTRACT

The human placenta of different gestational ages is a readily available source for isolation of adult mesenchymal stem cell (MSC) for potential use in regenerative therapies. The chorionic villous region, the largest component of a placenta that interfaces with the maternal circulation, is a rich source of placental MSCs (PMSCs). To remain multipotent, PMSCs are best maintained in culture conditions that mimic the *in vivo* microenvironment. Insulin like growth factors (IGFs, IGF-I and -II) and oxygen tension are two of the most important microenvironmental factors in the placenta. They are of low concentration or tension respectively, at early gestation, and increase as pregnancy progresses. In this thesis, we investigated the role and cellular mechanisms by which IGFs and low oxygen tension determine PMSC fate. The main hypothesis is that the interaction between IGFs and oxygen tension determines PMSC fate towards self-renewal or differentiation. We used cell proliferation assay, immunoblotting, real-time PCR, and cell monolayer staining to evaluate the role of IGF and oxygen tension on PMSC multipotency and differentiation. We found that low oxygen tension was a major determinant of PMSCs proliferation and multipotency, and to delay differentiation. Also, PMSC response to IGF stimulation and low oxygen tension was gestational age dependent — preterm PMSCs being more multipotent and proliferative than term PMSCs. IGF-I and IGF-II promoted PMSC proliferation and multipotency via IGF-IR or IR, depending on oxygen tension. IGFs enhanced PMSC differentiation towards the osteogenic lineage which was transduced by ERK1/2 and AKT signaling

cascades. We conclude that IGFs and oxygen tension act synergistically or antagonistically, mimicking *in vivo* microenvironmental conditions, to determine PMSC fate towards multipotency or differentiation. The appropriate combination of IGFs and oxygen tension can be used to maintain stem cells in multipotency, or to be induced towards a specific progenitor cell lineage for successful use in tissue regeneration therapies.

KEYWORDS

AKT

ERK1/2

Gestational age

Human placenta

Insulin-like growth factor (IGF)

Insulin-like growth factor-I receptor (IGF-IR)

Insulin receptor (IR)

Placental mesenchymal stem cell (PMSC)

Low oxygen tension

Microenvironment

Multipotency

OCT4

Osteogenic differentiation

Proliferation

Receptor tyrosine kinase

RUNX2

Signaling cascade

CO-AUTHORSHIP STATEMENT

Chapter two was written by me and modified based on review by Dr. Cristiana Iosef and then modified based on review by Dr. Victor Han.

All other chapters were written by me and modified based on review by Dr. Victor Han.

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°C	Degrees Celsius
α	Alpha
β	Beta
m	Milli
μ	Micro
n	Nano
λ	Wavelength
AKT	Serine/threonine protein kinase B
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bFGF	Basic fibroblastic growth factor
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Ca2+	Calcium
CD	Cluster of differentiation (cell surface marker)
cm	Centimeter
c-MYC	Myc proto-ocogene protein
DMEM-F12	Dulbecco's modified eagle medium and nutrient

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem cell
ETC	Electron transport chanin
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGR	Fetal growth restriction
g	Gram
GH	Growth hormone
GRB-2	Growth factor receptor-bound protein 2
HB-EGF	Heparin-binding EGF-like growth factor
HGF	Hepatic growth factor
HIF	Hypoxia inducible factor
hr	hour
HR	IGF-IR/IR Hybrid receptor

HRE	Hypoxia responsive element
HRP	Horseradish Peroxidase
Igf1	Murine insulin-like growth factor I gene
Igf2	Murine insulin-like growth factor II gene
Igf1r	Murine insulin-like growth factor I receptor gene
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGF-IIR	Insulin-like growth factor receptor II
IGF-IR	Insulin-like growth factor receptor I
iPS	Induced pluripotent stem cell
IR	Insulin receptor
IR-A	Insulin receptor isoform A
IR-B	Insulin receptor isoform B
IRS	Insulin receptor substrate
IVS	Intervillous space
JAK	Janus-associated tyrosine kinase
kDa	Kilodalton
KIT	Stem cell factor receptor tyrosine kinase
KLF-4	Krueppel-like factor-4
KOSR	knock-out serum replacement

L   Litre

LIF   Leukemia inhibitory factor

LIN-28   Lin-28 homology A / Zinc finger CCHC domain containing protein 1

LY294002   2-morpholin-4-yl-8-phenylchromen-4-one

M   Molar

MAPK   Mitogen-activated protein kinase

MEF   Mouse embryonic fibroblast

MEK   MAP/ERK kinase

min   Minutes

mL   Milliliter

mm Hg   Millimeter of mercury

MMP   Matrix metallopeptidase

mol   Mole

MSC   Mesenchymal stem cell

NANOG   Homeobox protein Nanog

NBT/BCIP   Nitroblue tetrazolium 5-bromo-4-chloro-3-indolylphosphate

ng   nanogram

nm   nanometre

OCT-4   Octamer-binding transcription factor-3/4

OPN   Osteopontin

p   Significance value

p85	Phosphatidyl-inositol-3-kinase regulatory subunit
PAS	PER-ARNT-SIM subfamily of basic helix-loop-helix (bHLH)
PBEF1	Pre-B-cell colony-enhancing factor 1
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PGF	Placental growth factor
pH	*potentia hydrogenii* (potential of hydrogen)
PHD	prolyl hydroxylase domain
PI3K	Phosphatidyl-inositol-3-kinase
PLCγ	Phospholipase C gamma
PMSC	Placenta mesenchymal stem cell
PO_2	Partial pressure of oxygen
POU	Pituitary-specific/Octamer/Uncoordinated-like protein family of transcription factors
POU5F1	POU domain, class 1, transcription factor 1
PTB	Phospho-tyrosine binding domain
PVDF	polyvinylidene fluoride
RTK	Receptor tyrosine kinase
ROS	Reactive oxygen species
RUNX-2	Runt-related transcription factor-2
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SH-2	Src-homology 2
SHC	Src-homology 2 containing protein
SOX-2	SRY-related HMG box 2
SSEA	Stage specific embryonic antigen
STAT	Signal transducer and activator of transduction
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline supplemented with 0.05% Tween-20
Tyr	tyrosine amino acid
U0126	1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene
VEGF	Vascular endothelial growth factor

CHAPTER ONE

INTRODUCTION

1.1. Stem Cell Origin

1.1.1. Stem cell classification and potency

Stem cells are *naïve* unspecialized clonal cells that have the capacity for self-renewal and differentiation (1). Cells from an early embryo (e.g. morula) have the greatest potential to differentiate and can give rise to the whole embryo or organism, including germ cells and extraembryonic tissues. Most stem cells are derived from later gestation embryos and are classified according to their differentiation potential into pluripotent, multipotent, oligopotent and unipotent cells. Also, stem cells are classified according to their origin and derivation procedure into embryonic (ESCs), adult mesenchymal (MSCs), tissue-resident and induced pluripotent stem cells (iPSCs), where the first three are naturally occurring and are found *in vivo*, while the latter are generated *in vitro* from reprogramming terminally differentiated cells. ESCs are pluripotent, isolated from the inner cell mass of the blastocyst and can differentiate into any cell type of the three germ layers; adult MSCs are multipotent stem cells and have a limited proliferation capacity and a more restricted differentiation lineage (2). Recently, the development of iPSCs, as autologous ES-like cells that can be an alternative to allogeneic ESCs for tissue regeneration therapies, has generated much excitement. They are developed by somatic cell nuclear transfer (somatic nucleus transfer into anuclear oocyte) or reprogramming (using a combination of 4 transcription factor OCT4, SOX2, KLF4, c-MYC, NANOG, and microRNA binding protein LIN28) (2-4).

The three transcription factors OCT4, SOX2 and NANOG are major regulatory triad of human ES cell fate and major transcription factors that are induced to develop iPSCs (3-6). Each of these transcription factors has a major role in repressing the differentiation process towards a specific lineage: OCT4 represses extraembryonic and epiblast-derived lineages, SOX2 represses mesendoderm differentiation and NANOG represses embryonic ectoderm differentiation (5). Among the three transcription factors, OCT4 is the only indispensible transcription factor to generate iPSCs, and its transfection into neural progenitor cells as a single factor is sufficient to generate iPSCs (3, 4, 7-9). OCT4, a POU homeodomain transcription factor, is regarded as the master regulator and gatekeeper of pluripotency to keep stem cells in an undifferentiated state (8, 10-12). For this critical role in maintaining pluripotency, OCT4 expression is tightly regulated, whereas under- or over-expression leads to differentiation (10). Also, OCT4 is not lost in committed lineage progenitor cells of the three germ layers beyond gastrulation suggesting that it plays an important role in the commitment of pluripotent stem cells into somatic lineages (8). Indeed, pluripotent ESCs/iPSCs with low OCT4 levels fail to exit the pluripotent state upon the induction of differentiation, and were rescued to differentiate into three germ layers by the restoration of OCT4 expression (13).

1.1.2. Adult mesenchymal stem cells

Mesenchymal stem cells (MSCs) or adult mesenchymal stem cells are multipotent cells that have the capacity for self-renewal and differentiation into different cell types of one or more lineages of three germ layers. The first discovery of the existence of multipotent stem cell population was by Till and McCulloch in 1961 (14), which was followed by the first bone marrow MSCs isolated in 1968 by Friedenstein (15). To date, MSCs have been isolated from every mature organ and tissue including skeletal muscle (16), adipose tissue (17), deciduous teeth (18), umbilical cord blood (19), peripheral blood (20), brain (21), etc. Several biological markers characterize stem cells of different origins, and are positive for CD73, CD105, CD117, CD29, CD44, CD71, CD90, CD106, CD120a, and CD124, and negative for hematopoietic markers CD34, CD45 and CD14 (22-24). It has been demonstrated that MSCs have the potential to differentiate into several major mesodermal cells including osteogenic, chondrogenic, adipogenic (25) and endothelial (26) cells, and ectodermal cells, including neuronal cells (27) in a microenvironment-dependent manner.

1.2. Human Placenta – A Source of Stem Cells

1.2.1. Placental development and the fetal compartment

The human chorioallantoic placenta is the main organ that supports fetal growth and survival by offering nutrient supply and eliminating waste products (28). The optimal growth and development of the placenta is essential, and any

abnormality in development is responsible for many pregnancy complications including miscarriages, preterm birth, preeclampsia and fetal growth restriction (FGR) (28, 29). In a normal pregnancy, the human placenta is developed as single organ in which maternal and fetal compartments are brought together, and is delivered after the delivery of the baby at term gestation.

The chorionic villi are the functional units of the placenta where oxygen/nutrient/waste exchange occurs between the uteroplacental and fetoplacental circulations without intermixing of blood. Each chorionic villus is composed of three layers: villous trophoblast (syncytiotrophoblasts/ cytotrophoblasts), villous stroma (mesenchymal cells/Hofbauer cells/fibroblasts), and fetal vascular endothelium (vascular smooth muscle cells/ perivascular cells/endothelial cells) (30). Trophoblasts are the first cells to be in direct contact with the maternal uterine environment, and therefore play a critical role in preventing an immune rejection of the conceptus and facilitating implantation. To form placental blood supply, trophoblasts invade the decidua and remodel spiral arteries in the uterus (28-30). By 10-12 weeks of gestation, maternal blood flows from the spiral arteries to fill up the intervillous space surrounding the villi for oxygen/nutrient/waste exchange. The stromal layer of the chorionic villous is a rich source of mesenchymal stem cells (MSCs), available for vasculogenesis and angiogenesis, and is located between the syncytiotrophoblasts/cytotrophoblasts layer that is in direct contact with maternal blood and the fetal endothelium that is in direct contact with fetal circulation (30). Also within the stromal layer, Hofbauer

cells, the antigen-presenting macrophages, are abundantly present to maintain host defense as they lie closely to cytotrophoblast cells and fetal endothelial cells (31). Fetal derived endothelial cells, surrounded by pericytes, form the capillary network of the chorionic villi. These pericytes play a significant role in blood vessel regeneration and are therefore important to maintain micro-vessel integrity and stability (30). Overall, the placenta's vital role in supporting fetal development is to offer the embryo and fetus an optimal environment for growth and development.

1.2.2. Stem cell populations in the placenta

Placenta is a readily available, non-invasive and ethically less controversial source of adult mesenchymal stem cells (MSCs). These stem cells have been isolated successfully from the umbilical cord blood, umbilical cord, amniotic fluid, fetal membranes and placental tissue (chorion) (32). Placental MSCs were also isolated from the tissues of maternal origin, the decidua basalis and the decidua parietalis (32). Although, MSCs can be isolated from fetal or maternal tissues, placental MSCs are fetal in origin, and have the ability to expand at a higher proliferation rate and passage number *in vitro* than bone marrow MSCs (32), the more common MSC used in tissue regeneration therapies.

Terminal Chorionic Villous

Endothelial Cell

Pericyte

Mesenchymal Stem Cell

Hofbauer Cell

Cytotrophoblast

Stromal Fibroblast

Capillary

Syncytiotrophoblast

Fetal Membranes

Umbilical Cord

Intervillous Space

Decidua

Chorionic Villi

Placenta

Fetus
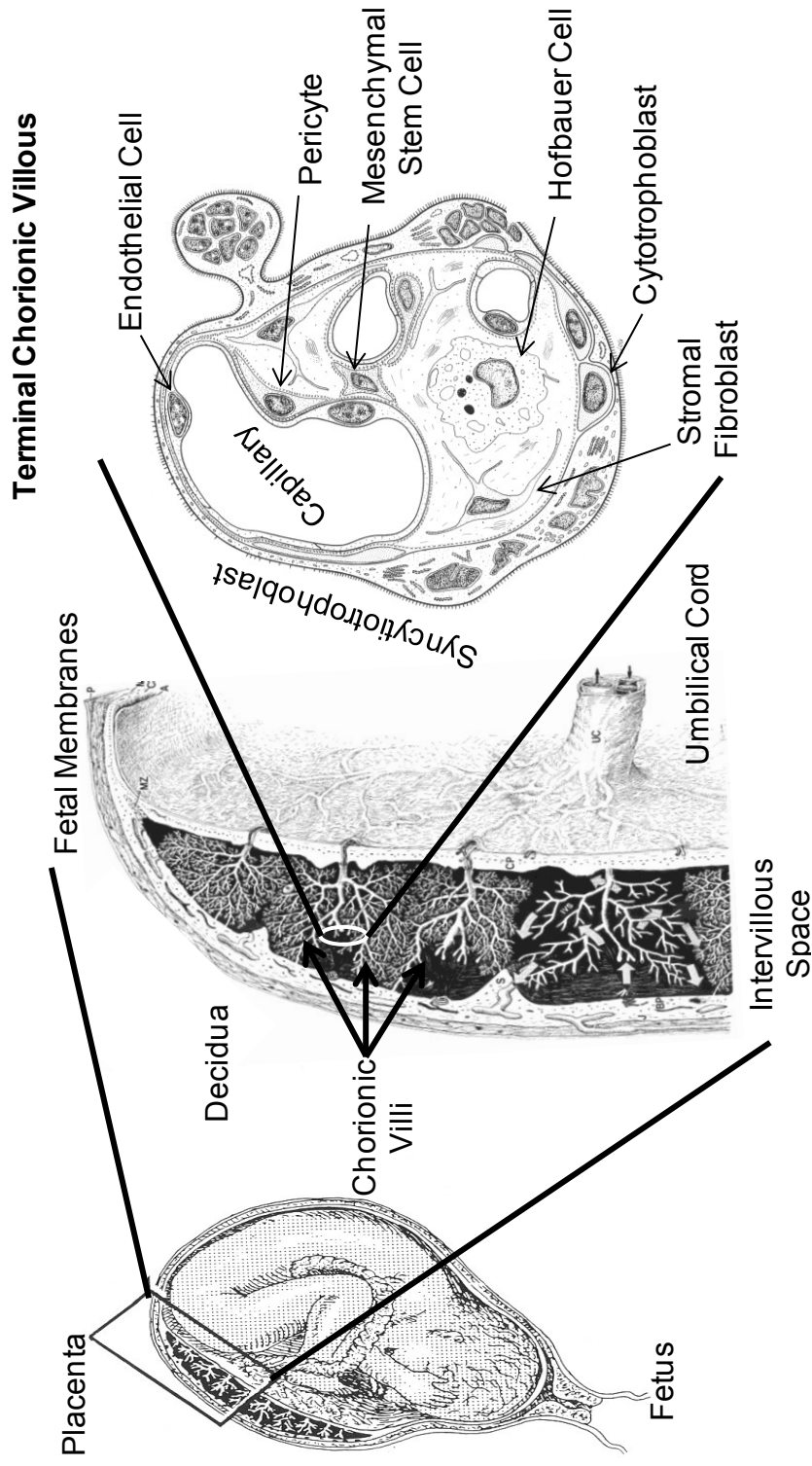
Figure 1.1. Mature Human placenta in situ. The human placenta is a discoidal type multivillous hemomonochorial structure. The intervillous space is filled with maternal blood delivered by the spiral arteries allowing nutrient and waste exchange between the fetal and maternal circulations. The chorionic villous is the functional unit of the placenta from a fetal origin that is made up of several cell types (syncytiotrophoblasts, cytotrophoblasts, Hofbauer cells, stromal fibroblasts, pericytes, endothelial cells, and mesenchymal stem cells).

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1.2.3. Placental mesenchymal stem cells (PMSCs) from chorionic villi

In this thesis, placental mesenchymal stem cells (PMSCs) were isolated from the chorionic villi (33) (Fig. 1.1). PMSCs originate from a single cell population from the extraembryonic mesoderm of the developing totipotent fertilized egg, which invades the chorionic villous core along with cytotrophoblasts at 21 days post conception (33-35). These PMSCs play an important role early in placental vasculogenesis and angiogenesis as they differentiate into four cell types: macrophage-like mesenchymal cells (Hofbauer cells), haemangioblastic cords (multipotent precursor of hematopoietic and endothelial cells), pericytes (can be precursors of vascular endothelial cells) and stromal fibroblasts (34). These PMSCs are isolated from early and late gestation placentae (Fig. 1.1), using mechanical and enzymatic digestion with trypsin, collagenase and DNase (36) followed by Percoll density gradient separation similar to hematopoietic MSC isolation (37). PMSCs proliferate for at least up to 10 passages without alteration of phenotype and morphology *in vitro*, and have differentiation potential for all mesodermal lineages (adipogenic, chondrogenic, osteogenic, skeletal myogenic), ectodermal lineages (neuronal), and endodermal lineage (beta cells) (33). These cells are promising candidates for tissue regeneration therapy for their availability, abundance, ease of processing, and wide range of differentiation lineages. However, optimal culture conditions are required to maintain prolonged proliferation and maintenance of pluripotency and complete homogeneous differentiation *in vitro*. This can be achieved by understanding the

in vivo microenvironment of the placenta, and thereby, developing *in vitro* culture conditions that mimic *in vivo* conditions.

1.3. Stem Cell Microenvironment

1.3.1. Physical compartment

Stem cell microenvironment or "niche" is an anatomical structure, including cellular and acellular components, that integrates local and systemic factors to regulate stem cell pluri- or multi-potency, proliferation, differentiation, survival and localization (Fig. 1.2) (38). Proliferation and differentiation of stem cells are maintained by the surrounding microenvironment *via* several clues including physical, structural, neural, humoral, paracrine, autocrine, and metabolic interactions (39). Therefore, a combination of different microenvironmental signals would determine the proliferative state of stem cells depending on the active process of tissue regeneration causing either cell proliferation or quiescence (40). In addition, the components present in the microenvironment, such as growth factors, cytokines, extracellular matrix, chemokines and oxygen levels, are critical for limiting or facilitating the MSC survival and propagation of stem cells (symmetric division) or differentiation to more specialized committed cells (asymmetric division).
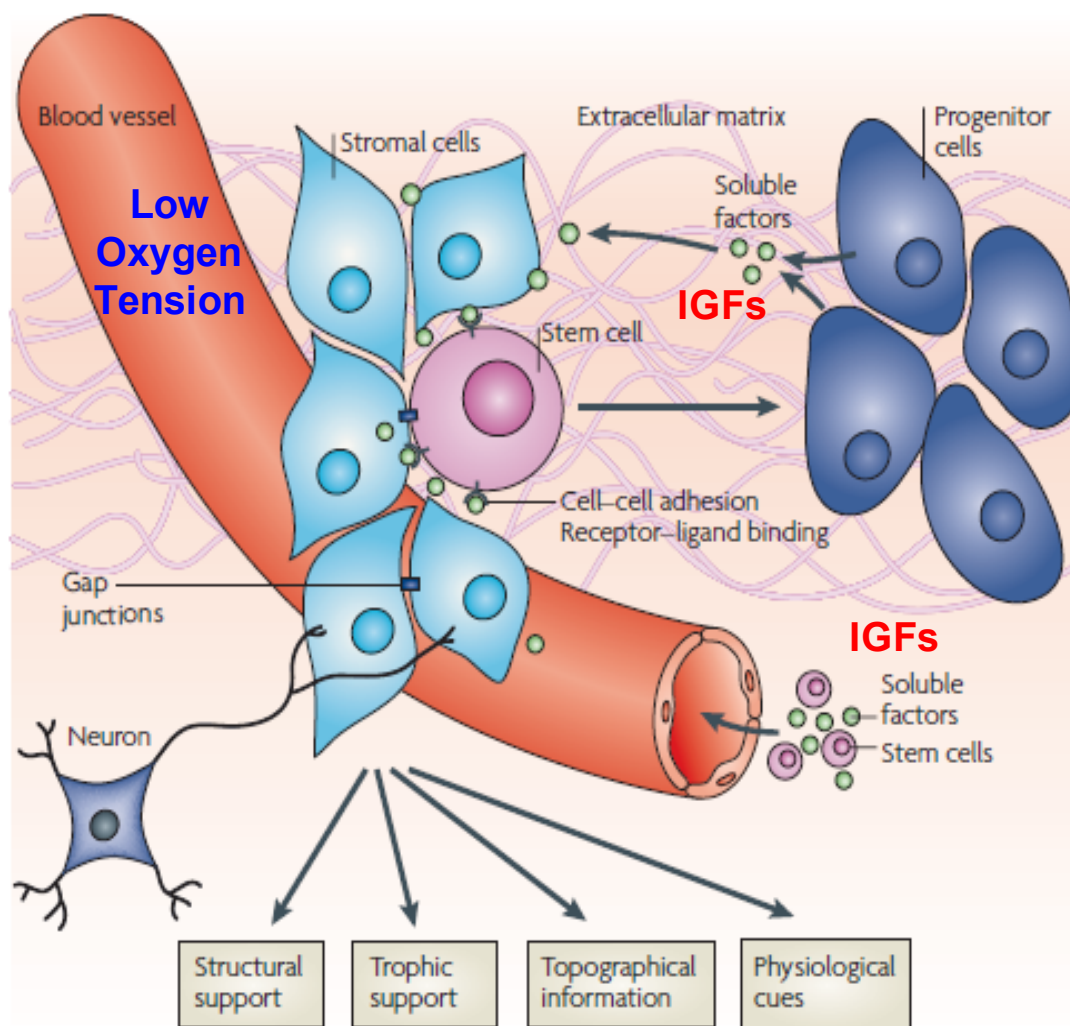
Figure 1.2. Stem cell niche *in vivo*. The stem cell niche is a complex compartment surrounding stem cells directing their identity preservation via cellular and acellular components. Various clues are exchanged between cells, extracellular matrix, growth factors, soluble factors and oxygen tension to direct the stemness state depending on growth and regeneration demand. Among growth factors, IGFs are derived *via* either paracrine or endocrine sources. Oxygen tension supplied by blood circulation is usually low in stem cell niches. IGFs and low oxygen tension alone or in combination may be major determinants of stem cell fate.

1.3.2. Oxygen tension and growth factor modulation

Low oxygen tension in the stem cell microenvironment is being recognized as an important signal that regulates stem cell proliferation and differentiation (41). The notion of considering stem cell culture under room air (20% oxygen) in a cell culture incubator as "normoxia", compared with a decreased ambient oxygen as "hypoxia", has to be changed as oxygen tension in natural niches is low and termed "physiologic normoxia" (42). Oxygen tension, in many organs and tissues, ranges from 2-9% (14-65 mmHg) (43). In particular, oxygen levels reach 1.5-8% O_2 in the mammalian reproductive tract, 1-2% O_2 in bone marrow, and ~3% O_2 in adipose tissue (41). Therefore, maintaining stem cells in low oxygen tension *in vitro* is more physiological than 20% O_2 (room air condition) used in standard cell culture. Low oxygen tension can increase the rate of MSC proliferation and potentiate or diminish differentiation (depending on the differentiation lineage); and also a hypoxic preconditioning of MSC can improve engraftment and survival for transplantation (44).

Besides its direct effect on maintaining on undifferentiated state, low oxygen tension can alter growth factor expression and secretion. Recently, MSCs were described as "medicinal secretory cells" that increase the secretion of trophic factors and immunomodulatory cytokines offering a regenerative microenvironment for injured tissues (45). Low oxygen tension is shown to

increase MSC gene expression and secretion of several factors involved in cell growth and survival including FGF2, HGF, VEGF-D, PGF, PBEF1, HB-EGF and MMP9 (46). IGF-I is another growth factor synthesized by MSCs, which induces an anti-apoptotic and pro-survival signals (47). Therefore, in ischemic or hypoxic tissues, MSCs are recruited by secreted cytokines and chemokines modulate survival, growth and angiogenesis, and facilitate recovery and repair of injured tissues. Hence, MSCs are increasingly recognized as important candidates for tissue regeneration either by direct differentiation or by supporting the repair mechanism to correct organ physiology and function — where low oxygen tension plays a significant role.

1.3.3. Growth factor signaling

In the stem cell niche, a combination of growth factor signaling inputs can control the stem cell state between proliferation and differentiation. In one study, the signaling of TGFβ/activin/nodal was required to maintain an undifferentiated state of hESCs (48). Another study showed that IGF-II and its signaling via the IGF-IR are important to maintain self-renewal and pluripotency *in vitro* with a requirement of bFGF/FGF-2 to maintain a supportive fibroblast cell population (49). The expression and signaling of many RTKs, such as FGFR1-3, EGFR (ERBB2 and ERBB3), VEGFR2, KIT, RET, and the IGF-IR, are important for stem cell multipotency and fate changes (50), where significant signaling cascades, yet to be elucidated, can maintain stem cell character. For application

in tissue regeneration, the use of xeno-free and animal-free cell culture conditions are desired to eliminate rejection and an immune response. For this, knock-out serum replacement (KOSR) media is becoming a preference for culture of ESCs, supplemented with growth factors to maintain pluripotency (51). Growth factors used in combination with KSOR media, including but are not limited to, bFGF/FGF2, activin A, HRG1, IGF-II and IGF-I (51).

In addition to a combination of growth factor signaling required for stem cell pluripotency, the extracellular matrix (ECM) complex (e.g. matrigel) or a single component, (e.g. fibronectin, laminin, collagen, etc.) is used to support a feeder-free culture of hESCs (51). One of the benefits of the ECM is in facilitating stem cell attachment and spreading, therefore enhancing cell proliferation and migration (52). Also, the extracellular matrix in stem cell niches can act as reservoir of growth factors to regulate their bioavailability and generate a gradient important to direct stem cell proliferation *vs.* differentiation (53). In the neural stem cell niche, heparan sulfate proteoglycans are shown to capture and concentrate FGF-2 from the *milieu* to support neural stem cell function (54). Also, in the ECM, integrins function cooperatively with signaling pathways to mediate receptor transactivation, coordination, modulation and receptor compartmentalization (53). For example, a crosstalk occurs between the IGF-IR and β1-integrin to modulate cell proliferation, migration and differentiation (52). Therefore, growth factor signaling can be manipulated by the stem cell niches.

By deconstructing the stem cell niche, the individual role of signaling pathways –
such as the IGF axis – will need to be delineated.

1. 3.4. Placental microenvironment changes during gestation stages

1.3.4.1 Oxygen tension

Oxygen tension and growth factor availability in the microenvironment of the
developing human placenta continually change throughout gestation. The human
conceptus requires low oxygen tension for healthy implantation into the external
layer of the endometrium (55). Moreover, low oxygen tension is maintained
during the first 10 days post-fertilization until trophoplasts develop and dip their
processes in the maternal-blood. The placental tissue develops further and the
oxygen level fluctuates in both maternal (endometrium) and fetal sides. At 8-10
weeks of gestation, oxygen tension is very low, on the average of 17.9 mm Hg or
2.35% O_2 on the fetal side, which is lower than the maternal side at 39.6 mm Hg
or (5.21% O_2) (Fig. 1.3). Later, at 12-13 weeks of gestation the oxygen tension
rises to reach 60.7 mm Hg or (7.98% O_2) at the fetal side compared with 46.5
mm Hg or (6.12% O_2) at the maternal side (Fig. 1.3) (56). This change between
the first and second trimester occurs due to the rise in the oxygen tension (PO_2)
at the intervillous space (IVS) which is facilitated by the active arterial blood flow
between the IVS and the spiral arteries with low vascular resistance (55). Thus,
the human embryo exists in a low oxygen environment, at least in the first
trimester (0-12 weeks), when organogenesis occurs, indicating a major role for
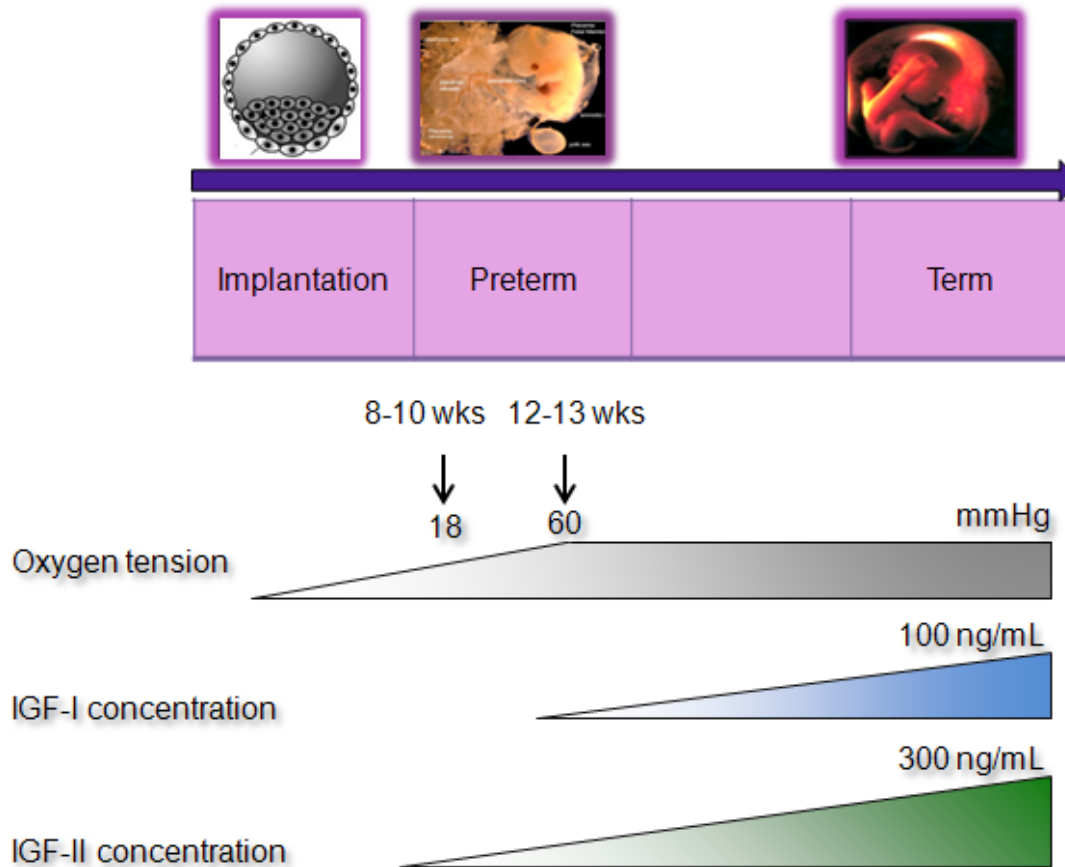
Figure 1.3. Low oxygen tension and IGF levels in the placental microenvironment during gestation. Oxygen tension and IGF concentration are constantly changing during gestation. Low oxygen tension starts at near anoxic levels during implantation and rises upon the placental growth and connection with the maternal spiral arteries to reach 60 mmHg at early gestation and is maintained through gestation thereafter. IGF-I is expressed around 12 weeks of gestation, while IGF-II is expressed earlier at 6 weeks. Both IGF expression gradually increases during gestation. At term gestation, IGF-I concentration is 100 ng/mL and IGF-II, 300 ng/mL, as measured in the umbilical cord blood.

low oxygen tension during development (55). Therefore, studying the behavior of placental mesenchymal stem cells (PMSCs) isolated at different gestation times (preterm *vs*. term) would unravel some aspects of placental development from the low oxygen tension microenvironment in the first trimester, to a well vascularized oxygenated tissue at the end of the third trimester (Fig. 1.3).

1.3.4.2. Insulin-like growth factors

Insulin-like growth factors (IGFs - IGF-I and IGF-II) represent one of the most important growth factor systems in cell growth and development, and cell-fate changes through several mitogen activation cascades (57-60). IGFs have a prominent role in pregnancy and fetal development by promoting proliferation, differentiation and survival of various types of placental cells (61). In human pregnancies, circulating IGF levels correlate proportionally with placental and fetal weights, where reduced levels due to nutrition can lead to fetal growth restriction (62). Both IGF-I and IGF-II are expressed in all cell types in the placenta as early as six weeks of gestation (63). IGF-II is important in facilitating the cytotrophoblast invasion of the maternal decidua early in human pregnancy and contributes to the placental vascularization, whereas, IGF-I is implicated with the placental development in the second trimester to term gestation (62). The exact IGF concentration in the immediate microenvironment of the chorionic villi during different gestational stages is not known, but the levels described are those measured in the umbilical cord blood. In a fully developed term placentae,

the IGF-I concentration is 71 – 132 ng/mL with IGF-II concentration being three-fold higher at 246 – 356 ng/mL in the umbilical cord blood (Fig. 1.3) (64). Therefore, the effect of IGFs on PMSCs during different gestational stages may change depending on IGF ligand abundance and IGF-IR.

1.4. Cellular Signaling and Mechanistic Regulation

1.4.1. Low oxygen tension and hypoxic regulation

Oxygen homeostasis is essential for the healthy physiology of an organism as well as to a single cell. Oxygen should be available in a narrow range to maintain the cellular health. The lower limit of this range is identified by the oxygen tension needed to maintain a reservoir of high-energy ATP molecules *via* aerobic respiration, which requires O_2 for the electron transport chain (ETC) (65). On the other hand, the oxygen tension should not exceed a threshold (the top limit) in order to prevent the generation of reactive oxygen species (ROS) that have a destructive effect on the structure and function of macromolecules (65). In low oxygen tension, reduced cellular energy demand is met by glycolysis, which is adequate for maintaining stem cell pluripotency and generating iPSCs (66, 67). At low oxygen tensions (hypoxia), oxygen-sensing mechanism stabilizes the formation of the hypoxia inducible factor (HIF). Mainly, the HIF-1 system can compensate for the production of ATP, under hypoxic conditions, by increasing the transcription levels of several genes that control glycolysis, angiogenesis and erythropoiesis, and therefore, indirectly promotes cell survival (68). Formed

under hypoxic conditions, HIF-1 is a heterodimer complex of α- and β- subunits, where both are basic-helix-loop-helix (bHLH) proteins containing PAS (PER-ARNT-SIM subfamily of bHLH) domains (69). At the protein level, the β-subunit (HIF-1β/ARNT) is continuously expressed and not degraded, while the α- subunit (HIF-1α) is regulated in the cytoplasm by regulatory proteins, such as prolyl hydroxylase domains -1, -2 and -3 proteins (PHDs), where PHD-2 utilizes molecular oxygen as a co-substrate and hydroxylates HIF-1α under normal oxygen tension oxygen (68, 70). Under low oxygen tension conditions, PHD-2 cannot deliver hydroxyl groups on proline residues, and therefore, HIF-1α is stabilized by preventing the interaction with the von Hippel–Lindau protein and ubiquitin. Hence, HIF-1α is not targeted to the 26S proteasome for degradation (68, 70-72). At this stage, HIF-1α accumulates in the cytoplasm, translocates into the nucleus and dimerizes with HIF-1β to form the nuclear dimeric HIF-1 protein (Fig. 1.4B). HIF-1 can bind to the hypoxia responsive element (HRE) defined by the core sequence 5'-RCGTG-3' (R = A or G), in promoters of target genes responsible for proliferation and survival (68, 71). At least seventy genes are regulated by HIF-1, encoding cell surface receptors, extracellular matrix proteins and enzymes, angiogenic growth factors, cytoskeletal proteins, glucose transporters and glycolytic enzymes, proapototic factors, and other transcription factors (73). In addition, insulin and IGF-I share with hypoxia, the ability to activate transcription through the HRE, where HIF-1 is their main target to induce gene transcription (74). HIF-1α is stabilized by its direct phosphorylation by ERK1/2 and not by p38 MAPK or c-Jun N-terminal kinase (75). Therefore,

manipulating PMSC microenvironment with IGFs can change the stem cell fate by the induction of various hypoxia genes, which can be achieved directly by low oxygen tension or indirectly by HIF-1α phosphorylation.

1.4.2. Insulin-like growth factor system

1.4.2.1 Ligands and receptors

Insulin-like growth factors (IGFs; IGF-I and -II) are polypeptides that stimulate the differentiation and self-renewal of many types of cells including stem cells (49), mainly through the interaction with the IGF-I receptor (IGF-IR). IGF-IR shares a structural homology domain with the insulin receptor (IR), which is expressed in two isoforms IR-A and IR-B, and can form hybrids (HR-A and HR-B) with the IGF-IR. IGF-II can also bind to its specific receptor, IGF-IIR, and with a higher affinity than IGF-I to the insulin receptor (IR). Both IGF-I and -II can bind to the hybrid receptor IGF-IR/IR with variable affinities (76). IR, IGF-IR and IR/IGF-IR hybrids are mitogenic RTKs, whereas IGF-IIR is not. IGF-II binding affinity to IR-A is similar to insulin, however, it can activate the mitogenic signaling cascade, which is a different response than activation of IR (77). IGF-IR is more sensitive to IGF-I with a Kd of 1 nM, whereas, with a 10-fold lower affinity to IGF-II (78).

Six IGF-binding proteins (IGFBPs, IGFBP-1 to -6) regulate the bioavailability of the IGF ligand in the extracellular vicinity of the receptors to sequester or release the IGFs, therefore, modifying the IGF actions (79).

Upon ligand binding, IGF-IR initiates mitogenic signals by activating the two main signal transduction pathways and effectors: the phosphoinositide 3-kinase (PI3K), AKT/PKB and the extracellular signal-regulated kinase (ERK1/2) (Fig. 1.4A) (80). Through these molecules, IGF-IR can induce transcriptional activity that would promote survival, self-renewal and differentiation (81, 82). The downstream signal of IGF-IR is dependent on the differential phosphorylation pattern of its β-subunit and the resultant tyrosine residues available to bind adaptor molecules or kinase effectors such as, PI3K, AKT, RAS/RAF and ERK1/2 (83, 84).

1.4.2.2. IGF-IR, adaptor molecules and signaling cascades

IGF-IR is a transmembrane tetramer receptor that exists as heterodimers composed of two *α* and *β* hemireceptors linked by disulfide bonds in a *β–α–α–β* structure (Fig. 1.5) (85). IGF-IR has three properties related to cell growth, where it (i) promotes survival and protects from apoptosis both *in vivo* and *in vitro*, (ii) stimulates cell-growth, and (iii) is required for the establishment and maintenance of a tumor-like phenotype (83).
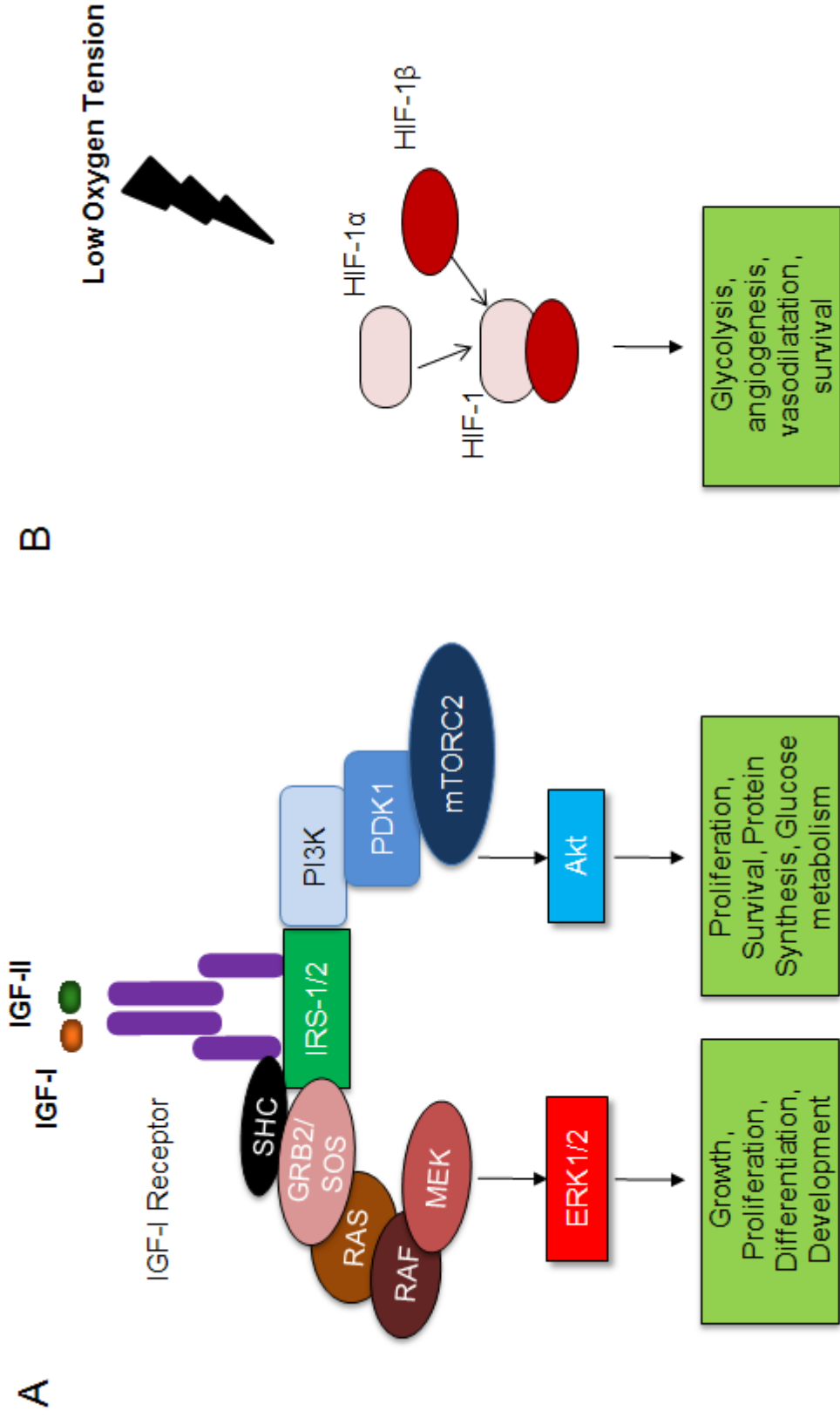
Figure 1.4. IGF signaling in low oxygen tension conditions. A) IGF-IR is the major signaling receptor to interact with IGF-I and IGF-II to trigger one of the two classical signaling cascades: the RAS/RAF/MEK/ERK or the PI3K/AKT pathways, which facilitate protein phosphorylation downstream in the cytoplasm and nucleus. B) In low oxygen tension, the hypoxia inducible factor system is stabilized and formed by the dimerization of the α and β subunits to form a transcription factor that translocates to the nucleus to induce gene transcription.

Upon activation of the extracellular α subunits of the IGF-IR, autophosphorylation of tyrosine residues (Y1131, 1135, 1136) on the *β*-subunits creates high affinity binding sites for signaling adaptor molecules and substrates. SHC and the insulin receptor substrate-1 (IRS-1) are two adaptor/substrate molecules interact with Tyr-950 at the NPXY motif of the *β* subunit of IGF-IR (Fig. 1.5) (83, 86). For the ERK1/2 signaling pathway, SHC interacts directly with the IGF-IRβ (at Tyr-950) which gets phosphorylated at tyrosine residues (Tyr-317 and/or Tyr-239/240) to recruit the SH2 domain binding of GRB2. In turn, GRB2 interacts via its SH3 domain with SOS that subsequently activates c-RAS by exchanging GTP for GDP leading to the sequential phosphorylation of RAF, MEK1/2 and then ERK1/2 (Fig. 1.4) (86-91). To activate the PI3K/AKT signaling, p85, the regulatory subunit of PI3K, interacts directly with IGF-IRβ at YAHM motif at Tyr-1316 independent of Tyr-950 — the site for IRS-1 and SHC binding (Fig. 1.5) (92). The C-terminal c-SH2-domain of p85 is responsible for binding with the carboxy-terminal of IGF-IRβ, while both n/c-SH2 domains of p85 bind to the IR corresponding C-terminal motif (YTHM at Tyr-1322) causing a more efficient and stronger interaction between IR and p85 than that with IGF-IR (92, 93). Therefore, the difference between the binding of p85 to either of IGF-IR and IR would exert different biological responses due to the dose of IGF-I or -II that can activate both IGF-IR and IR. However, IR has a 100-fold lower affinity to IGF-I than that for insulin to exert an effect (58). Besides the direct interaction with the
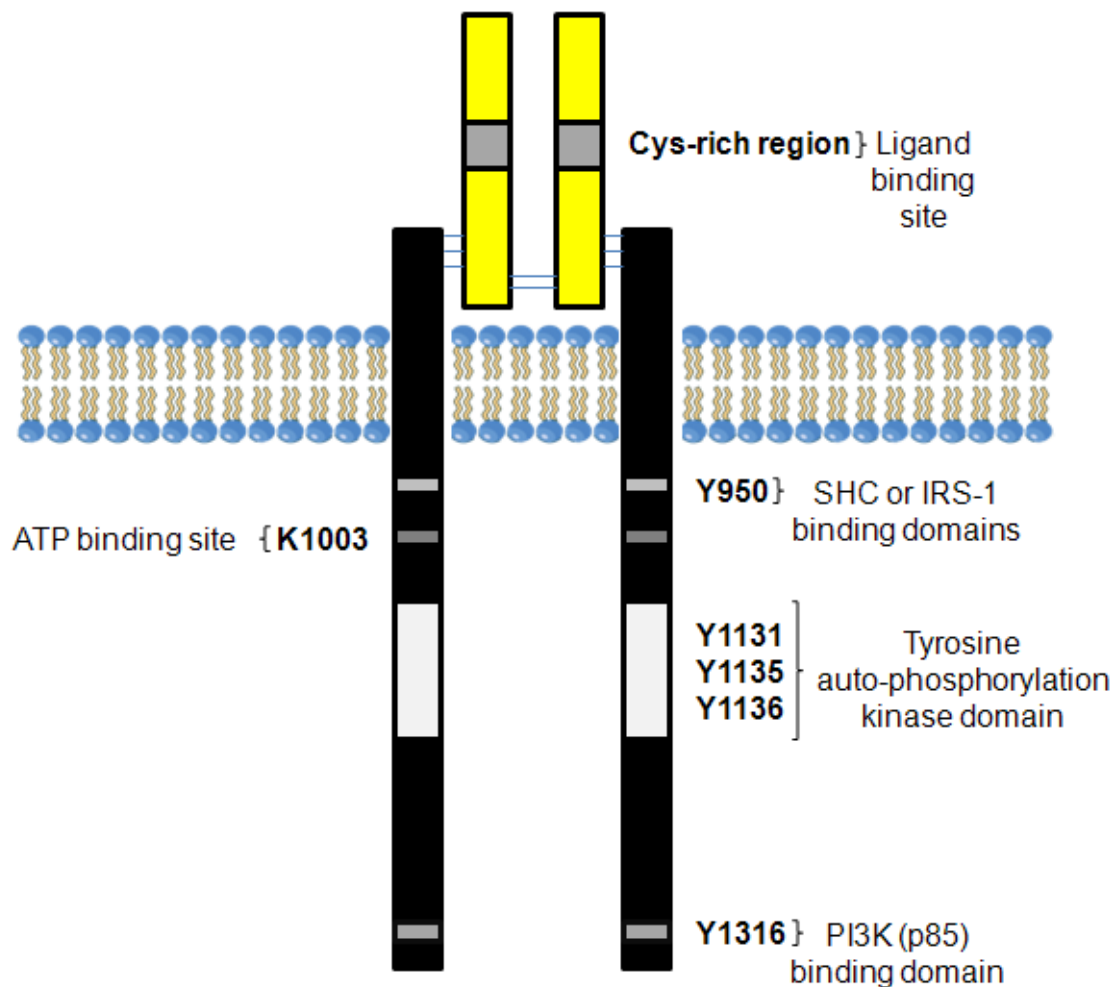
Figure 1.5. IGF-IR binding domains. IGF-IR is a class-2 transmembrane receptor tyrosine kinase with two extracellular α-subunits and two β-subunits that extends intracellularly spanning the membrane. The α-subunits contain the ligand binding domain, while the β-subunits contain the kinase function for autophosphorylation and binding to various adaptors.

IGF-IRβ, the ERK1/2 and the PI3K/AKT signaling is activated by the indirect interaction *via* IRS-1 recruited to IGF-IRβ. IRS-1 is a main adaptor of the IGF-IR implicated with the mitogenic effect of IGF-IR, inhibition of apoptosis and transformation of cells into the tumoral cells when over-expressed, whereas, its downregulation has been associated with the inhibition of differentiation and the induction of apoptosis (80, 94). It has been shown that the N-terminal domain of IRS-1 binds to the p-IGF-IRβ to induce over 20 potential phosphorylated sites available to recruit GRB2, NCK, the p85 regulatory subunit of PI3K, the tyrosine phosphatase SH-PTP2, SRC-like kinase FYN and the Ca^{2+}-ATPases SERCA1 and AERCA2 (86, 95). The phosphorylation of IRS-1 at Tyr-895 recruits GRB2 and transduces the signal through the ERK1/2 (87), whereas, the phosphorylation of Tyr-612/-632 would activate the PI3K signaling by recruiting p85 binding (96). Therefore, both ERK1/2 and PI3K/AKT signaling pathways are also activated by IGF-IR *via* the indirect binding of GRB2 and p85, respectively, to the IRS-1 (86, 87, 91), and therefore, a wider range of interacting adaptors can be mediated to define the signaling cascade. In the stem cell niche, the microenvironmental components (oxygen tension and IGFs) define the signaling cascades in stem cells to promote either self-renewal or differentiation depending on receptor activation and the combination of signaling cascade.

1.5. Stem Cell Differentiation and Fate Specification

1.5.1. Stem cell differentiation conditions

Two approaches are undertaken to induce stem cell differentiation using either intrinsic nuclear reprogramming, *via* transcription-factor-mediated reprogramming, or extrinsic, *via* growth-factor-mediated stimulation (97). Similar to iPSC generation, intrinsic nuclear reprogramming has been successful but is still limited to acquire only few lineages, such as cardiomyocytes, hepatocytes, and neurons due to the limitation of known reprogramming factors (97). For this purpose, reprogramming and de-differentiation to an iPSC is required. However, to cover a wider range of differentiation lineages, extrinsic growth factor stimulation is the solution to acquire cells from all three germ layers. *In vivo*, the natural route of stem cell differentiation is mediated via extrinsic growth factor signaling within the microenvironment. In their niche, ESCs are in quiescent dormant state that is maintained by three interactions with the niche components: the supportive cellular contact, the ECM, and the paracrine growth factor signaling (51). Thus, manipulating any of these conditions will disturb the natural niche of the stem cell causing asymmetrical division towards a differentiated phenotype. *In vitro*, differentiation is triggered by withdrawing maintenance growth factors or stimulating by differentiation promoting growth factors and molecules (98, 99). As shown in Fig. 1.6, differentiation of adult MSCs follow specific differentiation protocols using varying combinations of growth factors and chemical compounds, such as butylated hydroxyanisole and NGF for neuronal

differentiation; BMP-12 for tendocyte differentiation; dexamethasone, 3-isobutyl-1-methylxanthine, insulin and indomethacin for adipogenic differentiation; monothioglycerol, HGF, oncostatin, dexamethasone, FGF4, insulin, transferrin and selenium for hepatocytic differentiation; b-FGF and VEGF for endothelial differentiation; TGF-β1, insulin, transferrin, dexamethasone, ascorbic acid for chondrogenic differentiation; insulin, transferrin and selenium for skeletal myogenic differentiation; dexamethasone, ascorbic acid and β-glycerophosphate for osteogenic differentiation (Fig. 1.6) (100-105). Therefore, from a common MSC population in the mesoderm, receiving different extrinsic stimuli can initiate differentiation towards specific cell type by triggering a tissue specific transcription factor, as SOX5/6/9 for chondrocytes, PPARγ for adipocytes, MyoD family for myoblasts, and RUNX2/Osterix for osteoblasts (106).

1.5.2. Osteogenic differentiation

Bone tissue is a calcified connective tissue derived from the mesodermal lineage of the developing embryo. Osteoblasts, the mononucleated bone forming cells, are of a mesenchymal origin responsible for synthesis and mineralization of bone tissue initially at the forming stage and later at remodeling of the skeletal bone structure (107). During *in vitro* osteogenic differentiation, MSCs follow the intramembranous ossification mode starting by condensation of the stem cell
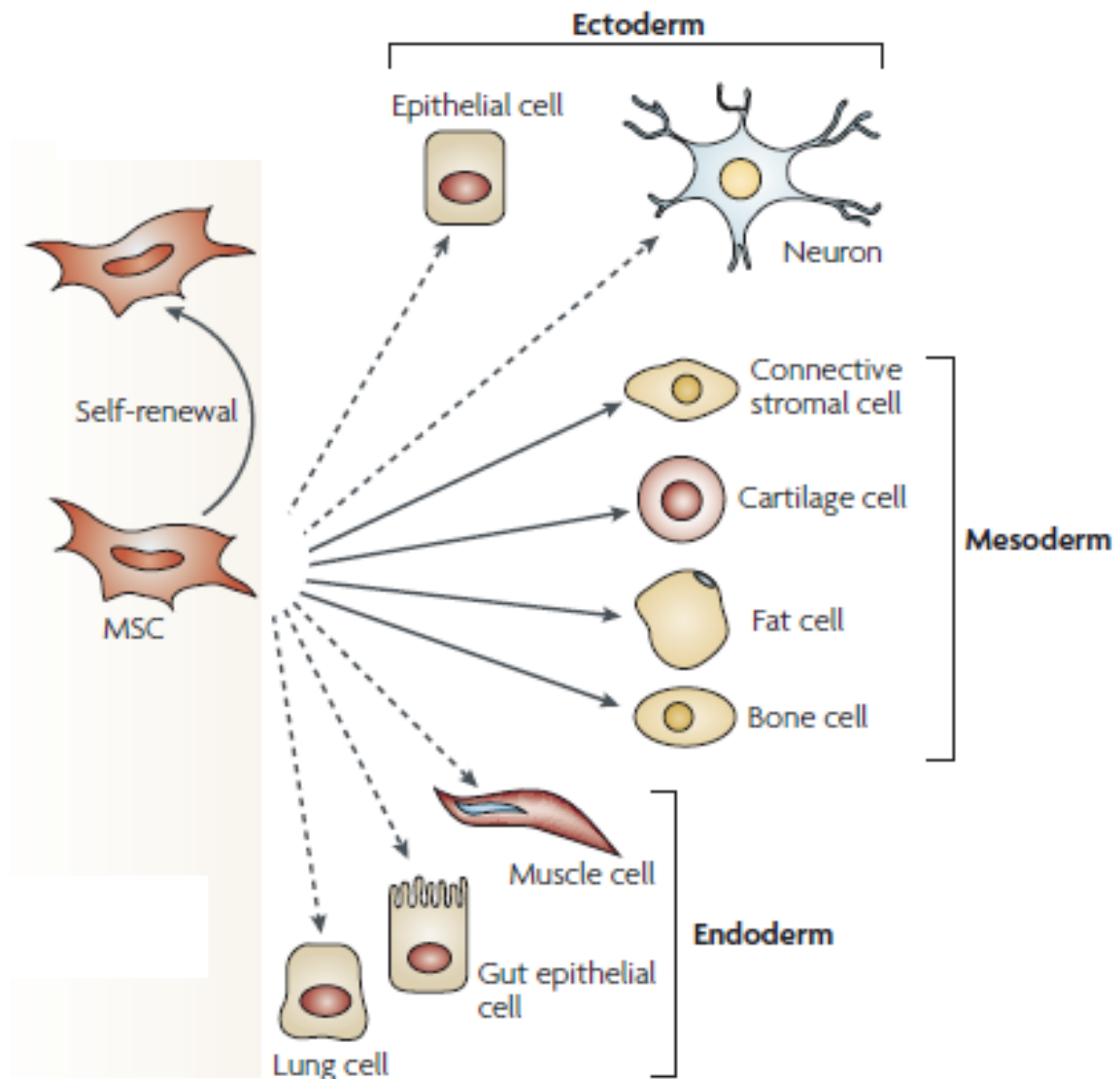
Figure 1.6. MSCs are multipotent *in vitro* and differentiate towards all three germ layer lineages. Mesenchymal stem cells including PMSCs are able to self-renew and differentiate towards all three germ layer lineages of ectoderm, mesoderm and endoderm. Transdifferentiation of MSCs *in vivo* is still controversial, but it has been achieved *in vitro* with different stimulation factor combinations which may not be present in the natural MSC niche *in vivo*.

population and direct differentiation into osteoblasts, without the need of cartilaginous template for ossification (108). Osteogenic differentiation has three phases: proliferation and condensation, extracellular matrix secretion and maturation, and matrix mineralization (109). The initiation of osteoblast formation is controlled by RUNX2, a lineage-specific transcription factor responsible for directing the multipotent MSC towards the osteoblast lineage (110). RUNX2 expression is essential for MSC proliferation and condensation with the highest expression just prior to differentiation; RUNX2 can inhibit differentiation if its level remains high (110-112). The lack of RUNX2 in mice causes bone malformation due to the absence of osteoblasts and death shortly after birth due to breathing dysfunction; also the lack of RUNX2 is manifested in human disease known as cleidocranial dysplasia (110, 112). Another known lineage-determining factor, Osterix, is downstream of RUNX2 and is also required for complete commitment of progenitor cells towards the osteoblast differentiation (113). At the early stage of osteoblast differentiation, RUNX2 is responsible for transcriptional upregulation of matrix deposition proteins (type I collagen, osteopontin, bone sialoprotein and osteocalcin), but is not essential to maintain these proteins in mature osteoblasts (107, 110, 112). During osteoblast maturation, higher expression of both RUNX2 and osteopontin higher expression is a marker of immature osteoblasts, whereas the upregulation of osteocalcin marks mature osteoblast with a loss of RUNX2 and osteopontin expression (114).

1.5.3. Role of low oxygen tension and IGFs in osteogenic differentiation

In a low oxygen tension microenvironment, osteogenic differentiation is severely inhibited with reduced matrix maturation and mineralization starting at an early stage, thereby keeping the cells in a stem cell like state (115). This inhibition is mediated by HIF-1α, under low oxygen tension or if stabilized in room air, to reduce RUNX2 expression and alkaline phosphatase activity and delay matrix production and mineralization (115, 116). However, stem cell preconditioning in low oxygen tension has shown an improved state of osteogenic differentiation. In their natural microenvironment, undifferentiated stem cells are in a steady dormant state with a low energy demand that is mostly dependent on glycolysis, with very low aerobic respiration and mitochondria count, even in room air conditions (117). On the other hand, osteogenic differentiation goes through several high energy demanding processes including matrix production, maturation and mineralization (115). Indeed, differentiating osteoblasts undergo a metabolic switch from anaerobic glycolysis to aerobic mitochondrial respiration that is inhibited under low oxygen tension (116). Generally, a metabolic switch is achieved by decreasing glycolysis enzymes and lactic acid production, upregulation of mitochondrial biogenesis and ATP production, and upregulation of antioxidant enzymes to eliminate reactive oxygen species (118, 119). By mathematical modeling, an oxygen gradient exists in the bone marrow where the most primitive stem cells are located in a very low oxygen tension microenvironment far from a bone marrow sinus (120), which suggests that differentiating MSCs move towards higher oxygen tension upon osteoblast

differentiation. Therefore, low oxygen tension can maintain stem cells in a higher multipotent proliferative state and inhibit differentiation *via* hypoxic molecular signaling and low energy supply that does not support the metabolic demand for a mature differentiated state.

In vitro, osteogenic differentiation is triggered by the combined signaling of 100 nM dexamethasone, 50 µM ascorbic acid followed by 10 mM β-glycerophosphate in presence of 10% fetal bovine serum (121). However, optimization experiments using lower concentration of dexamethasone (5-10 nM) and a higher concentration of ascorbic acid (150-250 µM) achieved a higher level of osteogenic differentiation that was significantly improved in presence of human serum or a defined xeno-free media — supplemented with specific growth factors such as insulin, FGF and activin A (121). Therefore, optimization of differentiation conditions can be achieved by changing the growth factor composition in the microenvironment. In immediate contact with bone cells *in vivo*, several growth factors, hormones and cytokines (BMPs, sonic hedgehog proteins, Wnt/β-catenin, FGF and IGF) mediate osteoblast commitment, growth and differentiation (107). IGFs (IGF-I and IGF-II) secreted by skeletal bone cells play an important role in paracrine/autocrine stimulation of bone formation, growth, and metabolism (122, 123). In osteoblast primary cultures, one of IGF-I functions is to exert an anti-apoptotic action and promote survival of differentiating osteoblasts especially at later stages (123). Also, IGF-I was shown to play a

chemotactic role that stimulates the recruitment and migration of osteoblasts (124). Local overexpression of IGF-I in osteoblasts is shown to accelerate the rate of bone formation and increase the pace for matrix mineralization (125). The function of IGF-I signaling is mediated via the IGF-IR, which upon its deletion caused a failure to mineralize bone matrix (125). In disease conditions such as osteoporosis, primary osteoblast cultures has an impaired IGF-IR signaling decoupled from IGF-I stimulation, which possibly causes lower proliferation rate and differentiation and therefore bone loss (126). Therefore, IGF has no effect on osteoblast commitment (no change in RUNX2 expression), but enhances cell maturation at later stages for matrix secretion (increased type-I collagen) and mineralization (127).

1.6. Scope of Thesis

1.6.1. Tissue regenerative therapy – stem cell promise

In the twenty first century, the burden of organ failure and degenerative diseases, which cannot be corrected by drugs alone, has been focused on the promise in cell replacement therapy and tissue regeneration (128). Some of the diseases that are targeted by stem cell therapy include hormonal dysfunction (diabetes and growth hormone), neurodegenerative diseases (Parkinson's, Alzheimer's and Huntington's) and cardiovascular diseases (myocardial infarction, peripheral vascular ischemia) as well as various injuries in the cornea, skeletal muscle, skin,

joints and bones (129). In this context, stem cells have a great potential for tissue/organ repair, replacement of dying cells and promoting the survival of damaged tissue (130). However, the specific use of stem cells from the sources available has their own risks and limitations that can restrict their use for tissue regeneration. ESCs, in principle, can be used to generate any cell type from the three germ layers; however, the uncertain result of tumorigenicity potential and the ethical and political controversies converge to an obstacle for successful use (128). To date, one clinical trial is ongoing to test the use of ESC in spinal cord injury (131). In line with pluripotent ESCs, iPSCs have the advantage of being generated from autologous sources that can eliminate graft versus host disease; however, they are not ready for clinical trials due to the lack of understanding of the optimal reprogramming effect on cells behavior *in vitro* and optimally for *in vivo* application. MSCs so far, show great promise as they have less ethical controversies and less tumorigenicity (do not form teratomas *in vivo*) due to less proliferative potential and a more restricted lineage differentiation. Also, MSCs have the immunomodulatory effect to reduce an immune response and were able to engraft successfully in a therapy resistant graft versus host disease patients (130). Among the adult MSC populations described to date, only the hematopoietic stem cells, isolated from bone marrow and umbilical cord blood, have been used in over 1,200 active open trials (128, 132). More trials are testing other sources of stem cells for clinical intervention, with 3 embryonic-, 36 adipose-, 280 neural- stem cells and 115 using MSCs (128). In Canada, there are 204 open or pending clinical trials using stem cells, as listed on the NIH

website clinicaltrials.gov (128). Therefore, research is still ongoing to optimize the use of MSCs from alternative sources for tissue regeneration therapies.

1.6.2. Hypothesis and objectives

In this study, we aim to obtain a homogeneous multipotent stem cell population from different gestations of the human placenta (PMSCs), optimize cell culture conditions and manipulate the stem cell fate to induce cell differentiation *in vitro*. By manipulating the stem cell microenvironment, my objective is to characterize the role of IGF-I and -II, abundant growth factors in the placenta, to regulate PMSC fate under physiological low oxygen tension by investigating, i) the signaling pathways that promote stem cell multipotency and proliferation and ii) the differentiation commitment of PMSCs towards osteogenic progenitor cells.

The *central hypothesis* is that the interactive signaling between IGFs and oxygen tension is required to alter gestational stage dependent PMSC fate towards self-renewal or differentiation.

The effect of low oxygen tension on IGF signaling in PMSC is investigated as follows:

1. To characterize the role of IGF-I on early and late gestation PMSCs multipotency and proliferation and the signaling cascade governed by ERK/2 and the AKT signaling pathways in low oxygen tension conditions (Chapter 2).

2. To compare and contrast the role of IGF-I and IGF-II in maintaining PMSC multipotency, proliferation and signaling in low oxygen tension conditions (Chapter 3).

3. To determine the role of IGFs on PMSC commitment and differentiation towards the mesodermal osteogenic lineage (Chapter 4).

In this study, we showed that the placenta is an abundant source of multipotent mesenchymal stem cells and that the physiological microenvironment is crucial for directing the stem cell fate to maintain multipotency or differentiation. This work is an antecedent investigation in understanding the niche conditions required to determine the specific fate of stem cells that will lead to improved success in tissue regenerative therapies.

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CHAPTER TWO

Low Oxygen Tension and Insulin-like Growth Factor-I (IGF-I) Promote Proliferation and Multipotency of Placental Mesenchymal Stem Cells (PMSCs) from Different Gestations via Distinct Signaling Pathways

2.1.   INTRODUCTION

Human placenta is a readily available source of adult stem cells, termed placental mesenchymal stem cells (PMSCs) that originate from the primitive allantoic mesenchyme derived from the embryoblast (1). Stem cells have been isolated from different parts of the placenta (chorionic villi, membranes, umbilical cord, chorioallantois and amniotic fluid) and the largest source is the chorionic villi (2, 3). Studies have shown that PMSCs are multipotent, can differentiate into cells of all three germ layers including cartilage, bone, endothelial, adipose, muscle or neuronal lineages, express embryonic stem cell markers such as OCT4, SSEA-4, TRA-1-61, TRA-1-80, and are devoid of trophoblastic and hematopoietic markers (4-7). PMSCs are potential candidates for regenerative therapy and tissue engineering, as they have significantly less or no allo- or xenogeneic immune response, and suppress alloreactive T cells in a mixed lymphocyte reaction (8, 9).

The stem cell microenvironment or niche maintains normal pluripotent capacity and enables cells to respond appropriately to cues when regeneration is needed. Abnormalities in this capacity can lead to disease states such as tumorigenesis or an opposite outcome, poor healing. During normal human pregnancy, the microenvironment of the developing placenta undergoes changes in oxygen tension and growth factor concentrations. IGF-I concentration is low at early gestation and increases at term to reach ~100 ng/mL in the umbilical cord venous blood (10). Similarly, oxygen tension is very low at implantation, near 0%,

reaches 2.4% O_2 at first trimester and increases gradually after the formation of the utero-placental circulation to reach ~5 - 8% O_2 similar to adjacent endometrium (11, 12). Hence, PMSCs isolated from early and late gestational stages may have different responses to IGF-I and low oxygen tension mimicking their natural microenvironment.

Insulin-like growth factors (IGFs; IGF-I and -II) are major autocrine/paracrine growth factors that regulate growth and differentiation during development of all tissues including placenta, and they are expressed as early as in preimplantation embryos in humans (13, 14). Circulating levels of IGF-I and -II correlate with placental and fetal weights, where both can regulate cell growth, differentiation and cell-fate changes through several mitogen activation cascades mainly through the IGF-I receptor (IGF-IR), a receptor tyrosine-kinase (15-17). Upon ligand binding, IGF-IR initiates mitogenic and metabolic signals by activating the two main signaling transduction pathways: the phosphoinositide 3-kinase (PI3-K)/AKT/PKB and the extracellular signal-regulated kinase (ERK1/2) (18, 19). In certain niche conditions, these molecules can induce transcriptional activity that would promote growth, differentiation, survival or self-renewal (20, 21). However, little is known about signaling mechanisms underlying IGF-I actions on MSC proliferation and fate changes.

Oxygen tension plays a major role in stem cell proliferation and differentiation, especially in the placenta. It was shown that cytotrophoblasts proliferate in low

oxygen tension (2% O_2), while they differentiate in high oxygen tension (20% O_2) (11). Also, low oxygen tension can retard the differentiation process of mesenchymal/progenitor stem cells into adipose and cartilage forming cells through changes in hypoxia inducible factor-1 (HIF-1) signaling (22-24). Under low oxygen tension, HIF-1α, the oxygen-sensitive α-monomer of the HIF-1, is stabilized and prevented from ubiquitination and proteosomal degradation (25, 26). HIF-1 can induce transcription of at least 70 genes containing functional hypoxia responsive element (HRE). However, it has been estimated that more than 200 genes and up to 5% of the human genes, depending on the cell-type, are induced by hypoxia, via HIF-1, but not necessarily all are regulated through HRE regions (27-29). HIF-1 can target major genes of diverse functions including cell surface receptors, angiogenic growth factors, glucose transporters, and glycolytic enzymes that primarily promote cell survival (25, 30). Interestingly, downstream of IGF-I signaling, p-ERK1/2 phosphorylates HIF-1α to increase its stability under room air by inhibiting its proteosomal degradation, thereby inducing transcription from HRE containing promoters (31, 32). Low oxygen tension is required to maintain stem cell pluripotency by HIF-2 interaction with the OCT4 promoter region to induce increased transcriptional activity (33). OCT4 is the master regulator of pluripotency in ESCs and is indispensible to generate iPSCs, and is lost upon differentiation (34-38).

To determine the developmental changes and signaling differences in the presence of IGF-I and low oxygen tension, we used both Preterm (P-) and Term

(T-) PMSCs to assess their role in determining stem cell proliferation and multipotency. We show here that the pattern of the IGF-IR, IRS-1, ERK1/2 and AKT levels and phosphorylation are gestational stage-dependent and are modulated by low oxygen tension. We conclude that IGF-I signaling is dependent on oxygen tension, and that the balance between the two factors regulates OCT4 levels, which is a marker for stem cell pluripotency.

2.2. MATERIAL AND METHODS

2.2.1. PMSC Isolation and Characterization

PMSCs were isolated from human placentae of two gestational ages, Preterm PMSCs from 10-13 weeks and Term PMSCs from 37-42 weeks (at least three PMSC primary cell populations from each gestational age). After informed consent, preterm placentae were collected from patients who underwent therapeutic pregnancy termination, and term placentae from healthy pregnant women who underwent elective Cesarean section. Immediately after surgery, small pieces of chorionic villi were dissected free of maternal decidua. Tissue samples were minced mechanically and enzymatically digested with collagenase IV/hyaluronidase and DNase I, followed by trypsin/EDTA for 20 min at 37ºC, followed by 10 min wash at 4ºC in 10% fetal bovine serum (FBS) in PBS (Gibco, Mississauga, ON). Cells were passed through a nylon tissue mesh (45 μm) to obtain a single cell suspension. Next, cells were separated on a Percoll (Sigma) discontinuous gradient (layers 4 and 5) modified from Worton et al. for hematopoietic stem cell isolation (39), and then seeded in tissue culture flask

(Falcon) using DMEM/F12 media (Gibco, Mississauga, ON) containing 10% FBS and antibiotic-antimycotic solution. After 4 days, media was changed and non-adherent cells were washed to leave behind adherent PMSCs able to form colonies.

Placental MSCs were characterized as mentioned previously (40-42), by flow cytometry for the absence of hematopoietic markers CD45 and CD34, and for cytokeratins (-8, -9, and -17), and the presence of MSC markers CD105 (SH2) and CD73 (SH3, SH4), and CD117 (c-Kit, stem cell factor receptor). In short, cell monolayer was trypsinzied by 1X TrypLE Express (Gibco, Mississauga, ON) for 5 min and centrifuged at 1000 rpm. Cell pellet was resuspended in 1x DPBS (Gibco, Mississauga, ON) and incubated with the primary Ab for 1-hr at 37° C, followed by Alexafluor 488 secondary Ab (A11029, Invitrogen) for 30 min at 37° C. Cells were then washed and resuspended in 1 mL of 1x DPBS and run using Beckman Coulter flow-cytometer reaching 10,000 counts.

By immunoblotting, PMSCs were positive for pluripotency markers OCT4, SOX2 and NANOG. Also, PMSCs were able to differentiate towards osteogenic, endothelial, adipose and muscle lineages from mesodermal lineage and neuron-like cells expressing Nestin from the ectodermal lineage.

2.2.2. Cell Culture and Incubation in Low Oxygen Tension

For each experiment, two preterm or term PMSC populations were used in triplicate. Cells were cultured and maintained using DMEM/F12 media with 10%

FBS and FGF-2 (100 ng/mL) (Gibco, Mississauga, ON). Before treatment, cells were cultured in DMEM/F12 with 10% FBS for 24-hrs. Upon treatment, PMSCs were switched directly to fresh serum free media containing IGF-I (0 – 100 ng/mL) in the presence or absence of either 5 µM U0126 or 10 µM LY294002 (Cell Signaling Tech., Danvers, MA). Proliferation was measured using the WST1 reagent assay (Roche) following manufacturer's protocol. PMSCs were seeded at 5,000 cells/100 µL in 96-well plates. All cell cultures were then placed in either a 5% CO_2 incubator or a hypoxia chamber—filled with a 1% O_2, 5% CO_2, balanced N_2 gas mixture (BOC Canada Ltd, Toronto, ON) for 15 min to ensure saturation. The saturation of the oxygen in the chamber was measured using a Hudson 5590 Oxygen Monitor (Hudson, Ventronics Division). Thereafter, the chamber was placed in a tissue-culture incubator at 37° C. At the end of the experiment, oxygen tension in culture media was measured using the ABL700 series blood gas analyzer (Radiometer, Copenhagen, Denmark).

2.2.3. Human Phospho-kinase Array

To screen for kinases phosphorylated upon IGF-I stimulation under room air or low oxygen tension, PMSCs were serum-deprived for 24-hrs under 20% or 1% oxygen. For IGF-I treatment, PMSCs from both oxygen tension conditions were washed with 1x PBS (x 3) and then stimulated with 100 ng/mL of IGF-I for 15 min., a proteome Profiler^{TM} Array (#ARY003, R&D system, Minneapolis, MN) Part A was used according to manufacturer's protocol. Treated cells were rinsed with PBS and then solubilized in lysis buffer and the protein concentration was

determined by Bradford Assay (BioRad laboratories, Hercules, CA). Kinase array was incubated with cell lysate of the same protein concentration, washed, and specific kinases phosphorylated were identified by detection biotinylated antibody cocktail A. Immunocomplexes were detected with a streptavidin-HRP binding and the signal was developed using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Perkin Elmer Life Sciences), and documented on Kodak Biomax film (Rochester, NY, USA).

2.2.4. Immunoblotting

Cell lysates were prepared using 1x cell lysis buffer for phospho-proteins according to manufacturer protocol (#9803, Cell Signaling Technologies, Burlington, ON, CAN). Protein samples (10-20 μg) were resolved by SDS-PAGE, and then transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 5% non-fat-dry milk in 1x TBS (Tris-buffered saline) for 1 hr at room temperature. Blots were then washed in 1x TBS 0.1% Tween 20 (TBS-T) (3x for 5 min) followed by incubation at 4°C overnight with primary antibodies as per manufacturers' protocols. Blots were then washed using TBS-T (3x for 10 min) and were incubated with secondary HRP-labelled antibody for 1 hr at RT. Immunocomplexes were detected by ECL and detected using VersaDOC^{TM} Imaging System (Bio-Rad).

2.2.5. Antibodies

For flow cytometry, the following antibodies were used: CD-73 (#550256) (BD Pharmingen, San Jose, CA), PE conjugated CD105 (#12-1057-73) (eBioscience, San Diego, CA) CD-117/c-Kit (sc-13508) (Santa Cruz Biotech., Santa Cruz, CA). To detect the IGF-I activated signaling molecules, the following antibodies were used: phospho-p44/42 MAPK (#4377), p44/42 MAPK (#9102) phospho-AKT (Ser473, #4051), AKT (#9272), PCNA (PC10, #2586), and IRS-1 (#2382) from Cell Signaling Technologies (Danvers, MA, USA). For OCT3/4, antibody (N-19, sc-8628) (Santa Cruz Biotech., Santa Cruz, CA, USA) was used. For the detection of hypoxia markers, HIF-1 alpha antibody (H206, sc-10790) (Santa Cruz Biotech., Santa Cruz, CA, USA) was used. For loading control, pan-Actin Ab-5 (#MS-1295) (Thermo Fisher Scientific, Fremont, CA) was used. The secondary antibodies used for immunoblotting were goat anti-rabbit (#170-6515) or anti-mouse (#170-6516) HRP conjugated antibodies (BioRad laboratories, Hercules, CA) or donkey anti-goat antibody (sc-2020) Santa Cruz Biotech., Santa Cruz, CA, USA).

2.2.6. Statistical Analysis

All experiments were run in triplicates from three independent experiments each; whenever possible, three or more PMSC primary lines were used from each gestational stage. All graphs and analyses were generated using GraphPad Prism Software 5.0 (GraphPad Software, San Diego, CA). A Two-way ANOVA with Bonferroni *post hoc* test was used for the PMSC WST1 proliferation assay

and densitometry quantifications. Data are expressed as mean ± standard error of the mean (SEM); values were considered significant when p<0.05.

2.3.   RESULTS

2.3.1. IGF-I mediated PMSC proliferation response is dependent on oxygen tension and gestational age

In PMSC cultures, low oxygen tension (1% O_2) achieved 52.2 mmHg oxygen tension in the culture media. This oxygen tension is physiological during human pregnancy at 12-13 weeks (early second trimester), which ranges between 46.5 mm Hg at the endometrium (maternal side) to 60.7 mm Hg in the placenta (fetal side) (12). This low oxygen tension stabilized HIF-1α confirming the hypoxic condition (Fig. 2.1A) and increased OCT4 levels in both types of PMSCs confirming enhanced multipotency (Fig. 2.1B). Low oxygen tension had no effect on the expression of surface markers (CD73, CD105 and CD117/c-kit) in both P- and T-PMSCs (Fig. 2.1C). However, gestational age differences were detectable between the two PMSCs, higher abundance of OCT4 in room air (one-way ANOVA, p<0.05) and higher CD117/c-kit expression (~15-fold) in P-PMSCs than in T-PMSCs.

To demonstrate the effect of IGF-I on PMSC proliferation, we subjected P- and T-PMSCs to increasing concentrations of IGF-I and low oxygen tension. P-PMSCs exhibited continued proliferation in serum-free media, which was increased in a dose-dependent manner by addition of IGF-I (plateau at 50 ng/mL) (Fig. 2.2A).
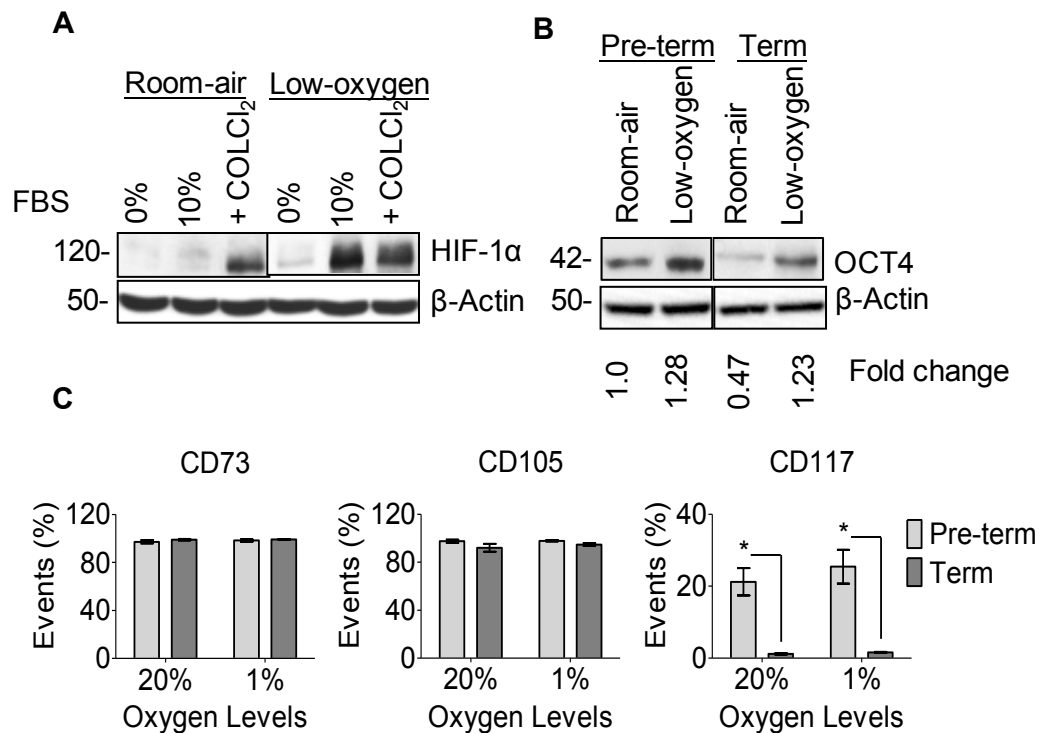
FIGURE 2.1. Low oxygen tension increases OCT4 levels in PMSCs and maintaining stemness. (A) HIF-1α levels in P-PMSCs in10% fetal bovine serum (FBS) in room air or low oxygen tension at 48 hrs (T-PMSCs show similar levels). The positive control is PMSCs in 10% FBS treated with 0.1 mM cobalt chloride in room air. β-Actin levels indicate equivalent protein loading. (B) OCT4 levels in P- and T-PMSCs in 10% FBS cultured in room air or low oxygen tension for 48 hrs. β-Actin levels indicate equivalent protein loading. (C) Stem cell surface markers (CD73, CD105, CD117) of P- and T-PMSCs in 10% FBS in room air or low oxygen tension expressed as % of total positive cells (measured in 10,000 cells, n=3; one-way ANOVA, p<0.05, * = significant difference between low oxygen and room air). Low oxygen tension increased OCT4 levels in preterm and term PMSCs. Stem cell surface marker expression was not changed by low oxygen tension in preterm or term PMSCs.

However, low oxygen tension together with IGF-I (100 ng/mL) further increased P-PMSC proliferation (Fig. 2.2A). On the other hand, T-PMSCs had not significantly increased proliferation in response to IGF-I in room air that was further increased by low oxygen tension (highest at 10 ng/mL and decreased at higher concentrations) (Fig. 2.2B). Since the proliferation of P-PMSCs and T-PMSCs in low oxygen tension was noticeably different at 100 ng/mL of IGF-I, a time course experiment (over 12, 24 and 48-hrs) was conducted. In room air, PMSC proliferation was significantly increased by IGF-I in both P- and T-PMSCs after 24-hrs (3.5-fold and 2.5-fold, respectively) (t-test, $P = 0.0005$) (Fig. 2.2C and D). Low oxygen tension enhanced the IGF-I stimulated proliferation of both PMSCs (up to 4.6-fold in P-PMSCs compared with 3.1-fold in T-PMSCs) (t-test, $P = 0.0001$). However, at 48-hrs, the proliferation subsided in both P-PMSCs and T-PMSCs to ~ 0.5-fold in both oxygen tensions (Fig. 2.2C and D). Overall, the response to IGF-I was gestational age-dependent and enhanced by low oxygen tension—P-PMSCs having a higher proliferation response.

2.3.2. IGF-I mediated PMSC OCT4 expression is dependent on oxygen tension and gestational age

Similar to the proliferation response, we checked for multipotency regulation by IGF-I concentration and exposure time in low oxygen tension. In P-PMSCs, IGF-I (100 ng/mL) increased OCT4 levels, in both oxygen tension conditions (Fig. 2.3A), whereas in T-PMSCs, OCT4 only increased in response to IGF-I in room air (Fig. 2.3B). OCT4 was also temporally regulated; in P-PMSCs
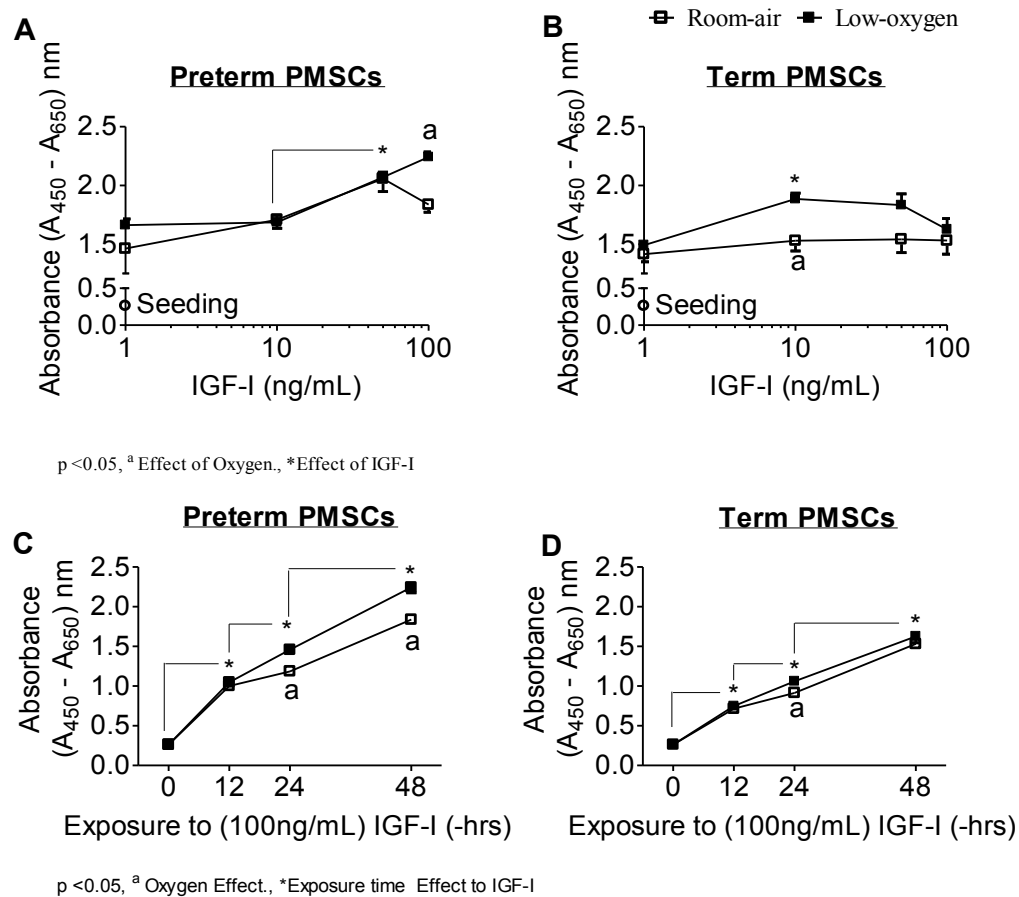
FIGURE 2.2. **Low oxygen tension increased PMSC proliferation in response to IGF-I**. PMSC proliferation was assessed using the WST-1 colorimetric proliferation assay using absorbance at 450 nm with a background reference of 650 nm. (A) P-PMSCs and (B) T-PMSCs were treated with increasing concentration of IGF-I concentrations (0, 10, 50 and 100 ng/mL) in room air or low oxygen tension for 48-hrs. (C) P-PMSCs and (D) T-PMSCs were treated with IGF-I (100 ng/mL) under room air or low oxygen tension in a time course from 0 to 48-hrs. Two-Way ANOVA, $P<0.05$, n=8; a = significant difference between room air and low oxygen tension, * = significant difference by different IGF-I concentration. A cell counting experiment has been done as shown in Figure S2.1A and B. In both P- and T-PMSCs, low oxygen tension enhanced the effect of IGF-I on cellular proliferation.

under room air, IGF-I gradually increased OCT4 levels that peaked at 12-hrs, and was stable up to 48-hrs (Fig. 2.3C). Low oxygen tension induced a similar expression pattern with a maximal increase at 24-hrs. In T-PMSCs, OCT4 expression increased rapidly after IGF-I exposure in room air, but not in low oxygen tension (Fig. 2.3D). The maximal increase in OCT4 expression (up to 1.5-fold) occurred earlier in T-PMSCs (6-hrs) compared with P-PMSCs (12 hrs) suggesting that IGF-I effect is gestational age-dependent—increased sensitivity in T-PMSCs.

2.3.3. ERK1/2 and AKT signaling downstream of IGF-IR

To map the downstream effectors of IGF-I signaling, we used a phospho-kinase array (Fig. 2.4A). Short-term exposure of P-PMSCs (15 min) in room air to IGF-I increased phosphorylation of ERK1/2 (spots A5, A6) (Fig. 2.4B). Increased p-ERK1/2 by low oxygen tension was not increased further by IGF-I (Fig. 2.4B). AKT phosphorylation (spots B9, B10) was increased by IGF-I in both room air and low oxygen tension conditions (Fig. 2.4B). Similarly, IGF-I increased p-ERK1/2 in room air, and low oxygen tension increased the phosphorylation independent of IGF-I (Fig. 2.4C and D). In addition, p-AKT was dependent on IGF-I for phosphorylation and was less so by low oxygen tension (Fig. 2.4C and D). The change in signaling proteins was not associated with changes in IGF-IR or in OCT4 levels (Fig. 2.4C). Overall, IGF-I increased ERK1/2 phosphorylation under room air and less under low oxygen tension, which was already induced
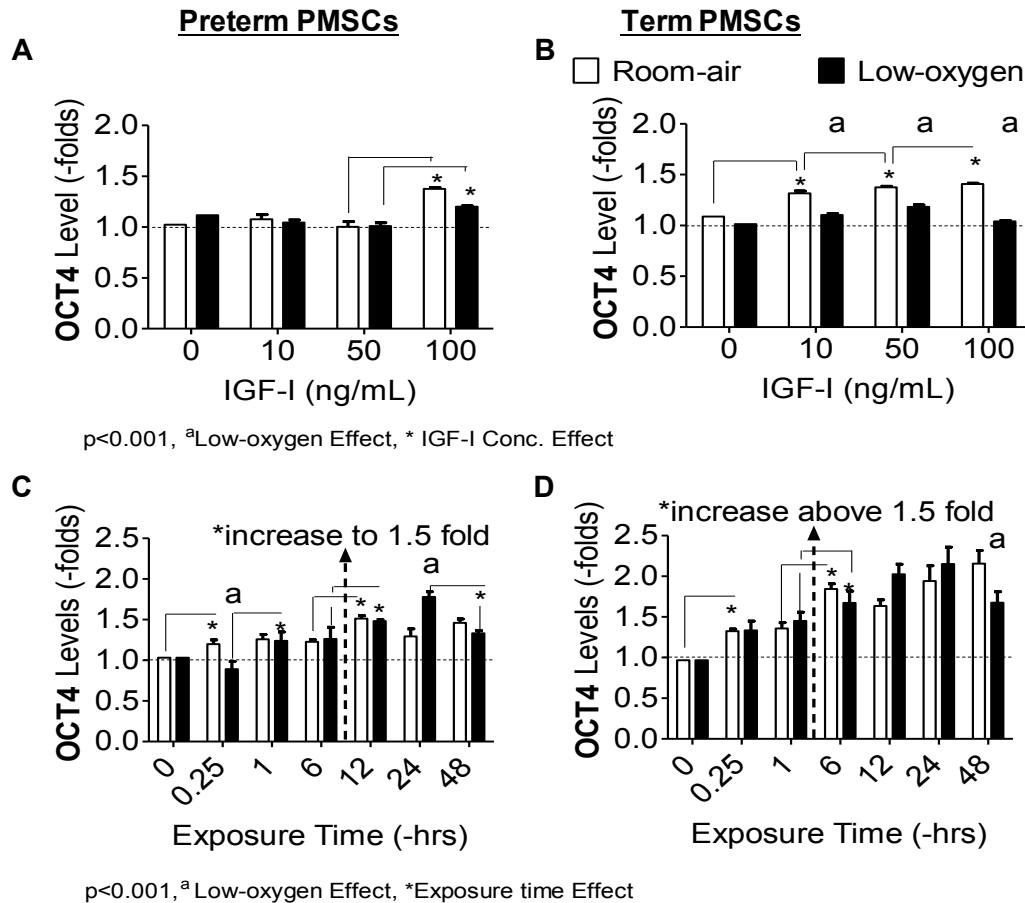
FIGURE 2.3. OCT4 levels in PMSCs in response to IGF-I and low oxygen tension. Levels of OCT4 were quantified by densitometry of immunoblots in P-PMSCs and T-PMSCs. (A) P- and (B) T-PMSCs were stimulated with increasing concentration (0, 10, 50 and 100 ng/mL) of IGF-I for 48-hrs under room air or low oxygen tension. Also, (C) P- and (D) T-PMSCs were stimulated over time with 100 ng/mL over 48-hrs. Quantification by densitometry was normalized to β-Actin for protein load and DNA content to account for stem cell heterogeneity. Two-Way ANOVA, P<0.001, n=3, a = significant difference between room air and low oxygen tension; * = significant difference by different concentration of IGF-I or stimulation time. IGF-I increased OCT4 levels in both P- and T-PMSCs. OCT4 levels increased slowly over 48-hrs in both P- and T-PMSCs.

independent of IGF-I; however, IGF-I was required for AKT phosphorylation under both oxygen tensions.

2.3.4. Phosphorylation of AKT and ERK1/2 is IGF-I-concentration and exposure-time dependent

IGF-I or low oxygen tension stimulated ERK1/2 phosphorylation in both types of PMSCs; however, low oxygen tension reduced levels of the IGF-I mediated increase (Fig. S2.1C). p-ERK1/2 levels in P-PMSCs were unchanged with increasing concentrations of IGF-I in room air but decreased slightly under low oxygen tension (Fig. 2.5A). In T-PMSCs, ERK1/2 phosphorylation was increased by IGF-I in a dose-dependent manner ($p<0.01$, Pearson r $=0.993$) in room air (Fig. 2.5A, right), whereas the opposite was observed in low oxygen tension ($p<0.05$, Pearson r $= -0.953$). The phosphorylation pattern of ERK1/2 was time dependent, in P-PMSCs, the maximal increase in p-ERK1/2 occurred 1-hr after IGF-I stimulation under low oxygen tension (two-fold higher than in room air), and declined at 6-hrs (Fig. 2.5B, left). p-ERK1/2 levels increased again after 48-hrs if cells were maintained in low oxygen conditions (Fig. 2.5B, left). In T-PMSCs, p-ERK1/2 levels were similar to P-PMSCs but at higher levels demonstrating a greater IGF-I response (Fig. 2.5B, right).

Unlike p-ERK1/2, AKT phosphorylation was increased only by IGF-I especially under low oxygen tension in both types of PMSCs (Fig. 2.5C, left). p-AKT

A

	1 2	3 4	5 6	7 8	9 10
A	Positive control	p38α	ERK1/2	JNK	GSK-3
B		MEK	MSK	AMPKα1	AKT
C	TOR	CREB	HSP27	AMPKα2	β-Catenin
D	Src	Lyn	Lck	STAT2	STAT5a
E	Fyn	Yes	Fgr	STAT3	STAT5b
F	Hck	Chk-2	FAK	STAT6	STAT5
G	Positive control		Negative control		

B

Room-air

IGF-I (ng/mL): 0   100

Low-oxygen

IGF-I (ng/mL): 0   100

C Room-air   Low-oxygen

0 100 0 100 IGF-I (ng/mL)

p-AKT
AKT
p-ERK1/2
ERK1/2
IGF-IRα
OCT4
β-Actin

D

p-ERK1/2 levels (Arbitrary units) — Room-air / Low-oxygen

p-AKT levels (Arbitrary units)

IGF-I (ng/mL)

FIGURE 2.4. The effect of low oxygen tension and IGF-I on phosphorylation of specific kinases. (A) The layout of major human cellular kinases in a phospho-kinase array, spotted in duplicates. (B) Phosphorylation changes in P-PMSC whole cell lysate upon stimulation with or without IGF-I under room air or low oxygen for 15 min. (C) Immunoblots of IGF-IRα subunit, OCT4 and the two major phospho-kinases, ERK1/2 and AKT, following stimulation of PMSCs with IGF-I (100 ng/mL) for 15 min. (D) Quantification by densitometry of p-ERK1/2 and p-AKT normalized to the respective total kinase and β-Actin. One-way ANOVA, $P<0.05$, n=3. * = significant difference by IGF-I. Similar results were seen with T-PMSCs. Low oxygen tension alone increased phosphorylation of ERK1/2 but not AKT. IGF-I increased phosphorylation of both ERK1/2 and AKT in both room air and low oxygen tension.

reached its maximal activation at 100 ng/mL IGF-I in P-PMSCs, and at 50 ng/mL in T-PMSCs, again showing sensitivity of T-PMSCs (Fig. 2.5C). Under room air, AKT phosphorylation was significantly higher in T-PMSCs, whereas the effect of low oxygen tension was observed predominantly in P-PMSCs. These results demonstrate a predominant effect of IGF-I on AKT activation compared to ERK1/2 under low oxygen. Concurrently, maximal AKT phosphorylation level was achieved at 15-60 min post IGF-I exposure in both PMSCs under room air (Fig. 2.5D). Low oxygen maintained higher levels of p-AKT, especially in P-PMSCs, before reaching its lowest level at 48-hrs (Fig. 2.5D).

Thus, in low oxygen tension, we observed a correlation between IGF-I mediated increase of OCT4 levels with a decrease in p-ERK1/2 and an increase in p-AKT. This can also be temporally regulated by the increased p-AKT and decreased p-ERK1/2, especially seen in low oxygen tension in P-PMSCs. Therefore, IGF-I concentration and stimulation time control the signaling *via* p-ERK1/2 and p-AKT, which may directly correlate with multipotency and proliferation.

Preterm PMSCs   **Term PMSCs**
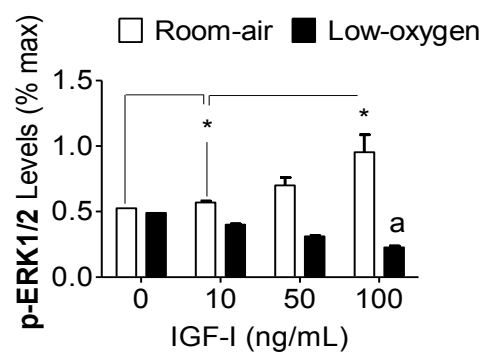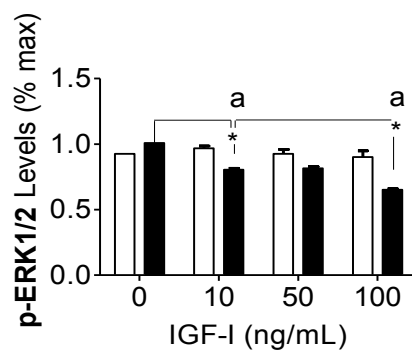
A

p<0.001, ^{a}Low-oxygen Effect, * IGF-I Conc. Effect

B

p<0.001,^{a} Low-oxygen Effect, *Exposure time Effect

Preterm PMSCs   **Term PMSCs**
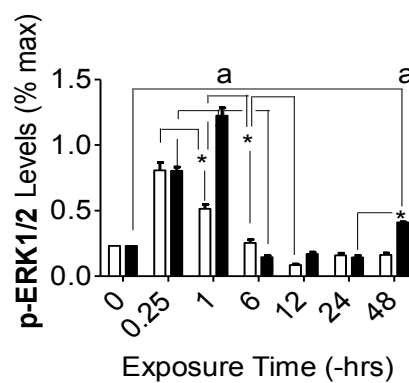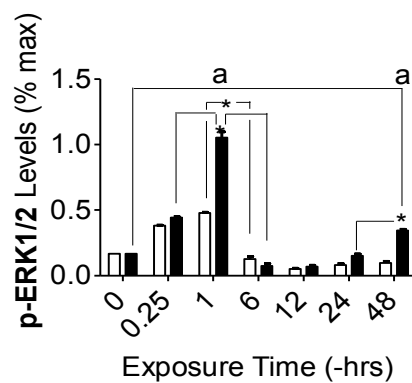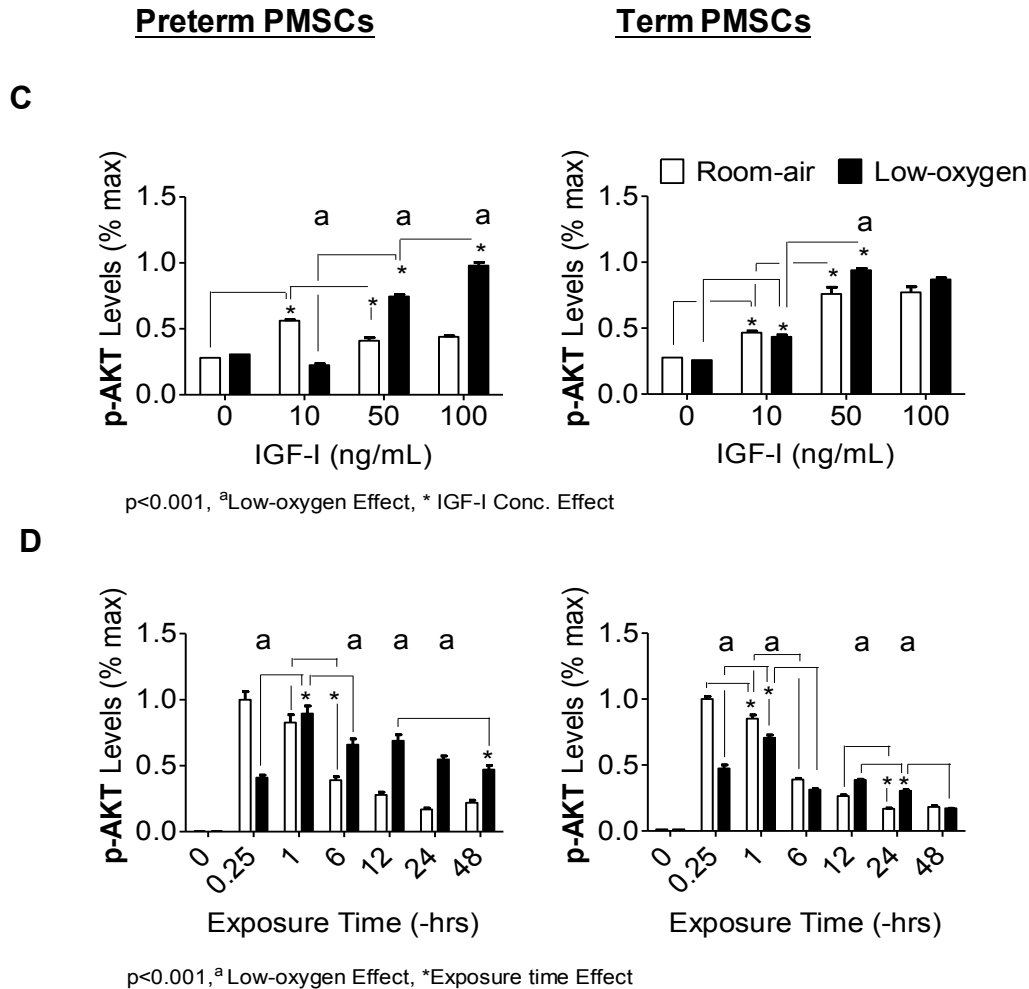
FIGURE 2.5. Oxygen tension modulates p-ERK1/2 and p-AKT in PMSCs in response to IGF-I concentration and exposure time. Phosphorylation of (A and B) ERK1/2 and (C and D) AKT were quantified by densitometry of immunoblots of P-PMSCs and T-PMSCs cell lysates. P- and T-PMSCs were stimulated with increasing concentration (0, 10, 50 and 100 ng/mL) of IGF-I for 48-hrs or with IGF-I (100 ng/mL) over time 0-48-hrs under room air or low oxygen tension. Quantification by densitometry was normalized to the respective total kinase, β-Actin for protein load and DNA content to account for stem cell heterogeneity. Two-Way ANOVA, $P < 0.001$, n=3, a = significant difference between room air and low oxygen tension; * = significant difference by different concentrations of IGF-I or stimulation time. IGF-I increased phosphorylation of ERK1/2 and AKT in both P- and T-PMSCs. Both ERK1/2 and AKT increased rapidly within 1 hr. p-ERK1/2 decreased acutely, whereas p-AKT response was more prolonged. Low oxygen tension prolonged ERK1/2 and AKT phosphorylation.

2.3.5. IGF-IR and IRS-1 levels and phosphorylation are modulated by oxygen tension and IGF-I concentration and stimulation time

IGF-I signaling starts at the level of the receptor, IGF-IR. As a result, levels of IGF-IRα, the IGF-I binding extracellular subunit, were increased in serum free conditions, and decreased by IGF-I in a dose-dependent manner in both P- and T-PMSCs (Fig. 2.6A). In low oxygen tension, IGF-IRα was maintained higher even in presence of IGF-I (Fig 2.6A). Also, IGF-IRα was decreased upon increased stimulation time by IGF-I (Fig. 2.6B). However, low oxygen tension maintained a more stable level of IGF-IRα in both P- and T-PMSCs than the sharp decrease by 48-hrs in room air. IGF-IRβ phosphorylation was also manipulated by low oxygen tension in PMSCs (Fig. S2.3). Levels of p-IGF-IRβ were increased with increasing concentration of IGF-I (Fig. S2.3 A and B), with a higher phosphorylation in P-PMSCs in low oxygen tension. However, increasing the stimulation time decreased IGF-IRβ phosphorylation with a more stable level in low oxygen tension (Fig. S2.3 C and D).

The signaling pathway *via* p-ERK1/2 or p-AKT is dependent on interacting adaptor molecules with the IGF-IR. IRS-1, a major interacting adaptor protein with p-IGF-IRβ, was not changed by increasing IGF-I concentration in P-PMSCs, whereas was increased in T-PMSCs in room air (Fig 2.6C). Low oxygen tension maintained higher IRS-1 levels independent of IGF-I in both PMSCs (Fig. 2.6C). IRS-1 levels were also dependent on the duration of IGF-I exposure time, which

Preterm PMSCs **Term PMSCs**

A

p<0.001, ^aLow-oxygen Effect, * IGF-I Conc. Effect

B

p<0.001,^a Low-oxygen Effect, *Exposure time Effect

Preterm PMSCs   **Term PMSCs**
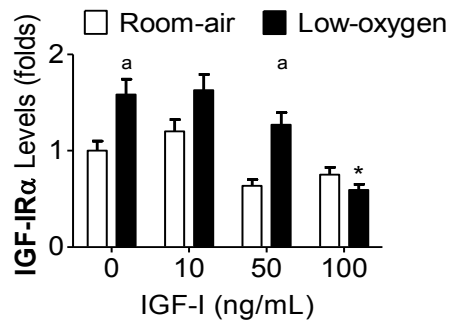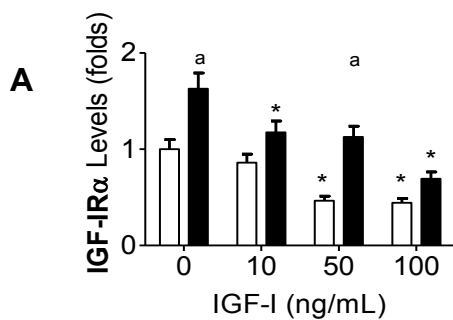
p<0.001, [a]Low-oxygen Effect, * IGF-I Conc. Effect
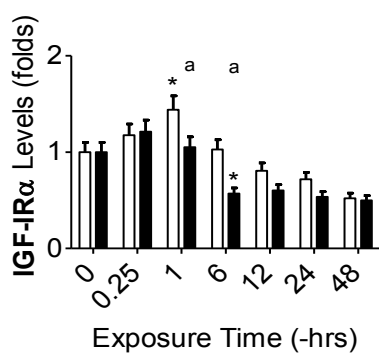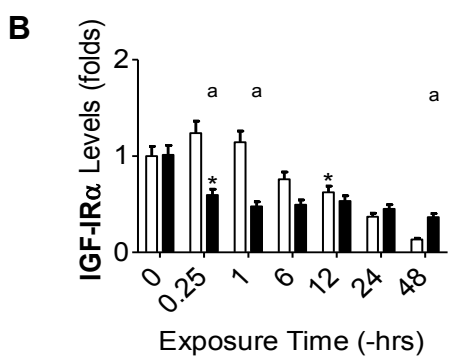
p<0.001, [a] Low-oxygen Effect, *Exposure time Effect
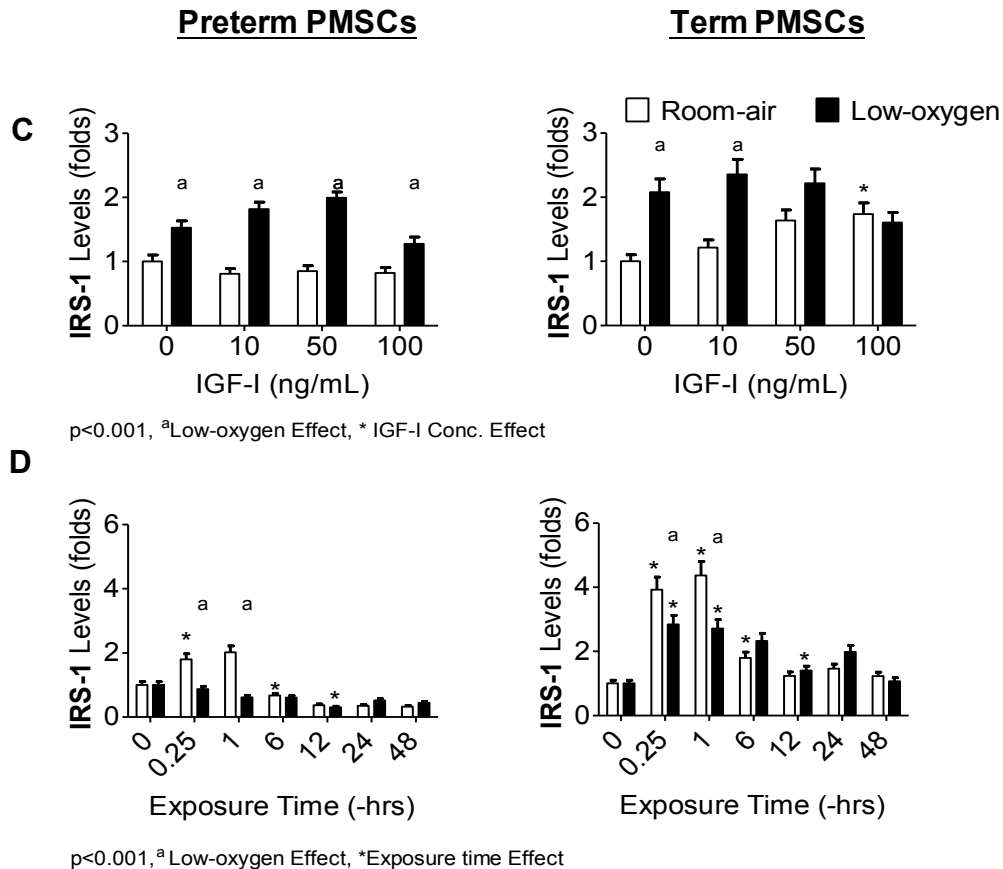
FIGURE 2.6. Oxygen tension modulates IGF-IRα and IRS-1 levels in PMSCs in response to IGF-I concentration and exposure time. Levels of (A and B) IGF-IRα and (C and D) IRS-1 were quantified by densitometry of immunoblots in P-PMSCs and T-PMSCs. P- and T-PMSCs were stimulated with an increasing concentration (10, 50 and 100 ng/mL) of IGF-I for 48-hrs or in presence of IGF-I (100 ng/mL) over 0-48-hrs under room air or low oxygen tension. Quantification of immunoblots (presented in Fig. S2.2) by densitometry was normalized to β-Actin for protein load and DNA content to account for stem cell heterogeneity. Two-Way ANOVA, P<0.001, n=3, a = significant difference between room air and low oxygen tension; * = significant difference by different IGF-I concentrations or stimulation time. Low oxygen tension maintained increased levels of IGF-IR and IRS-1 that were decreased by IGF-I and exposure time.

has peaked at 1 hr then decreased (Fig. 2.6D). Low oxygen tension, however, maintained a narrow decrease in IRS-1 levels.

To understand the role of IRS-1 in IGF-I signaling, we checked two phosphorylation sites on IRS-1, p-Y896 for GRB2 binding upstream of p-ERK1/2 (43), and p-Y612 for p85 subunit of PI3K upstream of p-AKT (44). Phosphorylation levels were different between pY612 and pY896. Low oxygen tension upregulated p-Y896 in P-PMSCs and less in T-PMSCs (Fig. S2.3 A and B), which correlated with a higher p-ERK1/2 and proliferation. The levels of p-Y612 were more responsive to increase with IGF-I concentration (Fig. S2.3 A and B), which are required for the increased p-AKT activation levels seen in figure 2.5 (C and D). With increased exposure time, p-Y896 was increased (peaked at 12 and 48 hrs) in both PMSCs, whereas p-Y612 was increased gradually (a major peak by 12 hrs) but decreased at 48 hrs with a higher level in P-PMSCs (Fig. S2.3 C and D). These findings indicate that low oxygen tension is a dominant regulator to increase IGF-IR and IRS-1 and their phosphorylation levels in presence of IGF-I. These changes may explain the induced phosphorylation levels seen with p-ERK1/2 and p-AKT levels that correlates with a higher proliferation and multipotency.

2.3.6. Inhibition of ERK1/2 and AKT suppresses PMSC proliferation and OCT4 levels

We inhibited signaling downstream of IRS-1 of either MEK1/2 or PI3K using U0126 and LY294002. In P-PMSCs, U0126 reduced p-ERK1/2 levels in the presence of IGF-I (100 ng/mL) (Fig. 2.7A, left), and increased p-AKT levels under low oxygen tension (Fig. 2.7B, left). LY294002 decreased p-AKT in both oxygen tension conditions, while IGF-I had no effect (Fig. 2.7D, left). LY294002 is not a strict inhibitor of PI3K/AKT pathway, because it also reduced the p-ERK1/2 levels (Fig. 2.7C, left). In T-PMSCs, U0126 had less inhibitory effect than in P-PMSCs in absence of IGF-I, while the inhibitory effect increased at higher concentrations of IGF-I (Fig. 2.7A, right). Also, LY294002 increased p-ERK1/2 levels and this effect was parallel to reduction in p-AKT levels, which were further downregulated by IGF-I (Fig. 2.7C and D, right).

The physiological role of activating ERK1/2 and AKT in PMSCs was evaluated by proliferation assay. Both U0126 and LY294002 abolished the IGF-I stimulated proliferation in both P- and T-PMSCs (Fig. 2.8A and B; C and D, respectively) that was not due to induced apoptosis (Fig. S2.4). Low oxygen tension increased cell proliferation in the presence of both inhibitors but did not restore normal proliferation response by IGF-I (Fig. 2.8 A – D). Interestingly, LY294002 caused a greater decrease in cell proliferation regardless of IGF-I or oxygen tension ($P <$ 0.0001) (Fig. 2.8C and D). The decreased proliferation by the inhibitors was greater in presence of IGF-I suggesting that PMSC proliferation is dependent on
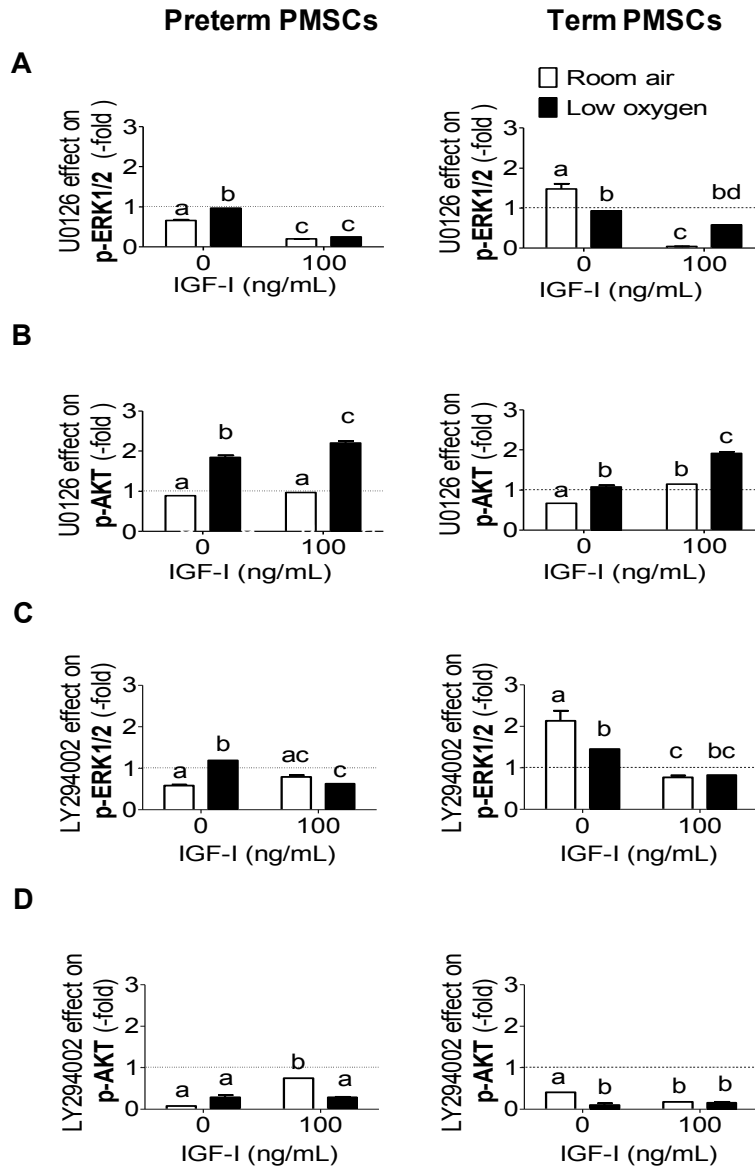
FIGURE 2.7. The effect of upstream MEK1/2 inhibition or PI3K inhibition on ERK1/2 and AKT phosphorylation PMSCs. (A and C) p-ERK1/2, (B and D) p-AKT levels in P-PMSCs and T-PMSCs in the presence of U0126 (5 µM) or LY294002 (10 µM). Cells were treated with the inhibitor with or without IGF-I (100 ng/mL) under room air or low oxygen tension. Phospho-kinase levels were normalized to both total level of kinase and to β-Actin. The dotted lines (set at 1) represent the levels of p-ERK1/2 and p-AKT in DMSO control. The comparison with DMSO control is present in the supplemental figures (S2.5 and S2.6). Two-Way ANOVA, $P < 0.001$, n=3. U0126 inhibited p-ERK1/2 while increased p-AKT in presence of IGF-I, especially under low oxygen tension. LY294002 inhibited p-AKT and reduced the levels of p-ERK1/2 in presence of IGF-I.
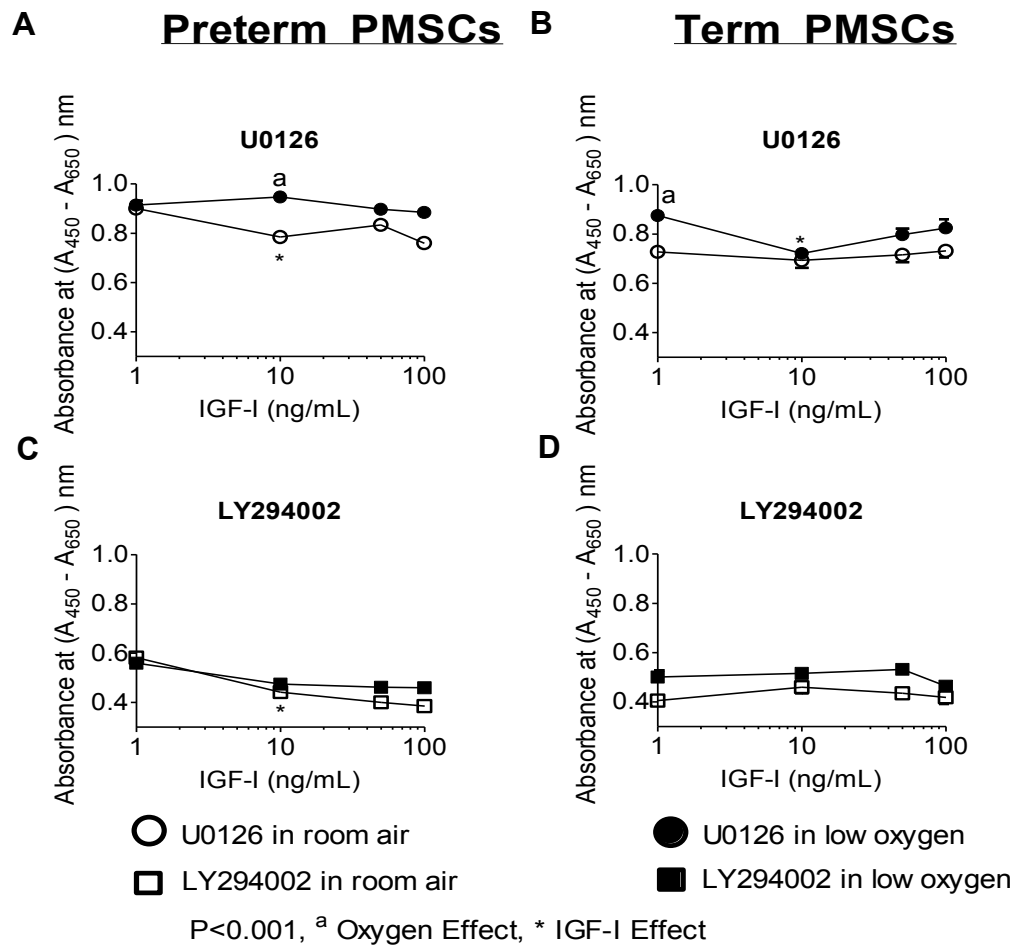
FIGURE 2.8. Proliferation of P- and T-PMSCs in the presence of ERK1/2 or AKT pathway inhibitors. Proliferation of both (A and C) P-PMSCs and (B and D) T-PMSCs is shown by the WST1 assay. P- and T-PMSCs were grown in the presence of (5 µM) U0126 (A and B) or (10 µM) LY294002 (C and D) in varying concentrations of IGF-I (0, 10, 50, 100 ng/mL) under room air or low oxygen for 48-hrs. The absorbance was measured at 450 nm with a background reference of 650 nm. Absorbance values were normalized to their matched DMSO controls. (Two-way ANOVA, $P<0.05$, n=8, a = significant difference between room air and low oxygen tension; * = significant difference by different IGF-I concentrations). PMSCs were not dying in response of inhibitor as shown in Figure S2.4. U0126 and LY294002 inhibited IGF-I increased P- and T-PMSC proliferation. Low-oxygen tension maintained a higher proliferation but could not rescue inhibition effect.

IGF-I signaling via MEK1/2 and PI3K, and eventually through p-ERK1/2 and p-AKT. The augmented effect of low oxygen tension on proliferation was dependent on both signaling pathways.

To determine if p-ERK1/2 and p-AKT are involved in multipotency, OCT4 levels were measured under low oxygen levels in presence of either U0126 or LY294002. Blocking p-ERK1/2 pathway did not affect OCT4 levels under low oxygen tension in both P- and T-PMSCs (Fig 2.9A), while blocking p-AKT decreased OCT4 levels in the presence of IGF-I (Fig. 2.9B). OCT4 was more sensitive to PI3K/AKT activation compared to MEK/ERK1/2 and it was restored in both types of PMSCs when p-AKT level was maintained or increased by U0126 under low oxygen (Fig. 2.7B and 9A). We concluded that p-AKT is important in maintaining OCT4 expression and therefore multipotency, by IGF-I.

2.4. DISCUSSION

Unlike Embryonic stem cells (ESCs), MSCs show great promise for tissue regeneration therapies as they have less ethical controversies and are less tumorigenic *in vivo* (45). Also, MSCs have the immunomodulatory effect to reduce an immune response and to successfully engraft in graft versus host disease resistant patients (45, 46). However, a large number of MSCs is required for tissue regeneration therapies; therefore, optimization of *in vitro* culture conditions are needed to maintain multipotency
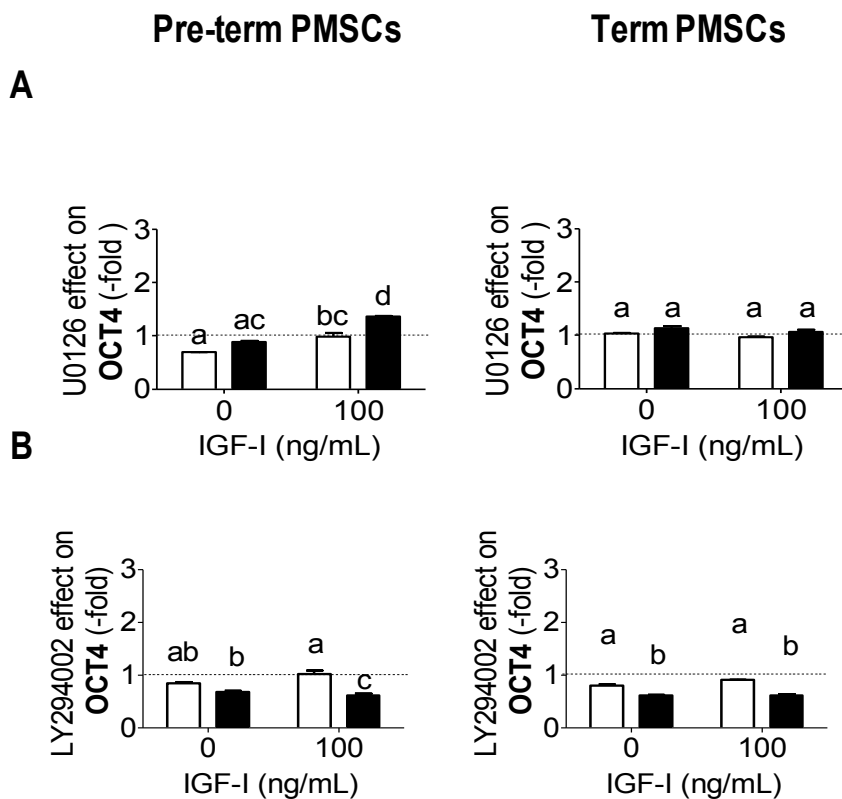
FIGURE 2.9. The effect of upstream MEK1/2 or PI3K inhibition on OCT4 levels in P- and T-PMSCs in the presence of IGF-I and low oxygen tension. OCT4 levels from immunoblots of P-PMSCs and T-PMSCs in the presence of (A) U0126 (5 µM) or (B) LY294002 (10 µM) are shown. P- and T-PMSCs were treated with the inhibitor with or without IGF-I (100 ng/mL) under room air or low oxygen tension. OCT4 levels were normalized to total β-Actin levels. The dotted lines (set at 1) represent the levels of OCT4 in their respective DMSO control. The comparison with DMSO control is present in the supplemental figures (S2.7). Two-Way ANOVA, P<0.001, n=3. OCT4 levels were not affected by U0126, but were reduced by LY294002 especially under low oxygen tension.

and proliferation without inducing differentiation. This can be manipulated by understanding the natural microenvironment of the stem cell niche, which defines the structural, chemical, and biochemical factors (47, 48). In this study, we focused on the role of low oxygen tension in controlling the IGF-I mediated proliferation and multipotency of PMSC from early and term gestation placentae. Here, we demonstrated that PMSCs from early gestation (P-PMSCs) had a greater proliferation response to IGF-I and was enhanced by low oxygen tension (1%) in contrast to term gestation PMSCs (T-PMSCs). IGF-I and low oxygen major signaling was mediated mainly *via* p-ERK1/2 and p-AKT. Phosphorylation of p-AKT but not p-ERK1/2 in low oxygen tension was IGF-I-dependent. Multipotency, *via* OCT4 levels, was positively regulated by IGF-I concentration and stimulation time mainly *via* the signaling of p-AKT. Thus, IGF-I can mediate PMSC proliferation and multipotency from different gestations which is enhanced in low oxygen tension.

Low oxygen tension culture conditions used in this study, which is usually defined as hypoxia in many *in vitro* experiments, is the normal physiological oxygen tension in stem cell niches, whereas room air (20% O_2) used for standard cell culture is hyperoxic. Low oxygen tension found in many tissues ranges from ~ 14.4 mm Hg to ~ 68.4 mm Hg (2 - 9% with a mean of 3%) (49-51) and can reach as low as 7.2 mm Hg (1%) in bone marrow (where hematopoietic stem cells reside), thymus or the kidney medulla due to atypical blood vessel network (51). In the placenta, oxygen tension at the endometrial surface starts low at 17.9 mm

Hg ($\sim$ 2.35% O_2) prior to the establishment of utero-placental circulation and then rises up to 60 mm Hg ($\sim$ 7.9% O_2) after connection with the maternal circulation (11). We, therefore, proposed that tissue culture conditions should physiologically mimic *in vivo* conditions in order to better understand PMSC proliferation and multipotency.

ESCs exist in low oxygen tension conditions, and fertilization occurs in a hypoxic microenvironment (12, 52, 53). Culturing embryonic stem cells at low oxygen (1%) maintained a slower proliferation rate with a higher number of cells in the inner cell mass (ICM) and, more importantly, maintained greater pluripotency (higher OCT4 expression and SSEA markers) as compared to those maintained in ambient room air (51, 54). Therefore, low oxygen tension supports the full maintenance of pluripotency state of mammalian ESCs. PMSCs are typically isolated from term pregnancies (55-57); here, we isolated PMSCs from preterm pregnancies (11-13 weeks) with a hypothesis that P-PMSCs can have a higher multipotent state. Just like in ESCs, our results showed that low oxygen tension enhance both proliferation and OCT4 expression in P- and T-PMSCs. However, P-PMSCs expressed higher levels of OCT4 and proliferate at a higher rate in response to low oxygen tension compared to T-PMSCs. Hence, P-PMSCs may have more stemness than T-PMSCs; however, PMSCs from different gestations have a preserved proliferation and multipotency as tested by self-renewal markers and differentiation potential into several lineages (bone, adipose, endothelial and neuronal) (data not shown).

IGF-I, an important growth factor in mammalian development including the placenta, stimulates cell mitogenesis, differentiation and survival, primarily *via* IGF-IR (16, 58). In the placenta, there is no knowledge of the IGF-I concentration in the chorionic villi during different gestations; however, IGF-I concentration was measured at 71 – 132 ng/mL in the umbilical cord blood (10); therefore we used a concentration between 10 and 100 ng/mL. IGF signaling via the IGF-IR has been demonstrated to be crucial to mediate ESC multipotency and proliferation (17). However, the effect of IGF signaling under low oxygen tension conditions is not understood. Here, in PMSCs, IGF-I increased the proliferation capacity in both P- and T-PMSCs that was enhanced by low oxygen tension. We monitored OCT4 level as a marker of multipotency, which was shown to change stem cell identity when is changed by 50% of the required level for pluripotency (35). In PMSCs, low oxygen tension alone caused an increase in OCT4 levels compared to room air (Fig. S1B), while IGF-I alone increased OCT4 levels similar to FBS in room air (unpublished data). Also, P-PMSCs were less sensitive to increase OCT4 levels than T-PMSCs (12- vs. 6-hrs) in response to IGF-I compared to T-PMSCs, which can be attributed to the already higher OCT4 expression in the more "naïve" P-PMSCs.

In ESCs, signaling pathways such as RAS/MEK/ERK and PI3K/AKT are specifically upregulated and are required to confer an undifferentiated state *via* maintaining levels of OCT4, SOX2 and NANOG (59). In PMSCs, we identified

that p-ERK1/2 and p-AKT were the two major kinases activated by both low oxygen and IGF-I. ERK1/2 is a mitogen kinase that translocates to the nucleus when phosphorylated to further phosphorylate target genes (c-jun, Elk-1, RSK90, Myc, MNK, BRF-1, UBF, etc) to promote proliferation or differentiation (60). A recent discovery of 14 phosphorylation sites on OCT4 has revealed three putative phosphorylation sites for ERK2 at S111, T118, and S355 (61), which may play a role to maintain multipotency of stem cells. Also, a chromatin interaction of ERK2 is shown to be involved in pluripotency transcriptional machinery to interact with OCT4 promoter region with no clear function (62). AKT, on the other hand, is responsible for maintaining survival by inhibiting apoptosis via phosphorylating several targets (c-RAF, BAD, caspase-9, etc.), promoting cellular growth and protein translation (via mTORC1), and regulating glycogen synthesis (63). We demonstrated that low oxygen tension alone was able to intrinsically upregulate the p-ERK1/2 levels (promoting growth), whereas IGF-I was required for the phosphorylation of AKT (promoting survival) regardless of the oxygen tension. Also, low oxygen tension caused a global reduction in p-ERK1/2 levels in presence of IGF-I and a lowered sensitivity to activate AKT. In P-PMSCs, p-ERK1/2 decreased within a narrow range while, in T-PMSCs, it decreased in a dose-dependent manner with increasing concentrations of IGF-I under low oxygen tension. These gestational stage-dependent differences in p-ERK1/2 and p-AKT activation patterns suggest that the maintenance of stemness of PMSCs, which is higher under low oxygen tension conditions, requires a steady active state of ERK1/2 and AKT signaling.

P- and T-PMSCs have similar abundance of IGF-IR and IRS-1 levels in response to IGF-I and low oxygen tension, however, they respond differently to IGF-I as demonstrated by p-ERK1/2 and p-AKT levels. It was shown previously in 293T, MEFs, 3T3-L1, and MCF-7 cells that IRS-1 is degraded through a caspase-mediated cleavage mechanism, following a transient (1-6 hrs) exposure to low oxygen (1%) in the presence of IGF-I (64). In PMSCs, low oxygen tension increased the levels of IGF-IR and IRS-1 in contrast to room air. However, the longer exposure to IGF-I in low oxygen tension conditions decreased IGF-IR and IRS-1 to lower levels than room air. This can be a negative feedback mechanism to downregulate the higher phosphorylation signal at IGF-IRβ and IRS-1 seen in low oxygen tension. Moreover, phosphorylation of IGF-IRβ in low oxygen tension was higher in P-PMSCs than T-PMSCs even at higher IGF-I concentration, which can explain the higher proliferation level at 100 ng/mL. IRS-1 was phosphorylated at Y896 and Y612 in response to IGF-I, providing docking sites for upstream signaling cascades for ERK1/2 and AKT activation, respectively. Low oxygen tension increased higher p-IRS-1 at Y896 and Y612 in P-PMSCs than in T-PMSCs. This demonstrates that low oxygen tension can mediate an increased availability of IGF-IR and IRS-1 with more binding sites for p-ERK1/2 and p-AKT signaling, especially in P-PMSCs, which have higher proliferation and OCT4 levels.

Inhibition of the activation of ERK1/2 and AKT led to decreased responsiveness to IGF-I in both P- and T-PMSCs, which was negatively impacted by low oxygen tension. It has been shown in ESCs that PI3K inhibition leads to differentiation by reducing OCT4 expression, while MEK1/2 inhibition caused the same effect, but less prominently (59). In addition, suppression of p-ERK1/2 sustains a basal state of pluripotency, and therefore conversely, increased p-ERK1/2 levels, should lead to differentiation (65). These previous findings support our findings in PMSCs, where we showed that inhibition of PI3K promoted the reduction of OCT4 levels and cellular proliferation that was greater than the inhibition of MEK1/2. The PI3K inhibitory effect could not be restored by IGF-I in both types of PMSCs and low oxygen tension enhanced the effect. As shown in ESCs (59), the MEK/ERK signaling pathway can potentially be downstream of the PI3K signaling in PMSCs, as LY294002 also reduced the levels of p-ERK1/2. The inhibition of MEK/ERK pathway increased p-AKT levels, especially under low oxygen tension conditions, demonstrating a possible compensatory mechanism for the reduced levels of p-ERK1/2 necessary to maintain PMSC proliferation and multipotency. These findings suggest that for the maintenance of multipotency, PMSCs should be maintained under low oxygen tension with activated PI3K/AKT signaling, perhaps by IGF-I.

This study provides an insight into the role of the microenvironment, especially low oxygen tension, in directing receptor tyrosine kinase signaling by modifying abundance of effector kinases and signal intensity in response to growth factors,

e.g. IGF-I. This knowledge of signaling pathways will allow for *in vitro* stem cell preconditioning by growth factors, oxygen tension and chemical molecules for a more successful use in tissue regeneration therapies mimicking that of *in vivo* conditions for better engraftment and survival.

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Appendix 1
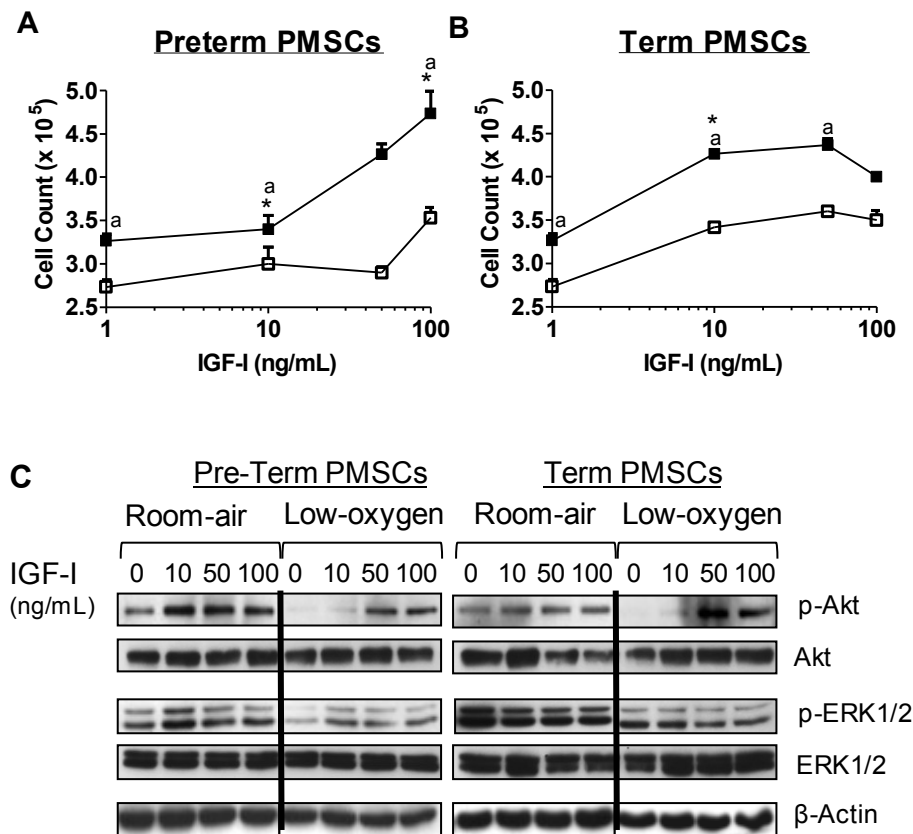
FIGURE S2.1. **Low oxygen tension increases PMSC proliferation in response to IGF-I**. (A) P-PMSC (B) T-T-PMSC proliferation was assessed using the Countess Cell counter. P- and T-PMSCs were treated with increasing IGF-I concentrations (0, 10, 50,100 ng/mL) in room air or low oxygen tension for 48-hrs. Two-Way ANOVA, $P<0.05$, n=6; a = significant difference between room air and low oxygen tension, * = significant difference by different IGF-I concentrations. In both P- and T-PMSCs, low oxygen tension enhanced the effect of IGF-I on cellular proliferation.

C) Immunoblotting of p-ERK1/2 and p-AKT in Pre-term and Term PMSCs. p-ERK1/2 is reduced in low oxygen tension below that in room air and sensitivity to induce p-AKT activation is reduced to higher IGF-I concentrations.

Appendix 1
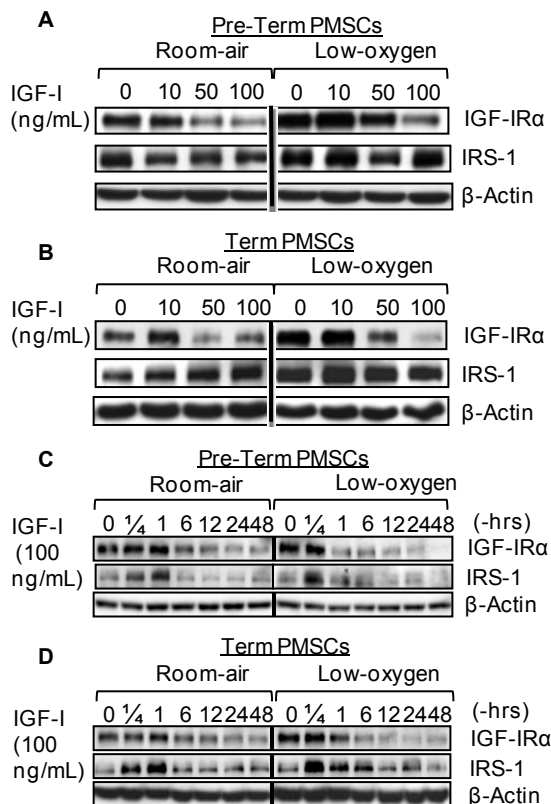
FIGURE S2.2. IGF-IR and IRS-1 levels in response to IGF-I stimulation under low oxygen tension is dose and time dependent. (A and B) Immunoblots of IGF-IRα and total IRS-1 levels following treatment with different concentrations of IGF-I (0, 10, 50, 100 ng/mL) in both P- and T-PMSCs for 48-hrs. (C and D) Immunoblots IGF-IRα and IRS-1 levels in P-PMSCs and T-PMSCs following treatment with IGF-I (100 ng/mL) over 48-hrs under room air and low oxygen tension (n=3). β-Actin levels indicate equivalent protein loading. Low oxygen tension enhance an increased level of IGF-IRα and IRS-1 in both PMSCs. IGF-IRα and IRS-1 levels decrease with increased concentration of IGF-I or stimulation time.

Appendix 1
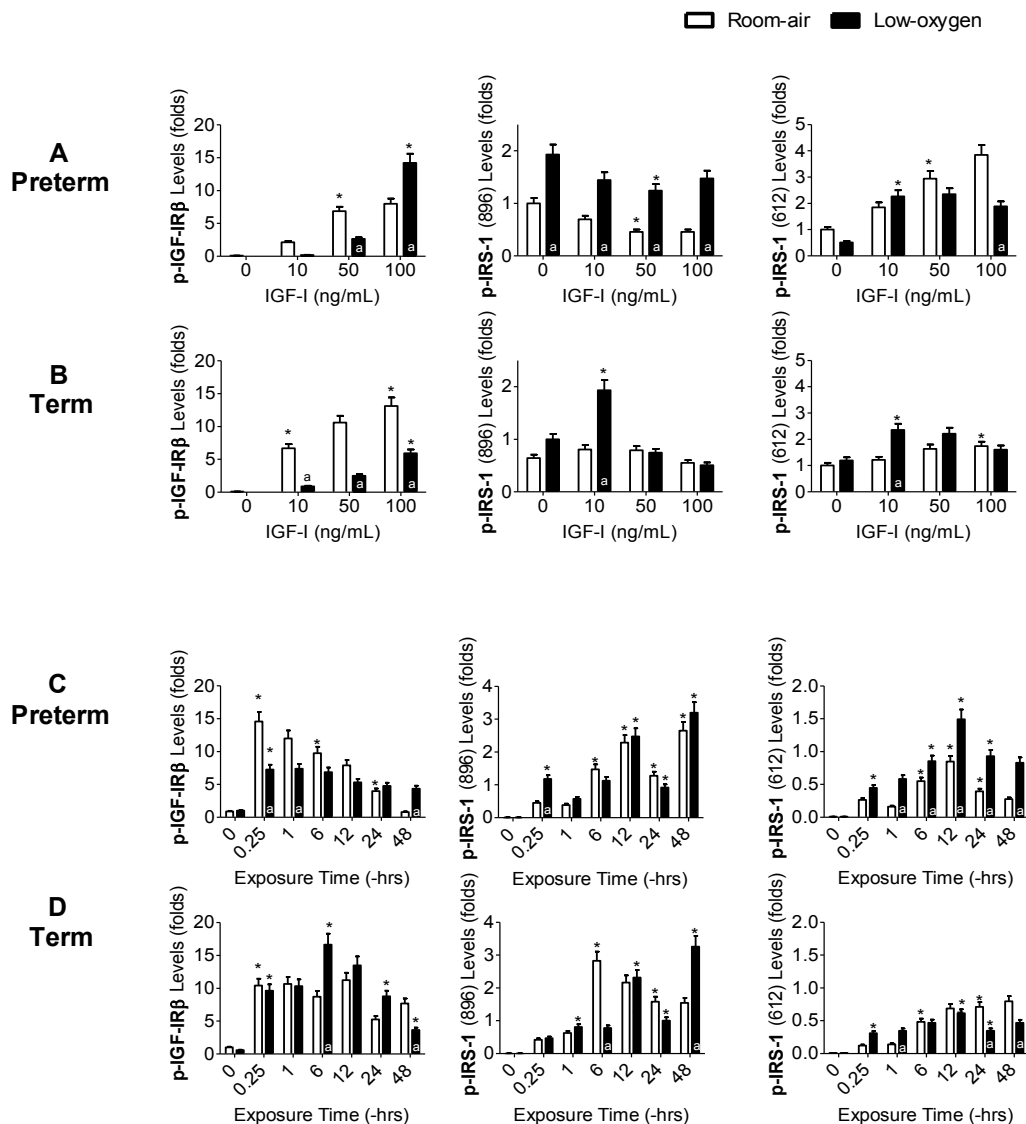
FIGURE S2.3. Oxygen tension modulates the phosphorylation of IGF-IRβ and IRS-1 (pY612 and pY896) levels in PMSCs. (A and C) P- and (B and D) T-PMSCs were stimulated with an increasing concentration (0, 10, 50, 100 ng/mL) of IGF-I or with IGF-I (100 ng/mL) over 0-48-hrs under room air or low oxygen tension, respectively. Levels of p-IGF-IRβ, p-IRS-1^{pY-896} and p-IRS-1^{pY-612} were quantified by densitometry of immunoblots in P-PMSCs and T-PMSCs. Quantification by densitometry was normalized to β-Actin for protein load and DNA content to account for stem cell heterogeneity. Two-Way ANOVA, P<0.001, n=3, a = significant difference between room air and low oxygen tension, * = significant difference by increased IGF concentration or stimulation time.

Appendix 1
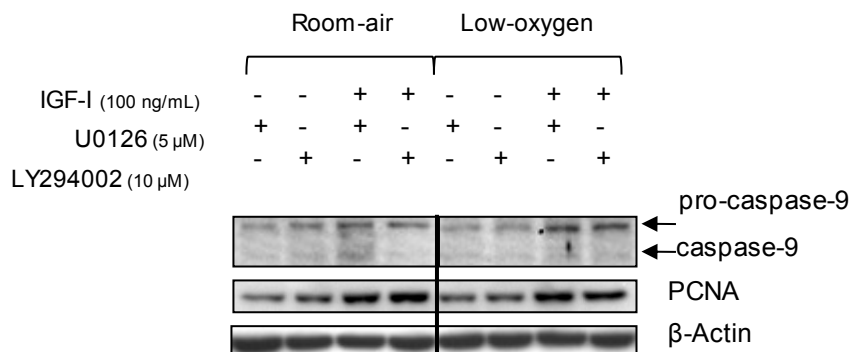
FIGURE S2.4. Assessment of use of MEK1/2 and PI3K inhibitor concentrations. Immunoblots of pro-caspase-9, activated caspase-9 and PCNA in preterm PMSCs after 48-hrs treatment. Cells were treated continually in presence of U0126 or LY294002 for 48-hrs in presence or absence of IGF-I (100 ng/mL) to assess apoptosis (caspase-9) and proliferation (PCNA) (n=3). Concentration of inhibitors was assessed based on highest concentration of inhibitor that does not induce apoptosis.

Appendix 1

A

B
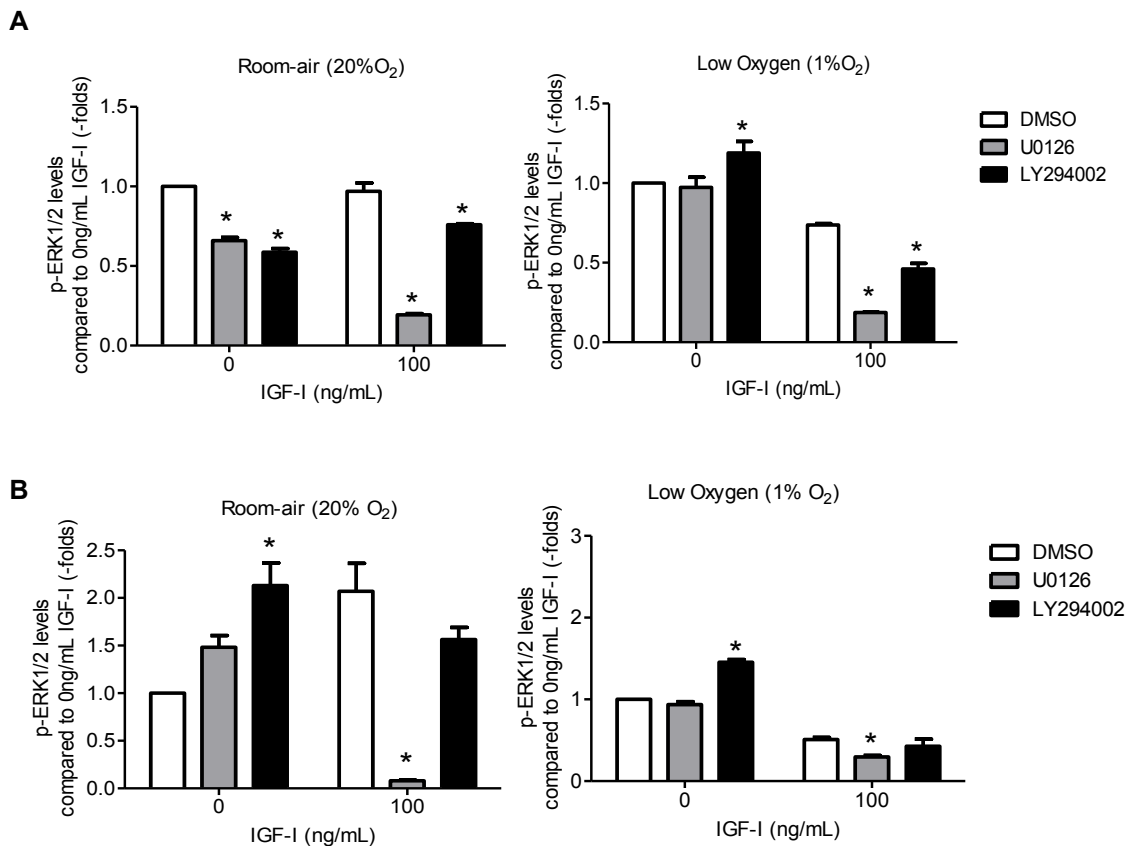
FIGURE S2.5. Effect of MEK1/2 and PI3K inhibition on p-ERK1/2 in PMSCs under low-oxygen. (A) P-PMSCs and (B) T-PMSCs (N=2) were treated in presence of U0126 (5 µM) or LY294002 (10 µM) in combination IGF-I (0 or 100 ng/mL) for 48-hrs under room-air or low-oxygen. Immunoblots of p-ERK1/2 were normalized to total kinase level and β-ACTIN. DMSO is a vehicle control. (One-way ANOVA, $p<0.05$, n=3, * = significant difference compared with DMSO control at 0 ng/mL IGF-I).

Appendix 1
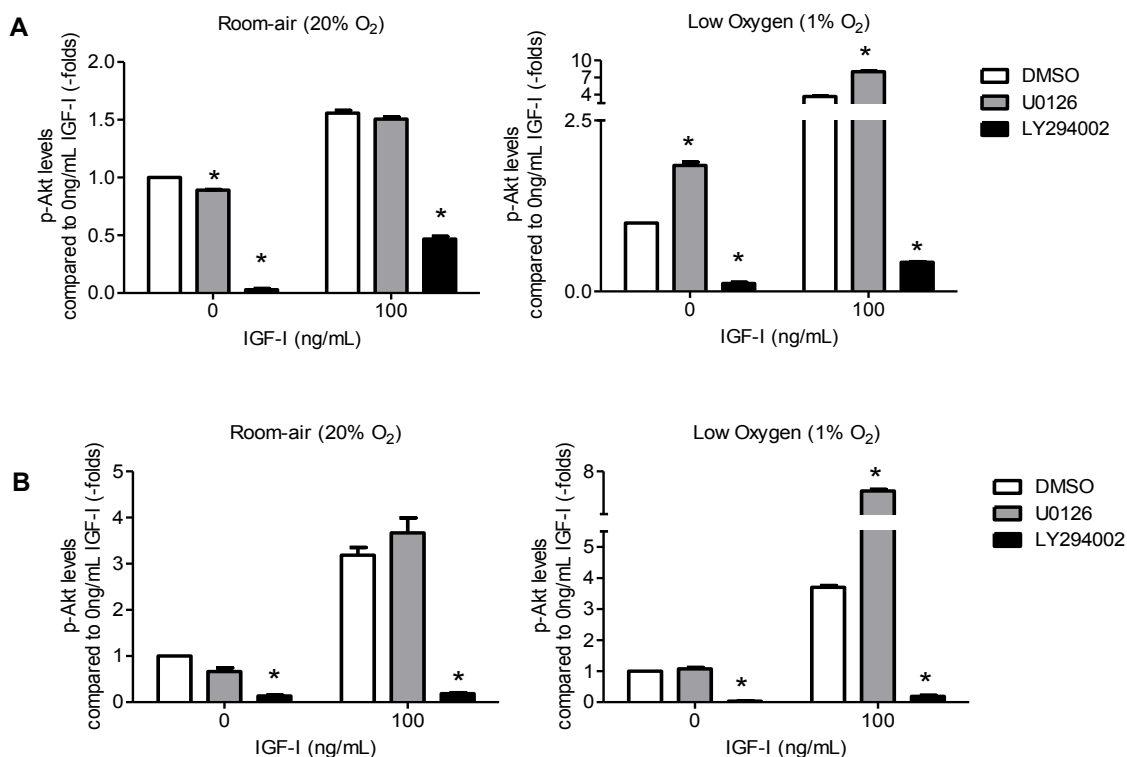
FIGURE S2.6. Effect of MEK1/2 and PI3K inhibition on p-AKT in PMSCs under low-oxygen. (A) P-PMSCs and (B) T-PMSCs (N=2) were treated in presence of U0126 (5 μM) or LY294002 (10 μM) in combination IGF-I (0 or 100 ng/mL) for 48-hrs under room-air or low-oxygen. Immunoblots of were normalized to total kinase level and β-Actin. DMSO is a vehicle control. (One-way ANOVA, p<0.05, n=3, * = significant difference compared with DMSO control at 0 ng/mL IGF-I).

Appendix 1

A
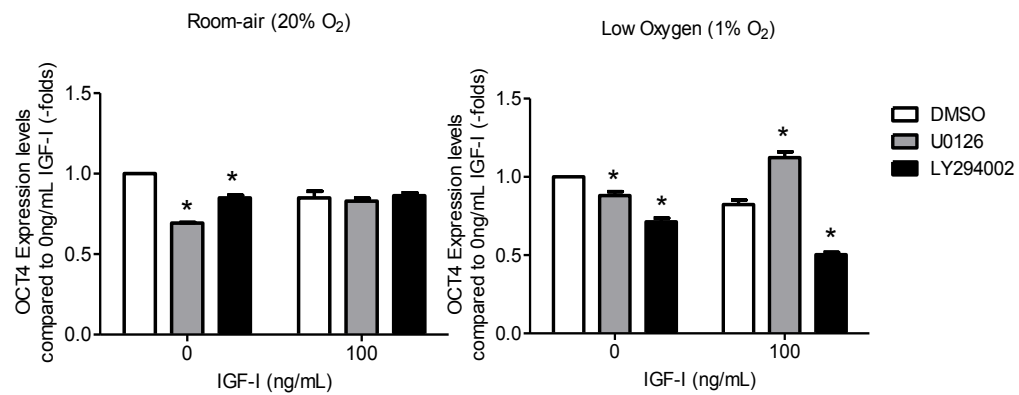
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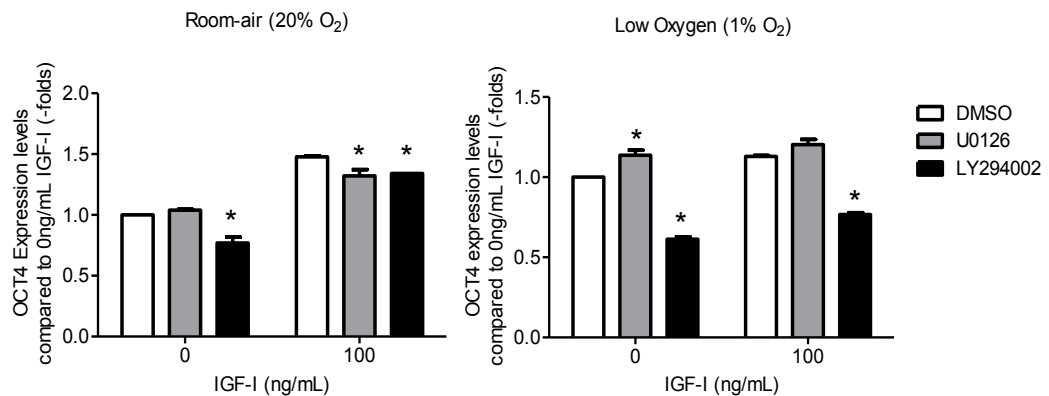
FIGURE S2.7. Effect of MEK1/2 and PI3K inhibition on OCT4 expression as a marker for stemness in PMSCs under low-oxygen. A) P-PMSCs and (B) T-PMSCs (N=2) were treated in presence of U0126 (5 µM) or LY294002 (10 µM) in combination IGF-I (0 or 100 ng/mL) for 48-hrs under room-air or low-oxygen. Immunoblots of were normalized to β-Actin. DMSO is a vehicle control. (One-way ANOVA, p<0.05, n=3, * = significant difference compared with DMSO control at 0 ng/mL IGF-I).

CHAPTER THREE

Low Oxygen Tension Modulates the Insulin-like Growth Factor Signaling via Insulin-like Growth Factor-I Receptor and Insulin Receptor to Maintain Stem Cell Identity in Placental Mesenchymal Stem Cells

3.1   INTRODUCTION

Manipulating stem cells *in vitro* is a critical process to preserve stem cell identity and induce fate changes towards self-renewal or differentiation (1). For tissue regeneration therapy, it is important to prolong the characteristics of stem cell *in vitro* which can be achieved by mimicking their natural microenvironment (growth factors, extracellular matrices, oxygen tension, etc.) before their use for successful *in vivo* applications (2, 3). Insulin-like growth factors (IGFs; IGF-I and IGF-II) and oxygen tension are major stem cell niche factors in the human placenta (4, 5). IGFs are known to manipulate stem cell fate to maintain multipotency or induce differentiation, while continuous low oxygen tension microenvironment inhibits stem cell differentiation and retain multipotency (6-9).

The use of IGF-I and IGF-II has become popular in cell culture systems to maintain self-renewal or induce differentiation. IGF-I and IGF-II are polypeptide ligands, similar to pro-insulin, that signal through the IGF-IR, a RTK, to trigger a mitogenic response through the ERK1/2 and the AKT pathways to promote growth and survival and inhibit apoptosis (4, 10, 11). Moreover, both IGF-I and -II can signal through the IR or hybrid IGF-IR/IR receptor (12, 13). IGF-IR is more sensitive to IGF-I with a Kd of 1 nM and with a 10-fold lower affinity to IGF-II (14). In ES cells, IGF-IR is highly enriched and is required to maintain stem cell self-renewal and pluripotency as blocking the IGF-IR reduced pluripotency and even induced differentiation (8, 9, 15). The use of IGF-I (200 ng/mL) in defined DC-

HAIF media (15) or IGF-II (30 ng/mL) in KOSR media (8) was sufficient to maintain undifferentiated hESCs *in vitro* without the need for a feeder layer.

OCT4, NANOG and SOX2 constitute a triad of transcription factors, which regulate human ES cell fate. They are also utilized to generate iPSCs (16-19). Each of these transcription factors has a major role in repressing the differentiation process towards a specific lineage: OCT4 represses extraembryonic and epiblast-derived lineages, NANOG represses embryonic ectoderm differentiation, and SOX2 represses mesendoderm differentiation (16). *In vivo*, stem cells reside in a compartment with low oxygen levels (3 - 5% O_2). Therefore, low oxygen tension is an important factor modulating stem cell identity. In hESCs, the hypoxia inducible factor 2 (HIF2), formed in low oxygen tension, can interact with the promoter regions of *OCT4*, *NANOG* and *SOX2* and enhance their expression (20, 21). Even, when generating iPSCs, low oxygen tension enhances reprogramming efficiency by increasing the number and size of colonies and their expression of pluripotency genes. It is possible to form colonies in absence of one or two of the four-transcription factors (Oct3/4, Klf4, +/- Sox2, +/- c-Myc) only if placed in low oxygen tension (22). Therefore, it is becoming important to culture ESCs, MSCs and iPSCs in low oxygen tension as it may modulate cell response to intracellular/extracellular growth factors in order to maintain stem cell character similar to *in vivo* conditions.

In this context, the interaction of IGFs with their receptors (IGF-IR vs. IR) in the stem cells is not clear and whether low oxygen tension can modify stem cell multipotency. For this purpose, we used placental mesenchymal stem cells (PMSCs) as a model to study the differences between IGF-I and IGF-II signaling at low oxygen tension, a microenvironment that mimic the placenta *in vivo*. We hypothesized that, under physiological low oxygen tension, IGF-I and IGF-II regulate PMSC self-renewal capacity by utilizing different signal transduction mechanisms *via* the IGF-IR. Here, we show how oxygen tension can distinctly upregulate IGF-II expression and abundance. Also, we demonstrate that IGF signaling *via* its receptor mediates PMSC signaling through ERK1/2 and AKT to promote increased PMSC proliferation. Low oxygen tension upregulated the expression of OCT4, NANOG and SOX2, which were lowered by IGF stimulation. We also propose that, besides the IGF-IR, an important role of the IR becomes prominent in enhancing PMSC proliferation under low oxygen tension conditions.

3.2. MATERIAL AND METHODS

3.2.1. PMSC Isolation

PMSCs were isolated from early gestation human placentae - from 10-13 weeks. For comparison between gestational ages, PMSCs were isolated from mid gestation 20-22 week or from late gestation 37-40 weeks. After informed consent, placentae were collected from patients who underwent therapeutic pregnancy termination. Immediately after surgery, placentae were dissected

under sterile conditions and small pieces of chorionic villi were collected. Tissue samples were minced mechanically and subjected to a process consisting of two steps of enzymatic digestion with (1) collagenase IV/hyaluronidase, and (2) DNase I followed by (3) trypsin/EDTA. Each enzymatic step was performed for 10 min at 37ºC, followed by 10 min wash at 4ºC in a solution of PBS supplemented with 10% fetal bovine serum (FBS) (Gibco, Mississauga, ON, CAN). Cells released during digestion were passed through a tissue mesh (45 µm) to obtain a single cell suspension. Next, cells were separated on a Percoll (Sigma) discontinuous gradient according to a modified protocol by Worton et al. for hematopoietic stem cell isolation (23) and then seeded in DMEM/F12 media (Gibco, Mississauga, ON, CAN) supplemented with 15% FBS, FGF-2 (100 ng/mL) and antibiotic-antimycotic solution (1x). After 4 days, media was changed and non-adherent cells were washed with media to leave behind adherent PMSCs able to form colonies.

3.2.2. Cell Culture and Incubation in Low Oxygen Tension

For each experiment, two preterm PMSC cell populations were used in triplicate. Cells were cultured and maintained using DMEM/F12 media supplemented with 15% FBS and FGF-2 (100 ng/mL) (Gibco, Mississauga, ON, CAN). Before treatments, cells were cultured in DMEM/F12 supplemented 10% FBS only. Upon treatment, PMSCs were switched to fresh media containing only IGF-I or IGF-II (0 – 100 ng/mL). For IGF-IR or IR neutralization experiments, cells were

grown in serum-free media for 24-hrs and then specific antibodies were added at a final concentration of 5 µg/ml in serum-free media. Then, 50 ng/ml of IGF-I or IGF-II final concentration was added to the cell-culture for 24-hrs. Cell cultures were then placed in either a 5% CO_2 incubator or a hypoxia chamber, which was filled with a mix of (1% O_2, 5% CO_2, Balanced N_2) (BOC Canada Ltd, Toronto, ON, CAN) for 15 min to ensure saturation using a Hudson 5590 Oxygen Monitor (Hudson, Ventronics Division, Temecula, CA, USA). Thereafter, the chamber was placed in a tissue-culture incubator at 37° C.

3.2.3. Real-time PCR

Total RNA was extracted from PMSCs using the PureLink RNA Mini Kit (Ambion, Burlington, ON) as per the manufacturer's protocol. Cells cultured in 60 mm dishes were lysed in lysis buffer with 1% β-mercaptoethanol. Lysates were scraped, collected in 1.5 mL tubes and homogenized using 21-gauge syringe needle (10 times). Lysates were then applied to the column for binding RNA, washed and eluted using RNase free water. 1-3 µg total RNA were reverse transcribed into cDNA using Superscript III first-strand synthesis system for RT-PCR (Invitrogen, Burlington, ON, CAN) and (25 µM) oligo (dT)$_{20}$ primers in 20 µL volume. Quantitative PCR was performed on 100 ng of cDNA using Platinum SYBR-Green qPCR SuperMix-UDG with ROX (Invitrogen, Burlington, ON, CAN) following the manufacturer protocol. PCR products were less than 200 bp and each primer concentration used was 0.4µM. All reactions were run in 10µL in

384-well plate format using the Applied Biosystems ViiA 7 System. Human RPL13a gene was used as the reference endogenous control for normalization of the target genes. Standard curves were generated with 5 points of cDNA (500-0.05 ng/µL) besides runs without template as a negative control (NTC). Amplifications conditions were run at 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 15 sec. The following primers were used: *IGF-I* sense (5'- CCT CCT CGC ATC TCT TCT ACC TG-3'), antisense (5'-CTG CTG GAG CCA TAC CCT GTG-3'); *IGF-II* sense (5'-CGG CTT CTA CTT CAG CAG GC-3'), antisense (5'-TGG CGG GGG TAG CAC AGT-3'); *IGF-IR* sense (5'-GAG CAG CTA GAA GGG AAT TAC-3'), antisense (5'-AAG TTC TGG TTG TCG AGG A-3'); *IR-A* sense (5'-GTT TTC GTC CCC AGG CCA TC-3'), antisense (5'-CCA ACA TCG CCA AGG GAC CT-3'); *IR-B* sense (5'-CAC TGG TGC CGA GGA CCC TA-3'), antisense (5'- GAC CTG CGT TTC CGA GAT GG-3'); *OCT4* sense (5'-GAT GTG GTC CGA GTG TGG TTC T-3'), antisense (5'-TGT GCA TAG TCG CTG CTT GAT-3'); *SOX2* sense (5'-TTG CTG CCT CTT TAA GAC TAG GA-3'), antisense (5'-CTG GGG CTC AAA CTT CTC TC-3'); *NANOG* sense (5'-ATG CCT CAC ACG GAG ACT GT-3'), antisense (5'-AAG TGG GTT GTT TGC CTT TG-3')RPL13a sense (5'-CAT AGG AAG CTG GGA GCA AG-3'), antisense (5'-GCC CTC CAA TCA GTC TTC TG-3').

3.2.4. Immunoblotting

Cell lysates were prepared using 1x cell lysis buffer for phospho-proteins according to manufacturer protocol (#9803, Cell Signaling Technologies, Burlington, ON, CAN). Protein samples (10-20 µg each) were resolved by 10% SDS-PAGE, and then transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 5% bovine-serum-albumin or in 5% non-fat-dry milk in 1x TBS (Tris-buffered saline) for 1 hr at room temperature. Blots were then washed in 1x TBS 0.1% Tween 20 (TBS-T) (3x for 5 min) followed by incubation at 4°C overnight with primary antibodies as per manufacturer's protocols. Blots then were washed using TBS-T (3x for 10 min) and were incubated with the corresponding secondary HRP-labelled antibody for 1 hr at RT. Immunocomplexes were detected by ECL and documented using VersaDOC™ Imaging System (Bio-Rad).

3.2.5. Antibodies

To investigate the IGF-I vs. IGF-II activated signaling proteins in PMSCs, we used the following antibodies: phospho-p44/42 MAPK (#4377), p44/42 MAPK (#9102) phospho-AKT (Ser473, #4051), AKT (#9272) and p-IGF-IRβ (#3918) from (Cell Signaling Technologies, Burlington, ON, CAN) and IGF-IRα (N-20, sc-712) and IGF-IRβ (C-20, sc-713) (Santa Cruz Biotech., Santa Cruz, CA, USA). For receptor neutralization, we used IGF-IRα (1H7, sc461-L) (Santa Cruz Biotech., Santa Cruz, CA, USA) and IRα (MA-20, NB400-142) (Novus Canada,

Oakville, ON, CAN). For multipotency markers, we used OCT3/4 antibody (N-19, sc-8628) (Santa Cruz Biotech., Santa Cruz, CA, USA), SOX-2 (2683-1) and NANOG (3369-1) (Epitomics, Burlington, ON, CAN). For loading control, we used pan-Actin Ab-5 (#MS-1295) (Thermo Fisher Scientific, Fremont, CA). The secondary antibodies used for immunoblotting were goat anti-rabbit (#170-6515), anti-mouse (#170-6516) HRP conjugated antibodies (BioRad laboratories, Hercules, CA) or donkey anti-goat antibody (sc-2020) Santa Cruz Biotech., Santa Cruz, CA, USA).

3.2.6. Statistical Analysis

All experiments were run in triplicates from three independent experiments each; whenever possible three or more PMSC primary lines were used. All graphs and analyses were generated using GraphPad Prism Software 5.0 (GraphPad Software, San Diego, CA). A Two-way ANOVA with Bonferroni *post hoc* test was used for the PMSC WST1 proliferation assay and densitometry quantifications. Data are expressed as mean ± standard error of the mean (SEM); values were considered significant when $p < 0.05$.

3.3. RESULTS

3.3.1 PMSC expression of IGF-I and IGF-II is controlled by oxygen tension and IGF stimulation

During placental development *in vivo*, IGFs are expressed differentially with gestation - IGF-II is expressed earlier and in higher abundance than IGF-I (24). This expression pattern was confirmed by PMSCs isolated from different gestational ages only when maintained under low oxygen tension to express higher IGF-II at early gestation and a later expression of IGF-I (Fig. S3.1). In this study, the experiments were conducted using preterm PMSCs isolated from early pregnancies only. They have higher levels of OCT4, the master regulator switch of pluripotency (Fig. S3.2). IGFs are mainly secreted by the liver as endocrine signaling factors but they are also produced in peripheral tissues as autocrine/paracrine factors, which contribute to tissue maintenance, growth and repair. In PMSCs, extracellular IGF-I stimulation increased IGF-I expression in room air at 100 ng/mL (positive feedback). There were no change in expression under low oxygen tension conditions, which demonstrated lowered basal IGF-I expression compared to room air (Fig. 3.1A). Similarly, extracellular IGF-II stimulation (10 and 50 ng/mL) increased IGF-I expression under room air (Fig. 3.1B). Low oxygen tension increased IGF-II expression ($\geq$ 2-fold) that was also increased by extracellular IGF-I (50 and 100 ng/mL) in low oxygen tension (Fig. 3.1C). With extracellular IGF-II stimulation, IGF-II expression increased in a dose dependent response in presence of IGF-II only in low oxygen tension (Fig. 3.1D). Therefore, expression of IGFs by PMSCs is dependent on oxygen tension - low
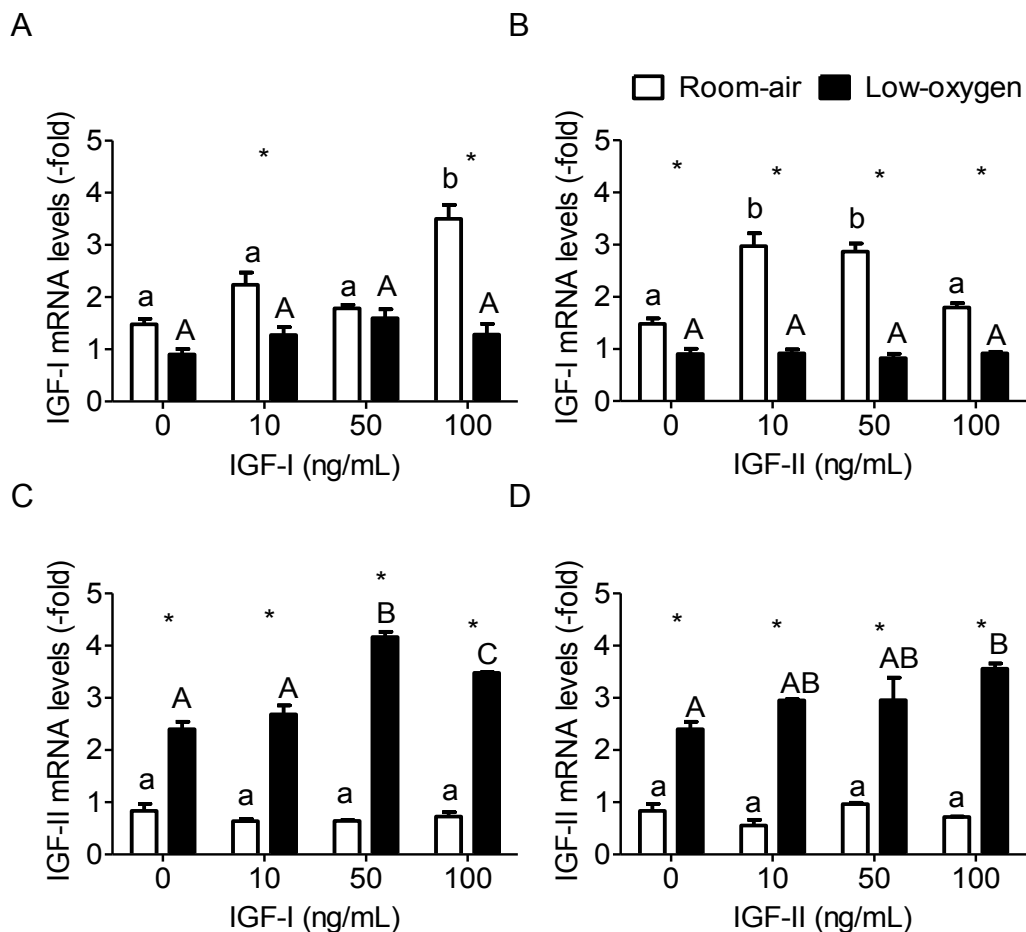
FIGURE 3.1. Expression of IGF-I and -II mRNA by PMSCs is regulated by exogenous IGF and low oxygen tension. PMSCs were cultured for 48-hrs in either room-air or low-oxygen tension in presence or absence of IGF-I or IGF-II (0, 10, 50, 100 ng/mL). By real-time PCR, expression level of IGF-I and -II mRNA were measured relative to RPL13a as an endogenous control. IGF-I levels were measured with increasing concentration of A) IGF-I or B) IGF-II. Similarly, IGF-II mRNA levels are shown in presence of increasing concentrations of C) IGF-I or D) IGF-II. (Two-way ANOVA, $p < 0.05$, n=3, *= significant difference between room air and low oxygen tension; a= significant difference of different IGF concentrations in room air, A= significant difference of different IGF concentrations in low oxygen tension). Low oxygen tension decreased IGF-I expression and increased IGF-II expression in PMSCs.

oxygen tension represses the expression of IGF-I, while it promotes the expression of IGF-II. Both are enhanced by extracellular IGFs in an oxygen tension-dependent manner.

3.3.2. IGF-IR and IR expression is dependent on oxygen tension and IGF concentration

PMSCs expressed both IGF-IR and IGF-IIR, and the IR, but the IGF actions are mediated through either the IGF-IR or IR to trigger mitogenic or metabolic actions. We noted that IGF-IR and IR (α-subunit) levels were upregulated in absence of IGFs and even more when combined with low oxygen tension (Fig. 3.2). In room air, the increased concentration of IGF-I or -II decreased both receptor (IGF-IR and IR) protein levels in a dose dependent manner (more with IGF-I than IGF-II) (Fig. 3.2 C-F), but low oxygen tension had a significant opposing effect maintaining higher levels even in presence of IGFs (less with IGF-I) (Fig. 3.2 C-F). The decrease in the IR levels was less than IGF-IR in response to IGFs and the IR was increased in low oxygen tension even in presence of IGFs. At the receptor expression level, IGF-IR receptor mRNA was increased in low oxygen tension which was also decreased by IGF-I and -II (Fig. S3.3A and B). PMSCs also expressed the two isoforms of IR (IR-A and IR-B) (Fig. S3.3 C-F); however, low oxygen tension increased IR-A expression several fold compared with the IR-B.
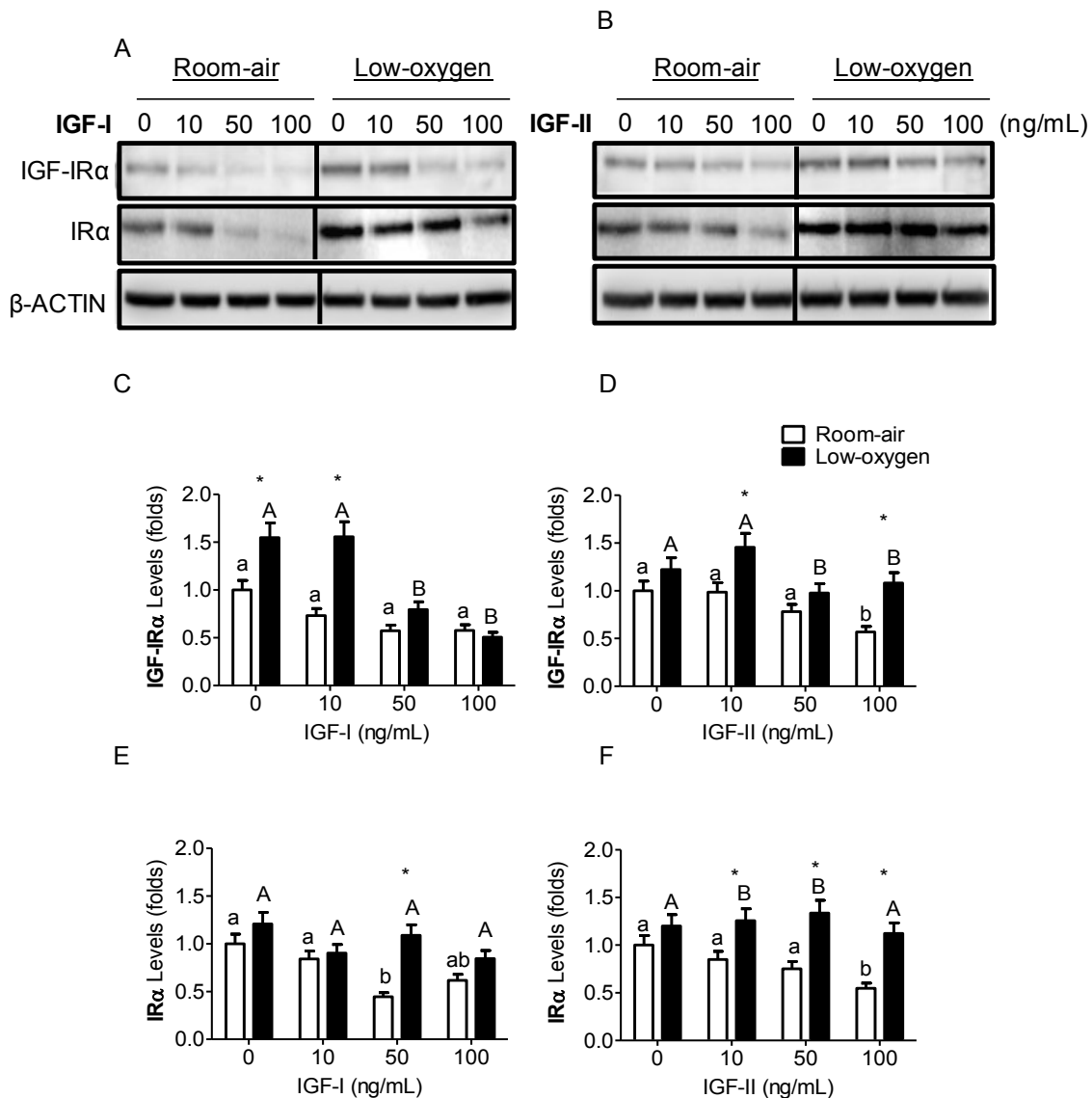
FIGURE 3.2. IGF-IR and IR levels are decreased by IGF-I and -II but elevated by low oxygen tension. PMSCs were cultured for 48-hrs in either room air or low oxygen tension in presence or absence of IGF-I or IGF-II (0, 10, 50, 100 ng/mL). By Immunoblotting, protein expression levels of IGF-IRα or IRα was detected in presence of increasing concentration of A) IGF-I or B) IGF-II. The β-subunits of both receptors showed similar protein levels with the different treatments. C-F) blot quantifications for both receptors are shown. β-Actin is used as a protein loading control. (Two-way ANOVA, $p < 0.05$, n=3, *= significant difference between room air and low oxygen tension; a= significant difference of different IGF concentrations in room air, A= significant difference of different IGF concentrations in low oxygen tension). Low oxygen tension maintained higher levels of both receptors that were otherwise decreased with increasing concentration of IGFs in room air.

In the presence of IGF-II (100 ng/mL), IR-A level was decreased in low oxygen tension; this was similar to IR-B abundance (Fig. S3.3D and F). Therefore, the expression profile and protein levels of the IR was similar to IGF-IR response in the presence of IGFs, indicating that similar signaling mechanism controls IGF-IR and IR in PMSCs from early gestation placentae. Also, low oxygen tension opposed the negative regulation of IGF-IR or the IR by IGFs; the increased IGF-IR or IR expression would allow increased responsiveness to IGFs.

3.3.3. Activation of IGF-IR signaling pathway in PMSCs is regulated by oxygen tension

IGF-I and IGF-II bind and activate the IGF-IR, but due to binding affinity, IGF-IR phosphorylation and activation varies especially in short term stimulation. In room air, the phosphorylation of the IGF-IR (β-subunit) was more sensitive to IGF-I than IGF-II (Fig. 3.3), whereas in low oxygen tension the sensitivity to IGF-I or IGF-II was decreased (Fig. 3.3A and B).

The propagation of IGF-IR activation following short-term stimulation was tested via the phosphorylation of major signaling kinases downstream, ERK1/2 and AKT. In room air, ERK1/2 phosphorylation was similar with either IGF-I or -II (Fig. 3.4A and B). However, low oxygen tension caused an increase in p-ERK1/2 levels in response to IGF-I greater than that induced in room air (Fig. 3.4A). The
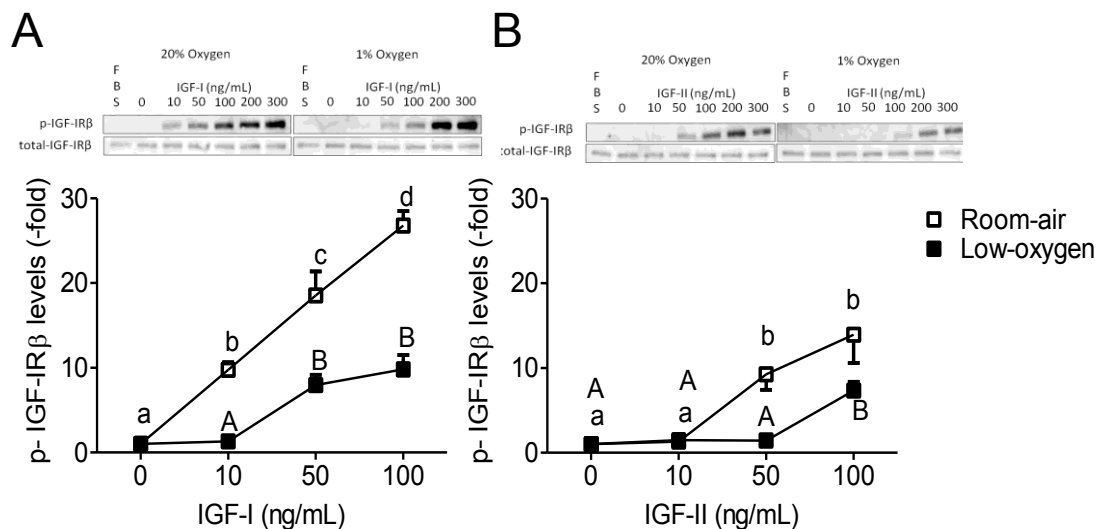
FIGURE 3.3. IGF-IR phosphorylation is dependent on IGF ligand, concentration and oxygen tension. PMSCs were stimulated for 15 min in either room air or low oxygen tension in presence or absence of A) IGF-I or B) IGF-II (0, 10, 50, 100 ng/mL) following serum deprivation for 24-hrs. From immunoblotting shown, levels of p-IGF-IRβ were quantified and normalized to total IGF-IRβ level followed by normalization to β-Actin, used as a protein loading control. (Two-way ANOVA, $p < 0.05$, n=3, a= significant difference of different IGF concentrations in room air, A= significant difference of different IGF concentrations in low oxygen tension). Low oxygen tension deceased the levels of IGF-IR phosphorylation/activation making it less sensitive to IGF-I or -II.

A

15 min

B

15 min

□ Room-air
■ Low-oxygen

C

48 hrs

D
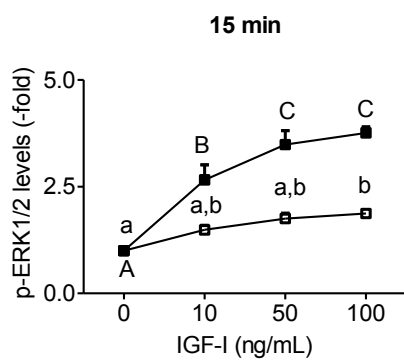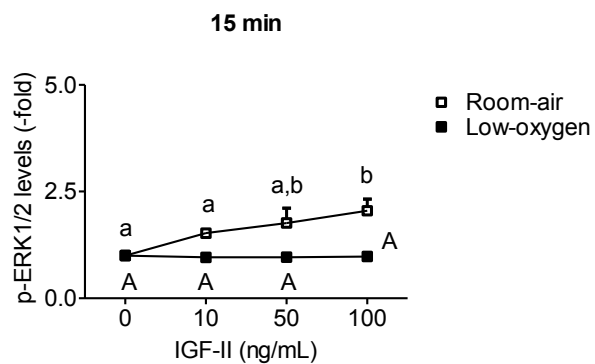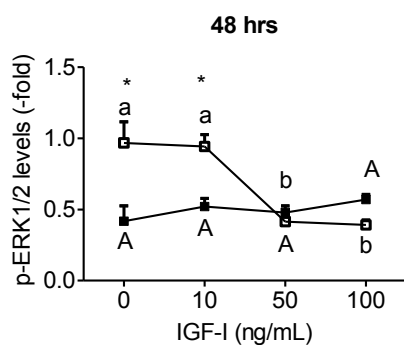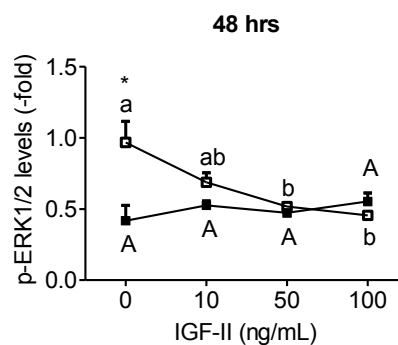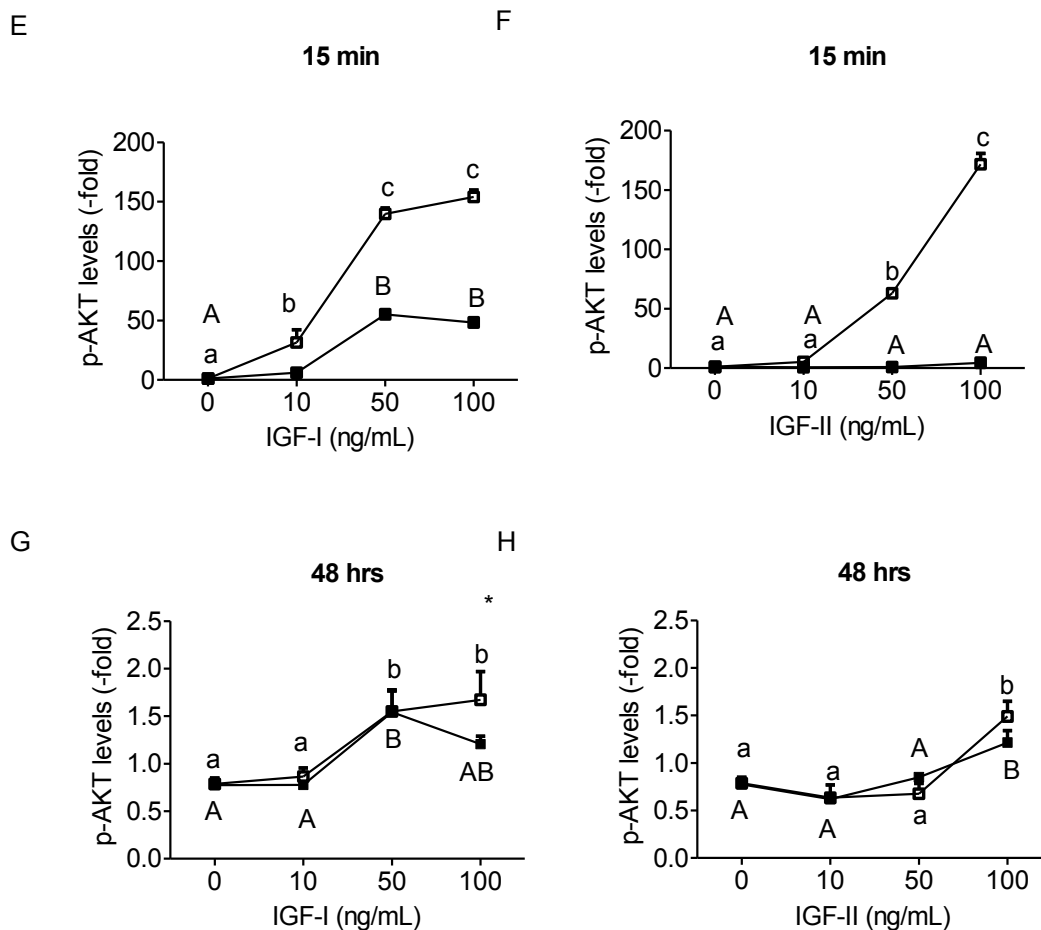
48 hrs

FIGURE 3.4. ERK1/2 and AKT phosphorylation in PMSCs is dependent on IGF concentration and oxygen tension. PMSCs were stimulated for 15 min or 48-hrs in either room air or low oxygen tension in presence or absence of IGF-I or IGF-II (0, 10, 50, 100 ng/mL). From Immunoblotting, detected levels of (A – D) p-ERK1/2 and (E – H) p-AKT were quantified and normalized to total kinase level and to β-actin, used as a protein loading control. (Two-way ANOVA, $p < 0.05$, n=3, *= significant difference between room air and low oxygen tension; a= significant difference of different IGF concentrations in room air, A= significant difference of different IGF concentrations in low oxygen tension). In short term, Low oxygen tension potentiates p-ERK1/2 signal and represses p-AKT. In long term, Low oxygen tension, maintains lower p-ERK1/2 than room air and elevated p-AKT levels similar to room air.

IGF-II effect was not seen in low oxygen tension (Fig. 3.4B). AKT phosphorylation was more sensitive to IGF-I, similar to IGF-IRβ phosphorylation. In room air, AKT phosphorylation plateaued at 50 ng/mL of IGF-I while IGF-II induced the highest activation at 100 ng/mL (Fig. 3.3E and F). On the other hand, low oxygen tension lowered the phosphorylation levels of AKT in a similar pattern to p-IGF-IRβ (Fig. 3.4E and F). Overall, low oxygen tension reduced the phosphorylation of IGF-IRβ and AKT in a similar manner, and increased the phosphorylation of ERK1/2 in presence of IGF-I.

Long-term phosphorylation of ERK1/2 and AKT can be indicators of stem cell state of proliferation. Continuous stimulation of PMSCs by IGF-I and -II in room air, caused a dose-dependent decrease in p-ERK1/2 levels similar to low oxygen tension (Fig. 3.4C and D). Phospho-AKT had an opposite activation pattern compared with p-EKR1/2 that caused a dose dependent increase with IGF-I or -II in both oxygen tensions (IGF-I being more potent than IGF-II) (Fig. 3.4G and H). Therefore, low oxygen can maintain a narrow range of p-ERK1/2 levels but does not interfere with p-AKT pathway that is dependent on IGF stimulation.

3.3.4. Expression of stem cell-associated genes in PMSCs is regulated by low oxygen tension and IGFs

Stem cell fate is determined by their microenvironment, and the expression of the pluripotency genes, OCT4, NANOG and SOX2, determines the character of stemness in hESCs and iPSCs. In PMSCs, all three genes are expressed and

regulated by low oxygen tension and IGFs. Low oxygen tension increased *OCT4*, *NANOG* and *SOX2* mRNA levels and was decreased by IGFs (Fig. S3.4). However, IGFs maintained a higher expression of the three genes in low oxygen tension condition only (IGF-II more prominent than IGF-I to enhance expression 72-hrs post stimulation) (Fig. S3.5). Transcription factors function at the protein level as they physically interact with DNA to regulate multipotency gene expression and fate changes. OCT4 protein levels did not change with increasing concentrations of IGF-I or IGF-II in either room air or low oxygen tension (Fig. 3.5A). However, SOX2 and NANOG expression was affected by IGF-I or IGF-II at both mRNA and protein. Levels of NANOG and SOX2 were increased at 10 ng/mL IGF-I and IGF-II then decreased in presence of increased concentrations of IGF-I or IGF-II (Fig. 3.5B and C). Therefore, OCT4 was unchanged by IGF and oxygen tension, although it was reduced at the mRNA level at increasing IGF-I. NANOG and SOX2 were elevated by low oxygen tension and decreased by IGF-I but not IGF-II stimulation. This shows that low oxygen tension can maintain or enhance multipotency but IGF-I and less with IGF-II (depending on concentration and stimulation time) oppose this effect.
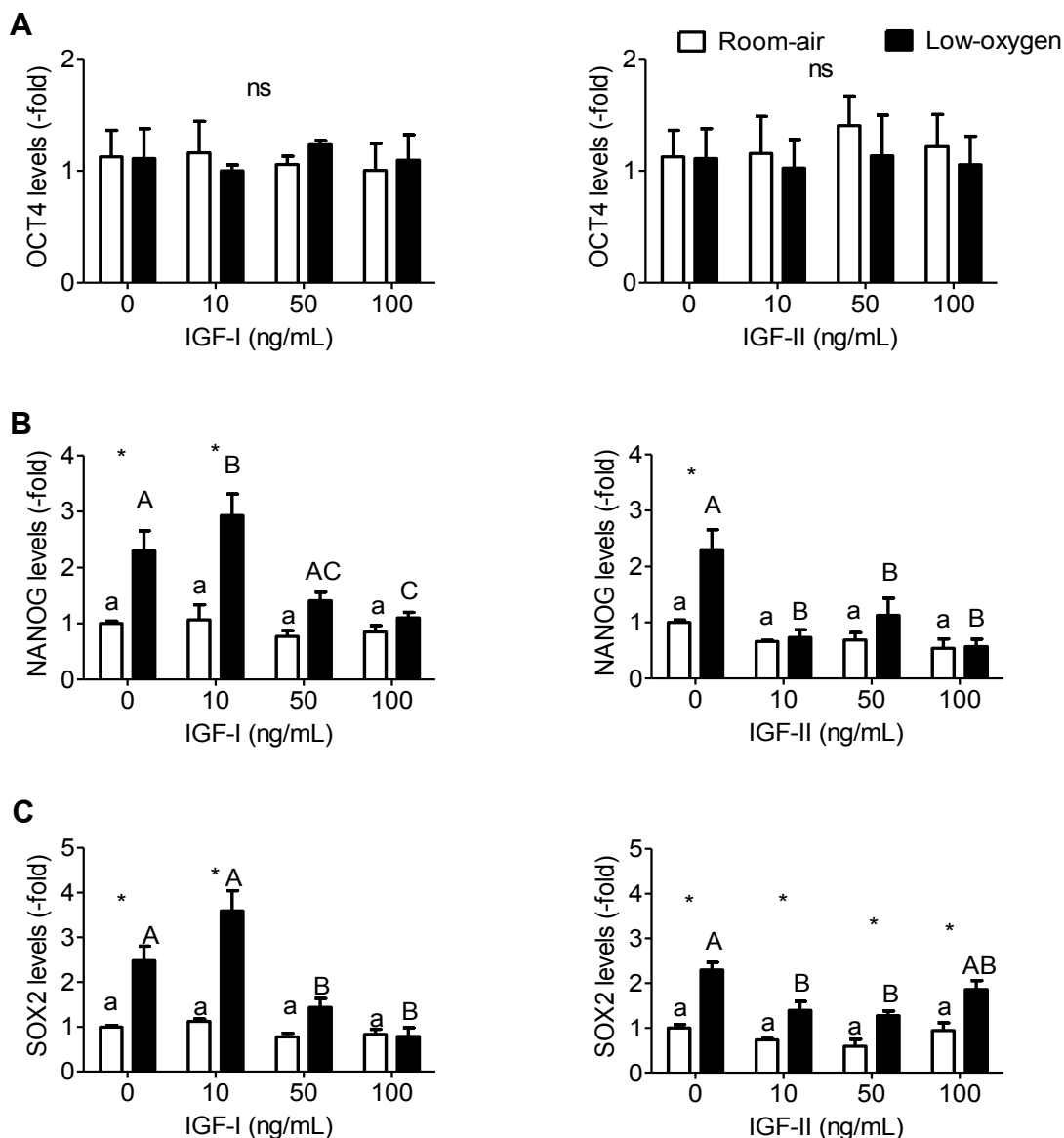
FIGURE 3.5. NANOG and SOX2 but not OCT4 are decreased by IGFs and increased by low oxygen tension in PMSCs. PMSCs were stimulated for 48-hrs in either room air or low oxygen tension in presence or absence of IGF-I or IGF-II (0, 10, 50, 100 ng/mL). From Immunoblotting, detected levels of A) OCT4, B) NANOG and C) SOX2 were quantified and normalized to β-actin, used as a protein loading control. (Two-way ANOVA, $p < 0.05$, n=3, *= significant difference between room air and low oxygen tension; a= significant difference of different IGF concentrations in room air, A= significant difference of different IGF concentrations in low oxygen tension). Low oxygen tension was able to elevate the levels of NANOG or SOX2 but not OCT4, whereas increased IGF concentration caused a decrease in total levels.

3.3.5. PMSC proliferation is enhanced by IGFs in low oxygen tension

Self-renewal is an important stem cell characteristic. We assessed PMSC proliferation as an indicator of self-renewal by oxygen tension and IGF-I or IGF-II concentration. In room air, IGF-I stimulated proliferation in a dose dependent manner that plateaued at (50 ng/mL), but was increased further if maintained in low oxygen tension (Fig. 3.6A). Interestingly, proliferation decreased when concentrations greater than 100 ng/mL of IGF-I was added in room air, but not in low oxygen tension (data not shown). In comparison, the effect of IGF-II was lower than that of IGF-I on PMSC proliferation in room air and was enhanced by low oxygen tension (Fig. 3.6B). Therefore, IGF-I is more potent in stimulating PMSC proliferation in room air, whereas, low oxygen tension potentiates both IGF-I and -II signaling to stimulate increased proliferation. These results show that a lowered p-ERK1/2 and increased p-AKT levels (Fig. 3.4) correlates with an IGF-stimulated proliferation.

3.3.6. Low oxygen tension mediates a differential IGF signaling through the IGF-IR and IR to stimulate proliferation

Low oxygen tension upregulated IGF-IR and IR expression. In room air, neutralizing the IGF-IR abolished the effect of IGF-I or -II stimulation; however, in low oxygen tension, the IGF-mediated proliferation remained elevated even in the presence of a 10-fold excess of antibody (Fig. S3.6). This finding suggests
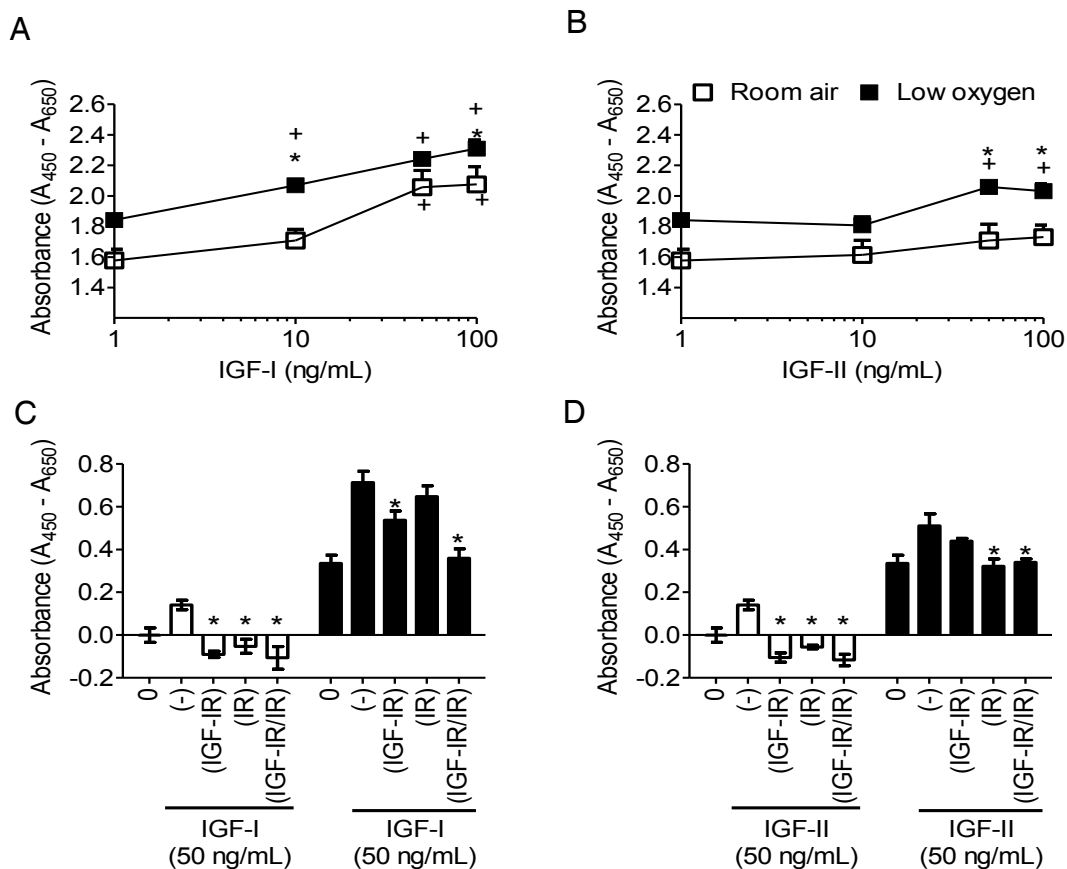
FIGURE 3.6. PMSC enhanced proliferation in response to IGF-I or IGF-II is dependent on oxygen tension and specific receptor stimulation. PMSC proliferation was assessed using the WST-1 colorimetric proliferation assay in presence of increasing concentration of A) IGF-I or B) IGF-II (0, 10, 50, 100 ng/mL) under room air or low oxygen tension for 48-hrs. Neutralization effect of IGF-IR or IR was measured in presence of 5 µg/mL of either neutralizing antibody in presence of 50 ng/mL of C) IGF-I or D) IGF-II in room air or low oxygen tension conditions for 24-hrs. The absorbance was read at 450 nm with a background reference of 650 nm. (Two-Way ANOVA, $P<0.05$, n=8, * = significant difference between room air and low oxygen tension, + = significant difference by IGF concentration in comparison with 0 ng/mL IGF). Low oxygen tension enhanced PMSC proliferation and increased the IGF effect in increasing PMSC proliferation. IGF-IR and IR are responsible for mediating IGF signaling to increase PMSC proliferation especially under low oxygen tension conditions. IGF enhanced proliferation in absence of exogenous IGF is mediated by endogenous IGF expression.

that the effect of IGF-I or -II was mediated additionally by another receptor. We tested whether IR was this receptor. In room air, IGF-I or IGF-II mediated proliferation was reduced by the IGF-IR neutralizing antibody and by IR neutralizing antibody (Fig. 3.6C and D). The reduction in proliferation was lower than without IGFs, suggesting that a portion of the proliferative response was due to endogenous IGF. On the other hand, low oxygen tension enhanced PMSC proliferation, and neutralizing the IGF-IR had a greater effect than IR neutralization (Fig. 3.6C). A combination of both receptor neutralizing antibodies completely abolished the IGF-I induced proliferation, suggesting a potential role for the hybrid receptor under this condition. Similarly in low oxygen tension, IGF-II stimulation was more reduced by neutralizing the IR (Fig. 3.6D). The PMSC IR-mediated proliferation was dependent on IGF stimulation as insulin stimulation alone did not increase proliferation regardless of oxygen tension (Fig. S3.7). Therefore, IGFs utilized both the IGF-IR and IR to promote PMSC proliferation, however, low oxygen tension can modulate IGF signaling response towards IGF-IR for IGF-I signaling and the IR for IGF-II signaling. As mediators of IGF-IR/IR signaling, we checked the ERK1/2 and AKT phosphorylation levels. The presence of either IGF-IR or IR neutralizing antibody negated the effect of IGF-I by decreasing ERK1/2 phosphorylation (Fig. 3.7A). No major changes in p-ERK1/2 levels were detected with IGF-II (Fig. 3.7B). AKT was more responsive to IGF stimulation, which was inhibited by IGF-IR and IR neutralizing antibodies.
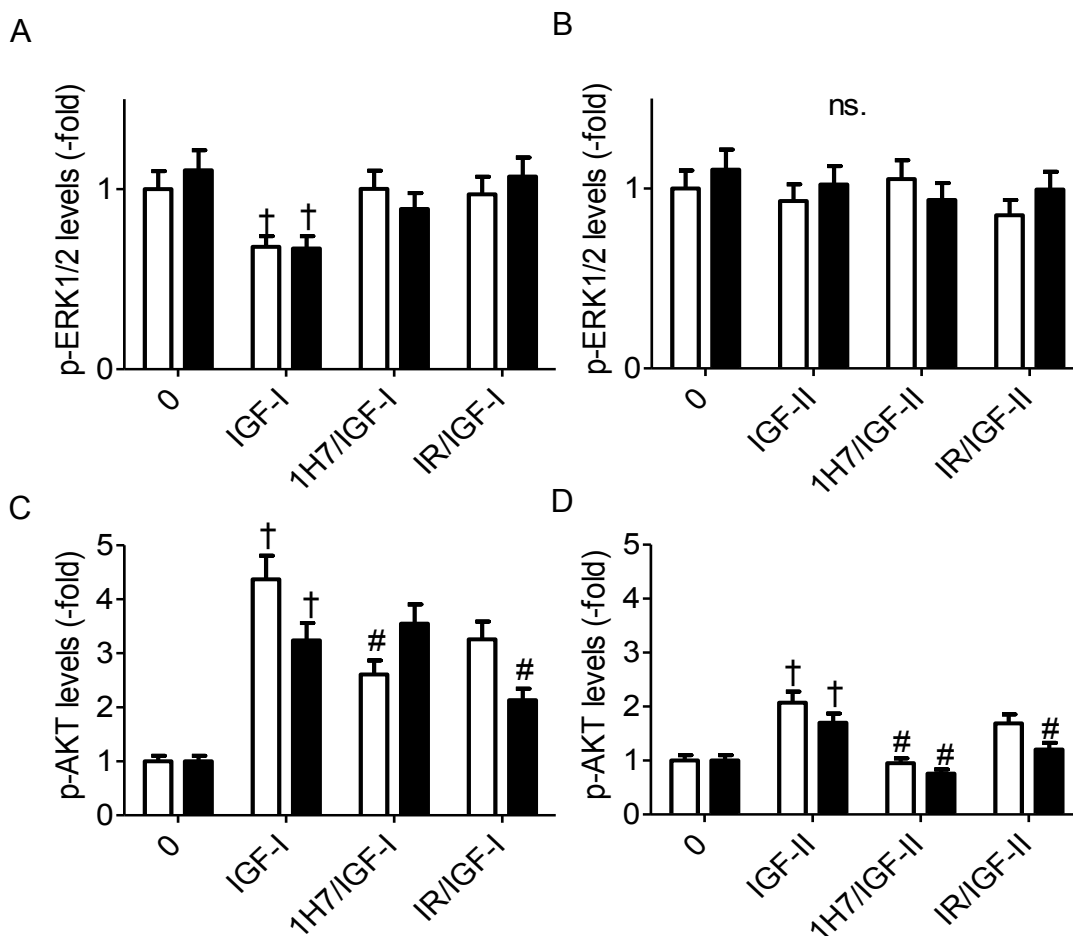
FIGURE 3.7. IGF-mediated p-ERK1/2 and p-AKT signaling in PMSCs under low oxygen tension is partially regulated through the IGF-IR giving the IR a role in low oxygen tension. PMSC were stimulated in presence of 5 µg/mL of IG-IR or IR neutralizing antibodies in presence of A) and C) IGF-I (50 ng/mL) or B) and D) IGF-II (50 ng/mL) under room air or low oxygen for 24-hrs. From Immunoblots, detected levels of A) and B) p-ERK1/2 and C) and D) p-AKT were quantified and normalized to total kinase level followed by normalization to β-actin used as a protein loading control. (Two-way ANOVA, $p < 0.05$, n=3, † = significant difference by IGF concentration compared with 0 ng/mL, # = significant difference by the neutralization antibody effect compared with IGF alone). ERK1/2 phosphorylation was not decreased by antibody neutralization of IGF-IR or IR in PMSCs in presence of IGF-I. p-AKT was dependent on IGF-IR in room air but more dependent on the IR in low oxygen tension conditions.

As demonstrated earlier, increased p-AKT by IGF-I or -II is required for proliferation. IGF-IR neutralization in room air mediated a decrease only in the IGF-I induced p-AKT, whereas the IGF-II induced p-AKT was decreased in both oxygen tensions. IR neutralization had a more significant effect in low oxygen tension regardless of IGF-I or -II (Fig. 3.7 C and D). These results confirm that phosphorylation of AKT is more dependent on IGF signaling, than ERK1/2, in PMSCs and that low oxygen tension can differently mediate the IGF signaling through either the IGF-IR or the IR indicating an important role of the IR in low oxygen tension to mediate higher p-AKT levels and higher proliferation.

3.4. DISCUSSION

In this study, we have demonstrated that low oxygen tension can modulate PMSC response to IGF mediated signaling, proliferation and multipotency. We showed that low oxygen tension and not room air promoted the increased expression of IGF-II and less of IGF-I, and enhanced IGF-IR and IR expression. Moreover, low oxygen tension repressed the short-term stimulation of p-IGF-IRβ and p-AKT, while it potentiated p-ERK1/2 in the presence of IGF-I. However, after a prolonged stimulation with IGFs, low oxygen tension maintained a low level of p-ERK1/2 and mediated a dose dependent increase in p-AKT levels. Among the pluripotency transcription factors, we found that OCT4 is more stably maintained and is less altered by IGFs and oxygen tension, unlike NANOG and SOX2. PMSC proliferation was enhanced in low oxygen tension that potentiated

the effects of IGF-I and -II for higher proliferation level via the cooperative action of IGF-IR and IR.

In the human placental mesodermal core, where placental mesenchymal stem cells (PMSCs) reside, IGF-II is more abundantly expressed as early as 6-weeks of gestation (first trimester) compared with IGF-I which begins after 12-weeks of gestation (second trimester onwards) (24). This suggests that IGF-II plays a major role in early placental development, possibly facilitating cytotrophoblast invasion and angiogenesis just preceding the formation of uterine circulation (25, 26). Oxygen tension is an important microenvironmental factor in influencing growth factor expression and signaling. In particular, low oxygen tension can induce IGF-II gene expression in primary rat osteoblasts or human hepatoma cells in culture (27, 28). As previously shown, IGF-II is important to maintain embryonic stem cell identity as it is expressed by the surrounding differentiated fibroblast cells (8). In this study, we demonstrated a relationship between oxygen tension and the type of IGF ligand expressed by PMSCs (IGF-II in low oxygen and IGF-I in room air). Although, in early gestation placentae, low oxygen tension is known to have an abundant expression of IGF-II *in vivo* (24), PMSCs from different gestational stages still expressed high IGF-II when maintained under low oxygen tension conditions. Therefore, the increased expression of IGF-II can be solely due to low oxygen tension. Hence, maintaining PMSCs in a microenvironment that mimics their natural conditions can program PMSCs to adapt and behave as *in vivo*.

Among the pluripotency triad of transcription factors, OCT4 is regarded as the master regulator and gatekeeper of pluripotency to keep stem cells in undifferentiated state and prevent differentiation of hESCs into trophoblast-like cells (29-32). OCT4 is an indispensible transcription factor to induce pluripotency in iPSCs (18, 19, 32, 33). Also, OCT4 alone was sufficient to generate iPSCs, if neural progenitor cells, which already express endogenous SOX2, c-MYC and KLF4, were used for reprogramming (34). In our PMSCs, OCT4 protein levels were less susceptible to change, unlike NANOG and SOX2, in the presence of IGF-I or -II regardless of oxygen tension. This could be due to OCT4 being central for pluripotency and as a master regulator to keep stem cells in undifferentiated state, as it is tightly regulated (within 0.5-fold) to prevent differentiation or de-differentiation of hESCs (29, 30). In support of IGF and low oxygen tension maintenance of multipotency, we showed that low IGF concentrations could maintain higher levels of NANOG and SOX2.

Activation of ERK1/2 and AKT signaling pathways is important in the maintenance of stem cell proliferation and multipotency. Interestingly, the role of ERK1/2 and/or AKT was dependent on oxygen tension. In differentiation studies, increased activation of ERK1/2 in low oxygen tension conditions promoted proliferation while AKT activation in room air promoted differentiation of myoblasts in culture (35). Inhibition studies done by others and us demonstrate that AKT activation is more important to maintain stem cell potency as measured by OCT4, NANOG and SOX2 levels (36). Also, the induction of PI3K/AKT

signaling pathway has been demonstrated to be central for pluripotency maintenance of hESCs by participating in promoting proliferation, preventing apoptosis and enhancing telomerase activity (37). The direct regulation of stem cell pluripotency by IGF signaling is not clear; however, it is proposed that IGF signaling can maintain pluripotency by phosphorylating the tumour suppressor p53, which acts as a negative regulator at *OCT4* and *NANOG* promoters, and therefore IGF actions would disallow senescence and pluripotency gene repression (37). Apart from direct phosphorylation of p53, AKT can antagonize p53 by the direct phosphorylation of Mdm2 that in turn facilitates p53 ubiquitination and degradation (38), hence decrease multipotency gene repression in stem cells. Therefore, a similar mechanism carried on by p-AKT can play a role in enhancing PMSC multipotency at the gene regulation level.

In PMSCs, low oxygen tension modified IGF signaling starting at initial stimulation and also at prolonged stimulation. Low oxygen decreased the phosphorylation/activation of IGF-IRβ, ERK1/2 and AKT although an increased expression of the IGF-IR was evident. These changes can be due to changes in the phosphorylation mechanism or IGF binding sensitivity to IGF-IR and combination with IR, which is still unclear in low oxygen tension. However, ERK1/2 phosphorylation by IGF-I was transiently higher in low oxygen tension than in room air. It was shown that transient high p-ERK1/2 levels promote proliferation (39). A prolonged stimulation (48-hrs) with increasing concentration of IGF-I and -II changed the levels of ERK1/2 and AKT phosphorylation in

opposite directions, where the latter increased in a dose-dependent manner. In low oxygen tension, ERK1/2 was lowered even with IGF stimulation, possibly to maintain higher PMSC proliferation and inhibit differentiation. Therefore, low oxygen tension maybe required to decrease the continuous p-ERK1/2 level, as it is demonstrated that prolonged p-ERK1/2 levels are required for differentiation (39). In PMSCs, ERK1/2 is less dependent on IGF stimulation than AKT as shown by receptor activation. This was also shown from the receptor neutralization that IGF signaling is more critical for AKT than ERK1/2 phosphorylation, which demonstrated the dependence of AKT on IGF stimulation, especially in presence of IGF-I, to mediate stem cell proliferation.

Signaling through the IGF-IR is important to maintain stem cells in self-renewal state (8, 9). PMSC proliferation was dependent on the IGF-IR stimulation in room air and partially when in low oxygen tension. Both the IGF-IR and IR, which were upregulated in low oxygen tension, participated in mediating the IGF actions to induce PMSC proliferation in low oxygen tension. Interestingly, in neural progenitor stem cells, proliferation was dependent on IGF-II, which was mediated *via* the IGF-IR, while the IR-A had a more important role in maintaining self-renewal than IGF-IR (40). Therefore, as PMSCs express both higher IGF-II and IR-A in low oxygen tension, it is possible that there is dependence on IGF-II/IR-A signaling to maintain PMSC multipotency and proliferation. IGF-II plays a major role in placental development, as the IGF-II knockout mice, but not the IGF-I or IGFIR knockout mice, caused a more significant growth reduction in mouse

placenta (10, 41). Therefore, our data suggests that human stem cells may also carry this dependence on IGF-II/IR when maintained in low oxygen tension conditions. Also, IGF signaling utilizes the IR in addition to IGF-IR, to maintain AKT phosphorylation, possibly due to the higher expression of the IR in low oxygen tension. We propose that the IR homodimer or the hybrid with the IGF-IR increases in low oxygen tension due to increased expression, and therefore can maintain stem cell character to in response of IGF-II signaling.

Overall, placental stem cells are sensitive to their microenvironment with a higher dependence on low oxygen tension. We showed that culturing PMSCs under low oxygen tension modifies IGF signaling through the IGF-IR *via* ERK1/2 and AKT, suggesting that those two kinases participate in the regulation of stem cell fate. Also, we show that low oxygen tension can modify the stem cell dependence on IGF-IR by utilizing other signaling pathways, such as the IR, to maintain PMSC proliferation and multipotency. Thus, maintaining stem cells under low oxygen tension, similar to *in vivo* conditions, may be important in maintaining stem cell natural behaviour in preparation for regenerative therapy.

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Appendix 2
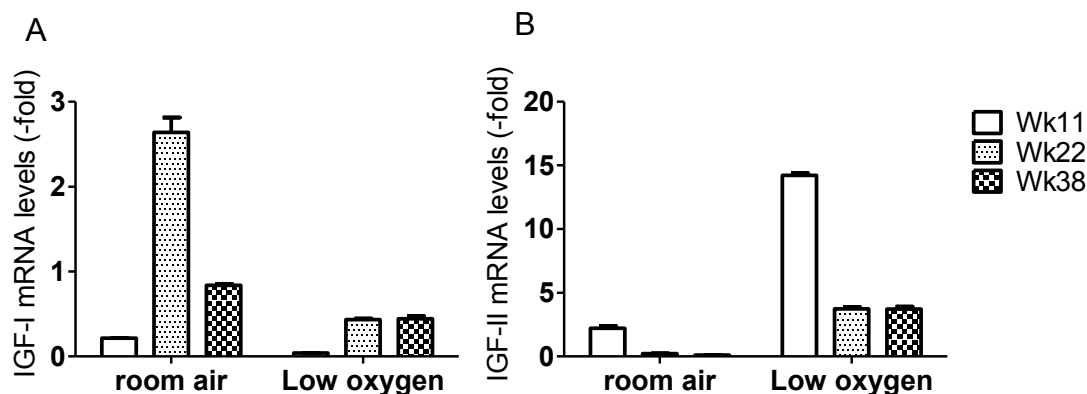
FIGURE S3.1. IGF ligand type expression in PMSCs is gestational age and oxygen tension dependent. PMSCs isolated from 11, 22, 38 weeks placentae were cultured for 48-hrs in either room-air or low-oxygen tension in serum and growth factor free conditions. By real-time PCR, expression level of IGF-I and -II mRNA were measured relative to RPL13a as an endogenous control. IGF-I levels were measured with increasing concentration of A) IGF-I or B) IGF-II. (Two-way ANOVA, p<0.05, n=3). IGF-I expression is elevated in room air at later gestation that is reduced further with PMSCs are maintained in low oxygen tension. IGF-II expression is higher at earlier gestation PMSCs and is enhanced by low oxygen.

Appendix 2
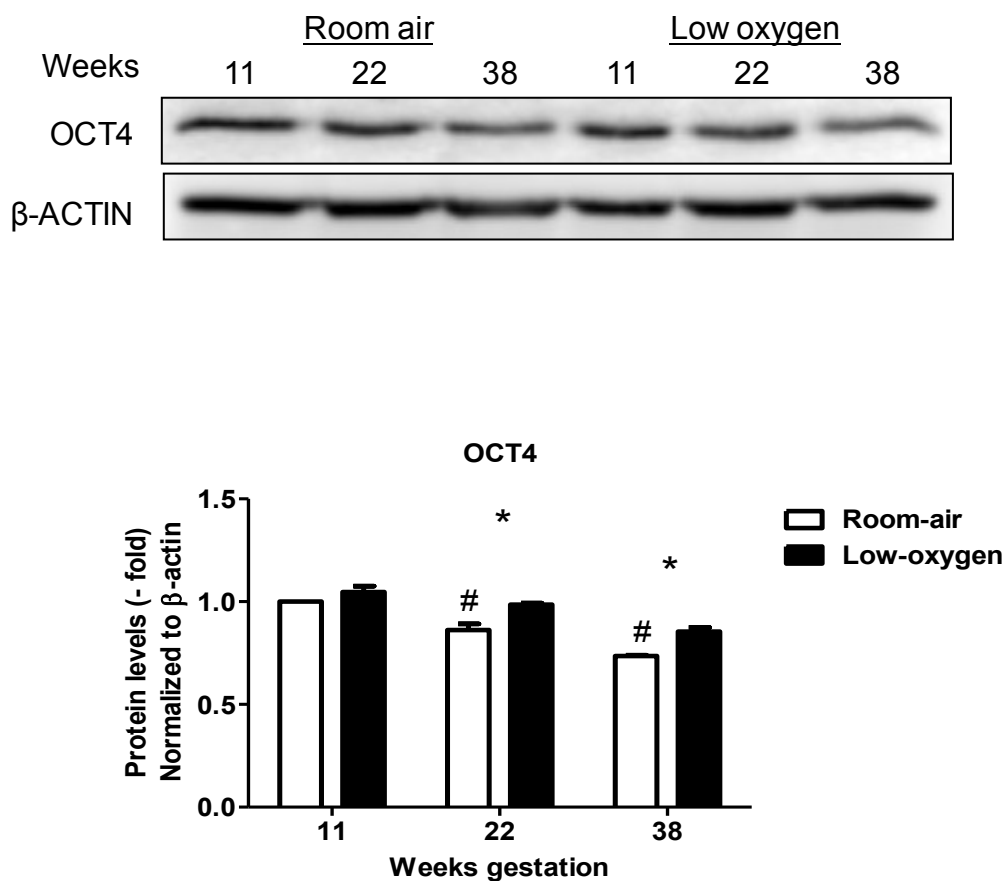
FIGURE S3.2. PMSC stemness as measured by OCT4 is gestational age and oxygen tension dependent. PMSCs isolated from 11, 22, 38 weeks placentae were cultured for 48-hrs in either room-air or low-oxygen tension in 10% fetal bovine serum conditions. By immunoblotting, level of OCT4 protein levels were measured and normalized against β-Actin as an endogenous control. (Two-way ANOVA, $p < 0.05$, n=3, * = significant difference between low oxygen tension and room air, # = significant difference by comparing gestational age). OCT4 from different gestations is maintained at higher levels by low oxygen tension.

Appendix 2
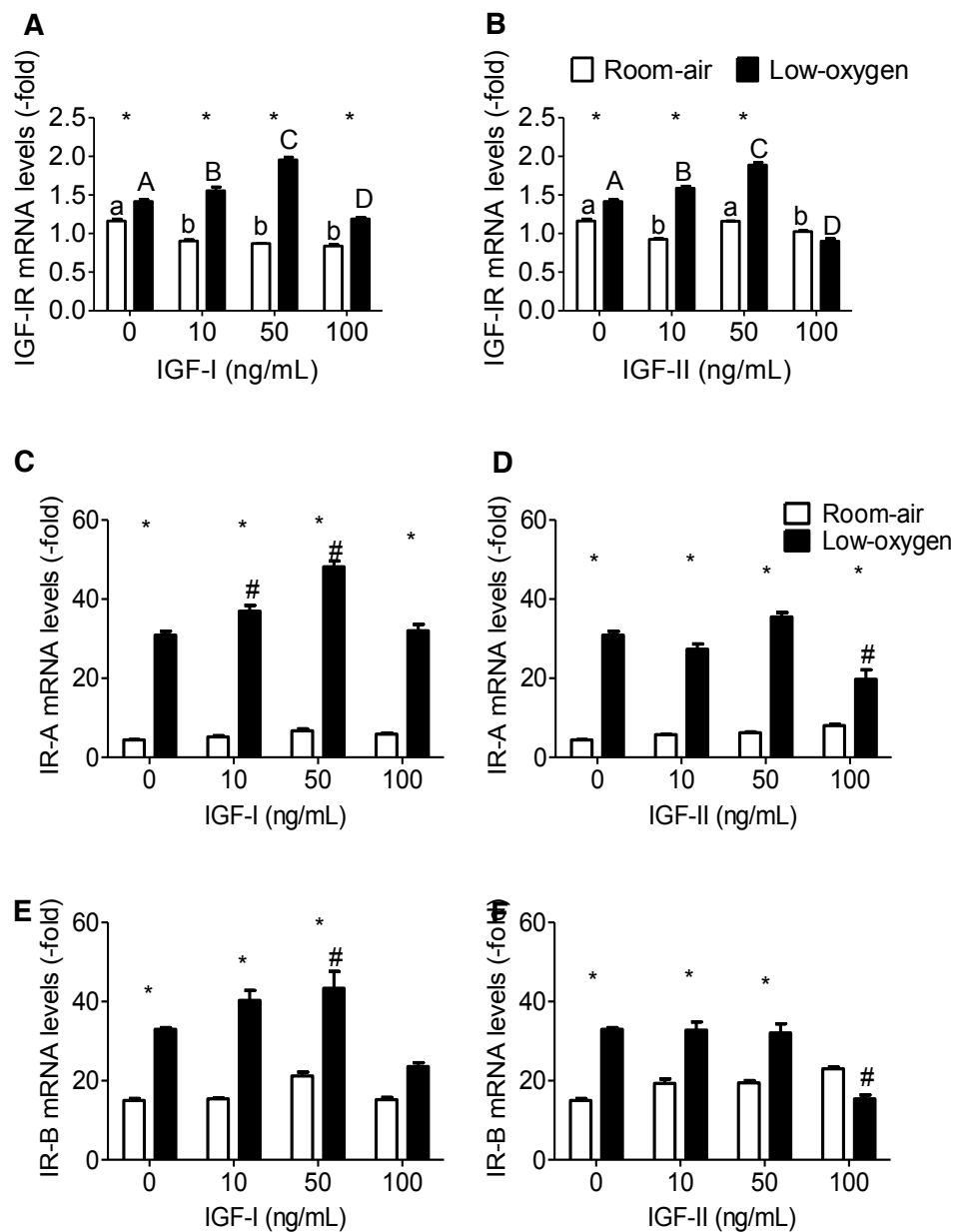
FIGURE S3.3. IGF-IR and IR mRNA expression are decreased by IGF-I and - II but elevated by low oxygen tension. PMSCs were cultured for 48-hrs in either room air or low oxygen tension in presence or absence of IGF-I or IGF-II (0, 10, 50, 100 ng/mL). By real-time PCR, expression level of A) and B) IGF-IR or C) and D) IR-A or E) and F) IR-B mRNA were measured relative to RPL13a as an endogenous control in presence of IGF-I or IGF-II. (Two-way ANOVA, $p<0.05$, n=3, * = significant difference between room air and low oxygen tension, # = significant difference by different IGF concentrations).

Appendix 2
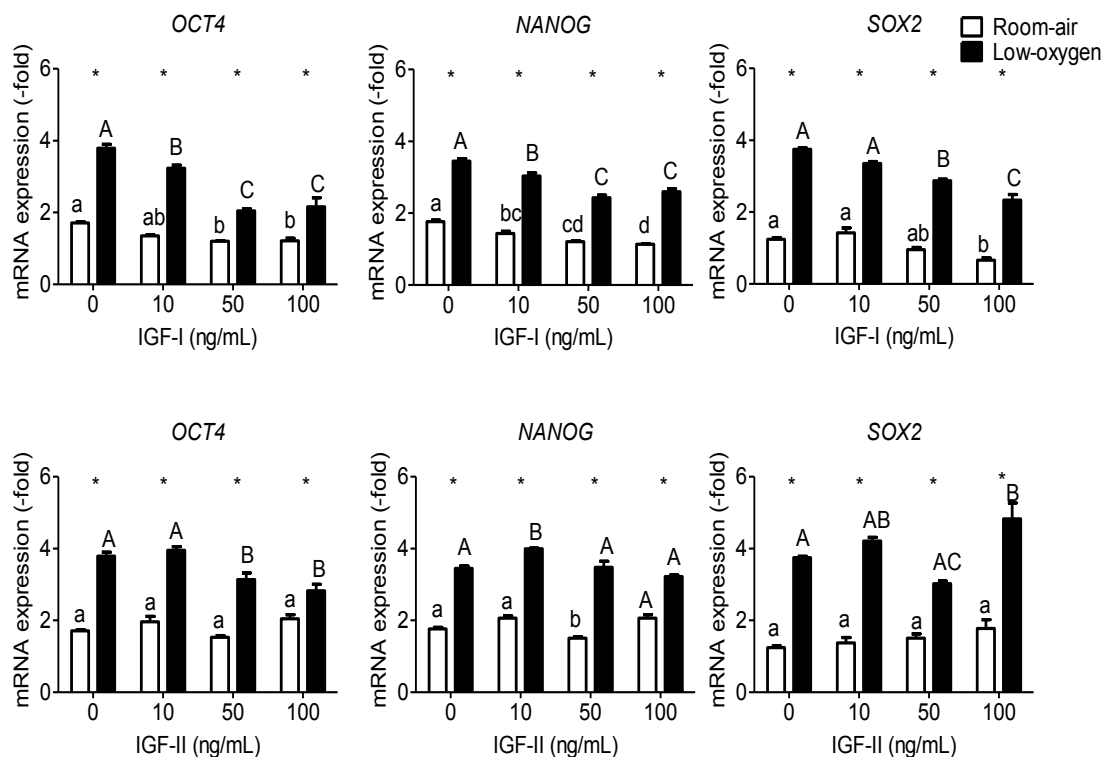
FIGURE S3.4. OCT4, NANOG and SOX2 expression is affected by IGF-I or -II concentration depending on oxygen tension. PMSCs were cultured for 48-hrs in either room air or low oxygen tension in presence or absence of IGF-I or IGF-II (0, 10, 50, 100 ng/mL). By real-time PCR, expression level of OCT4 or NANOG or SOX2 mRNA was measured relative to RPL13a as an endogenous control in presence of IGF-I or IGF-II. (Two-way ANOVA, $p < 0.05$, n=3, *= significant difference between room air and low oxygen tension; a= significant difference of different IGF concentrations in room air, A= significant difference of different IGF concentrations in low oxygen tension). Low oxygen tension increased pluripotency associated gene expression where IGF treatment reduces them (more with IGF-I than IGF-II).

Appendix 2

A

B

C
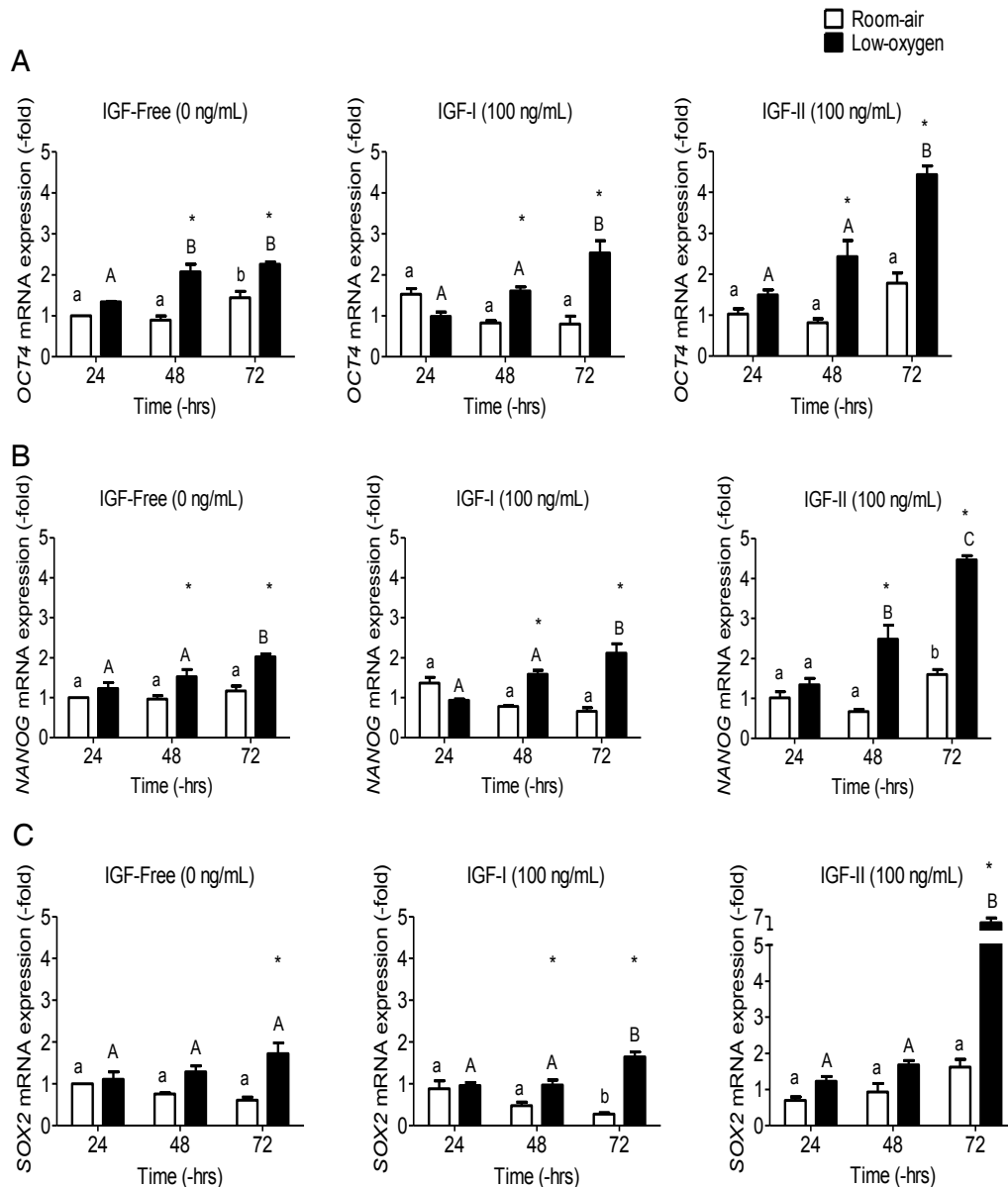
FIGURE S3.5. OCT4, NANOG and SOX2 expression is increased by IGF-I or -II oxygen tension. PMSCs (N=2) were cultured for 24, 48 or 72-hrs in either room air or low oxygen tension in presence or absence of IGF-I or IGF-II (100 ng/mL). By real-time PCR, expression level of A) OCT4 or B) NANOG or C) SOX2 mRNA was measured relative to RPL13a as an endogenous control in presence of IGF-I or IGF-II. (Two-way ANOVA, $p<0.05$, n=3, *= significant difference between room air and low oxygen tension; a= significant difference of different IGF concentrations in room air, A= significant difference of different IGF concentrations in low oxygen tension).

Appendix 2
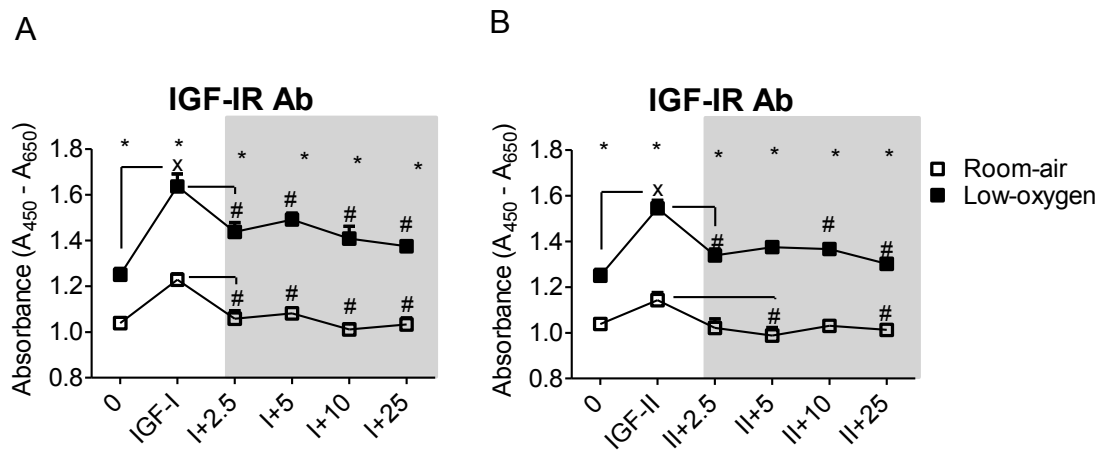
FIGURE S3.6. IGF-mediated PMSC proliferation under low oxygen tension is partially regulated through the IGF-IR. PMSC proliferation was assessed using the WST-1 colorimetric proliferation assay in presence of increasing concentration of 1H7 antibody (0, 2.5, 5, 10 , 25 µg/mL) in presence or absence of A) IGF-I (50ng/mL) or B) IGF-II (50ng/mL) under room air or low oxygen for 24-hrs. The absorbance was read at 450 nm with a background reference of 650 nm, (Two-Way ANOVA, $P<0.05$, n=8, * = significant difference between room air and low oxygen tension; X = significant difference of IGF-I or IGF-II, # = significant difference of of neutralizing antibody compared with IGF effect).

Appendix 2
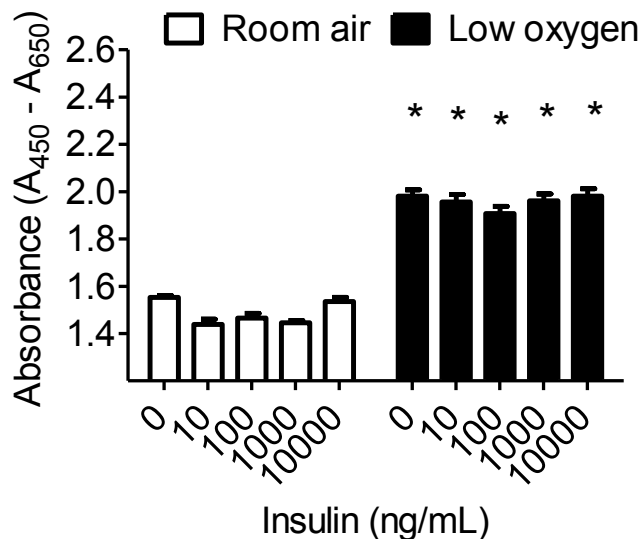
FIGURE S3.7. PMSC proliferation is not responsive to insulin stimulation even in low oxygen tension. PMSC proliferation was assessed using the WST-1 colorimetric proliferation assay in presence of increasing concentration of insulin (0, 10, 100, 1,000, 10,000 ng/mL) under room air or low oxygen for 48-hrs. The absorbance was read at 450 nm with a background reference of 650 nm. (Two-Way ANOVA, $P<0.05$, n=8, *= significant difference between room air and low oxygen tension). Low oxygen tension enhanced PMSC proliferation without an effect of increasing concentration of insulin.

CHAPTER FOUR

Insulin-like Growth Factor and Low Oxygen Tension Regulate Osteogenic

Differentiation of Placental Mesenchymal Stem Cell

4.1.   INTRODUCTION

Mesenchymal stem cells (MSCs) are found in many adult tissues and are responsible for tissue repair and homeostasis (1). Unlike ESCs, MSCs are less tumorigenic and have a more restricted mesendodermal lineage-specific differentiation (2) towards myocytes, osteoblasts, chondrocytes, adipocytes, stromal fibroblasts, and endothelial cells (1, 3, 4). In addition, MSCs have been demonstrated to form neuroblasts *in vitro*, and neuron-like cells bearing axons and dendrites *in vivo* (5). Also, MSCs can modulate the immune response and were successfully used in graft vs. host disease resistant patients (6). Therefore, MSCs are increasing in promise for stem cell-based therapies to treat many adult and paediatric diseases, such as sickle cell disease (7), rheumatic diseases (8), lymphoma (9), and heart failure (10).

In bone, MSC transplantation has been utilized to correct bone malformation and injury. Osteogenesis imperfecta (OI) is a severe pediatric genetic disorder of mesenchymal cells with a deficit for type-I collagen synthesis, which is critical for matrix deposition and mineralization in bone (11). There is no treatment for OI, however, an allogeneic bone-marrow transplantation was shown to successfully accelerate linear growth and increase total body bone mineral density in children with OI (11). Also in bone injury, MSCs have the potential to treat the more severe nonunion bone fractures and accelerate recovery of delayed union. In a mouse model, bone-marrow MSCs administered by systemic infusion

successfully homed to the fracture site and accelerated the initiation of a callus and improved the cartilage and bone content (12). Therefore, the use of MSCs for the treatment of bone diseases and injury is a promising regenerative therapy.

Although bone-marrow MSCs have been used for stem cell therapy (13, 14), the challenge has been their availability. Placental MSCs (PMSCs) are abundant, readily available and do not require invasive isolation techniques (15). PMSCs are isolated from different parts of the placenta (amnion, chorion and umbilical cord) and have a higher proliferation capacity than bone-marrow MSCs (15). In culture, MSCs are dependent on their surrounding microenvironment for maintaining stem cell identity, and they differentiate by the signaling by several growth promoting factors and the use of lineage specific cell culture conditions (16, 17). In this microenvironment, several clues regulate stem cell differentiation by a tight transcriptional network of losing multipotency and initiating linage-specific differentiation. In osteoblast differentiation, RUNX2 is known as a lineage-specific regulator transcription factor that upregulates transcription of genes required for bone matrix deposition and mineralization including osteopontin (OPN), osteocalcin, type-I collagen, alkaline phosphatase, etc. (18-21). In this process, RUNX2 is strongly detected in preosteoblasts, immature osteoblasts, and early osteoblasts. However, its continued expression at later stages of mature osteoblast differentiation inhibits complete differentiation (20, 22).

In the stem cell microenvironment, MSCs reside in low oxygen tension, which promotes stem cell proliferation, self-renewal and multipotency (23). However, low oxygen tension can severely inhibit osteoblast differentiation *in vitro* (24), while re-exposure to room air restores differentiation and may potentiate osteogenic differentiation (25, 26). In this process, the hypoxia inducible factor (HIF) system is responsible for downregulating the expression of osteoblast commitment genes, such as RUNX2 (27, 28), and a complete differentiation is obscured due to inhibition of expression of downstream genes responsible for mineralization and matrix formation (27). However, *in vivo*, inhibiting cellular responses in low oxygen tension by HIF1α knockout decreases trabecular bone volume, reduces bone formation rate, alters cortical bone architecture and reduces proliferation of osteoblasts during long bone development (29). In contrast, an over-expression of HIF-1α in osteoblasts leads to the development of extremely dense and heavily vascularized long bones (30). Therefore, the regulation of gene expression by low oxygen tension is required for healthy development of bone *in vivo* at least at the initial stages as confirmed by preconditioning experiments. In this context, long-term exposure to low oxygen tension is inhibitory to stem cell differentiation, although a short term exposure can play a role in directing stem cell fate towards osteogenic differentiation (25).

In addition to oxygen tension, stem cell differentiation conditions can be defined by soluble factors, small molecules, hormones, and growth factors. The insulin-like growth factors (IGFs, IGF-I and IGF-II) can promote and stimulate stem cell

differentiation towards hepatic (31), endothelial (32), adipogenic (33) and osteogenic (34, 35) lineages. In osteogenic tissues, the expression of IGF-I, IGF-II and their receptor IGF-IR, has a strong association with osteogenesis and are abundantly expressed specifically in mature osteoblasts and osteoclasts by autocrine/endocrine mechanisms (36). The IGF-I and IGF-IR null mice show short bone, low bone mineral density, and delayed calcification, whereas, IGF-II null mice show no major skeletal defects (37). IGFs have no effect on osteoblast differentiation commitment (no change in RUNX2 expression), but enhance the differentiation function by promoting growth, inhibiting apoptosis and upregulating matrix maturation (increased type-I collagen) and mineralization (38). Also, *in vivo*, the use of MSCs that overexpress IGF-I improves fracture healing by accelerating bone cell differentiation which is dependent on IRS1-PI3K pathway in mediating the IGF-I dependent osteogenic actions via paracrine and autocrine functions (39).

IGFs and low oxygen tension are endogenous components of the osteogenic microenvironment, which are shown to affect later stages of osteogenic differentiation during the mineralization period of MSC differentiation (27, 38). However, the combined effect of these two microenvironmental factors on the commitment and early differentiation stages has not been investigated. In this study, we used MSCs isolated from the chorionic villi of the human placenta (PMSCs) to study the role of IGF-I and IGF-II signaling in combination of low oxygen tension in osteogenic differentiation. We show that low oxygen tension

inhibits the differentiation of PMSCs towards the osteogenic lineage and IGF-I enhances differentiation *via* specific signaling pathways.

4.2. MATERIAL AND METHODS

4.2.1. PMSC Isolation

PMSCs were isolated from early gestation (10-13 weeks) human placentae. After informed consent, preterm placentae were collected from patients who underwent therapeutic pregnancy termination. Immediately after surgery, placentae were dissected under sterile conditions and small pieces of chorionic villi were collected. Tissue samples were minced mechanically and subjected to a process consisting of two steps of enzymatic digestion with (1) collagenase IV/hyaluronidase, and (2) DNase I followed by (3) trypsin/EDTA. Each enzymatic step was performed for 20 min at 37ºC, followed by 10 min wash at 4ºC in a solution of PBS supplemented with 10% fetal bovine serum (FBS) (Gibco, Mississauga, ON, CAN). Cells released during digestion were passed through a tissue mesh (45 μm) to obtain a single cell suspension. Next, cells were separated on a Percoll (Sigma) discontinuous gradient according to a modified protocol by Worton et al. for hematopoietic stem cell isolation (40) and then seeded in DMEM/F12 media (Gibco, Mississauga, ON, CAN) supplemented with 10% FBS and antibiotic-antimycotic solution. After 4 days, media was changed and non-adherent cells were washed with media to leave behind adherent PMSCs able to form colonies.

4.2.2. Cell Culture and Incubation in Low Oxygen Tension

For each experiment, two preterm PMSC cell populations were used in triplicate. Cells were cultured and maintained using DMEM/F12 media supplemented with 10% ES-FBS and FGF-2 (100 ng/mL) (Gibco, Mississauga, ON, CAN). Before treatments, cells were cultured in DMEM/F12 supplemented 10% FBS only. Upon treatment, PMSCs were switched to fresh media containing IGF-I or IGF-II (0 – 300 ng/mL). For IGF-IR neutralizing, cells were grown in serum-free media for 24-hrs and then switched for 2-hrs in serum-free media with 1.33x final concentration of 1H7 Ab (in 75% final volume). Secondly, Serum-free media containing 4x final concentration of IGF-I or -II (in 25% final volume) was added and incubated for the rest of 24-hrs. Cell cultures were then placed in either a 5% CO_2 incubator or a hypoxia chamber, which was filled with a mix of (1% O_2, 5% CO_2, Balanced N_2) (BOC Canada Ltd, Toronto, ON, CAN) for 15 min to ensure saturation using a Hudson 5590 Oxygen Monitor (Hudson, Ventronics Division, Temecula, CA, USA). Thereafter, the chamber was placed in a tissue-culture incubator at 37° C.

4.2.3. Osteogenic Differentiation

PMSCs were plated at 70% confluency in non-differentiation conditions (15% FBS/DMEMF12) or in presence of osteogenic stimulatory conditions (15% osteogenic differentiation FBS, 10^{-8} M Dexamethasone, 50µg/mL Ascorbic acid, and 3.5 mM β-Glycerophosphate) (Stem Cell Technologies, Vancouver, BC,

CAN). With IGF-I or -II treatments, 100 ng/mL of either IGF was added continuously to a reduced FBS level of 2% instead of 15% in either media. Relative effect of different IGF and oxygen tension treatments on calcium deposits was compared using alizarin red and alkaline phosphatase staining. The signaling of MEK/ERK pathway or the PI3K/AKT pathways was inhibited by the continuous presence of U0126 (5 µM) or LY294002 (10 µM), respectively. To assess these morphological changes of differentiated vs. non-differentiated, cells were cultured for 14-days and then fixed with 4% formaldehyde for 30 min at RT. Then cells were either stained with 1% Alizarin Red solution for 10 min at RT, or with NBT/BCIP reagents as per manufacturer's protocol. Both staining were then solubilized with 10% cetylpyridinium chloride in 10mM sodium phosphate buffer pH 7.0 as previously described (35). The absorbance of 200 µL solubilized staining was read at $\lambda=570$nm using a plate reader. The absorbance was then normalized to total protein content per well in micro-grams.

4.2.4. Immunoblotting

Cell lysates were prepared using 1x cell lysis buffer for phospho-proteins according to manufacturer protocol (#9803, Cell Signaling Technologies, Burlington, ON, CAN). Protein samples (10-20 µg) from each cell lysate were resolved by 10% SDS-PAGE, and then transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked with 5% bovine-serum-albumin or in 5% non-fat-dry milk in 1x TBS (Tris-buffered saline) for 1 hr at room

temperature. Blots were then washed in 1x TBS 0.1% Tween 20 (TBS-T) (3x for 5 min) followed by incubation at 4°C overnight with primary antibodies as per manufacturer's protocols. Blots then were washed using TBS-T (3x for 10 min) and were incubated with the corresponding secondary HRP-labelled antibody for 1 hr at RT. Immunocomplexes were detected by ECL and documented using VersaDOC^{TM} Imaging System (Bio-Rad).

4.2.5. Antibodies

To investigate the IGF-I activated signaling molecules in placental MSCs, we used the following antibodies: phospho-p44/42 MAPK (#4377), p44/42 MAPK (#9102) phospho-AKT (Ser473, #4051), AKT (#9272) from (Cell Signaling Technologies, Burlington, ON, CAN) and IGF-IRα (N-20, sc-712) and IR- α (N-20, sc-710) (Santa Cruz Biotech., Santa Cruz, CA, USA). For multipotency markers, we used OCT3/4 antibody (N-19, sc-8628) (Santa Cruz Biotech., Santa Cruz, CA, USA) and SOX-2 (2683-1) (Epitomics, Burlington, ON, CAN). For the osteogenic differentiation markers we used RUNX2 (#8486) from (Cell Signaling Technologies, Burlington, ON, CAN) and OPN (K-20, sc-1059) from (Santa Cruz Biotech., Santa Cruz, CA, USA). For loading control, we used pan-Actin Ab-5 (#MS-1295) (Thermo Fisher Scientific, Fremont, CA). The secondary antibodies used for immunoblotting were goat anti-rabbit (#170-6515), anti-mouse (#170-6516) HRP conjugated antibodies (BioRad laboratories, Hercules, CA) or donkey anti-goat antibody (sc-2020) Santa Cruz Biotech., Santa Cruz, CA, USA).

4.2.6. Statistical Analysis

All experiments were run in triplicates from three independent experiments each; whenever possible three or more PMSC primary lines were used from preterm placentae. All graphs and analyses were generated using GraphPad Prism Software 5.0 (GraphPad Software, San Diego, CA). A Two-way ANOVA with Bonferroni *post hoc* test was used for the PMSC WST1 proliferation assay and densitometry quantifications. Data are expressed as mean ± standard error of the mean (SEM); values were considered significant when $p < 0.05$.

4.3. RESULTS

4.3.1. Effect of low oxygen tension on PMSC osteogenic differentiation

We selected to use preterm PMSCs (11 – 13 wks gestation) to be studied for differentiation into osteogenic lineage. The progressive morphological changes over 14 days were greater in room air when maintained in osteogenic differentiation conditions. Low oxygen tension inhibited osteogenic differentiation over the same time period (Fig. 4.1A), and enhanced cell proliferation compared with room air (Fig. S4.1). Alizarin red staining showed that PMSCs could spontaneously differentiate into an osteogenic-like cells in room air beginning at day 3, and was limited by low oxygen tension (Fig. 4.1B). Therefore, low oxygen tension oxygen tension (Fig. 4.1B).
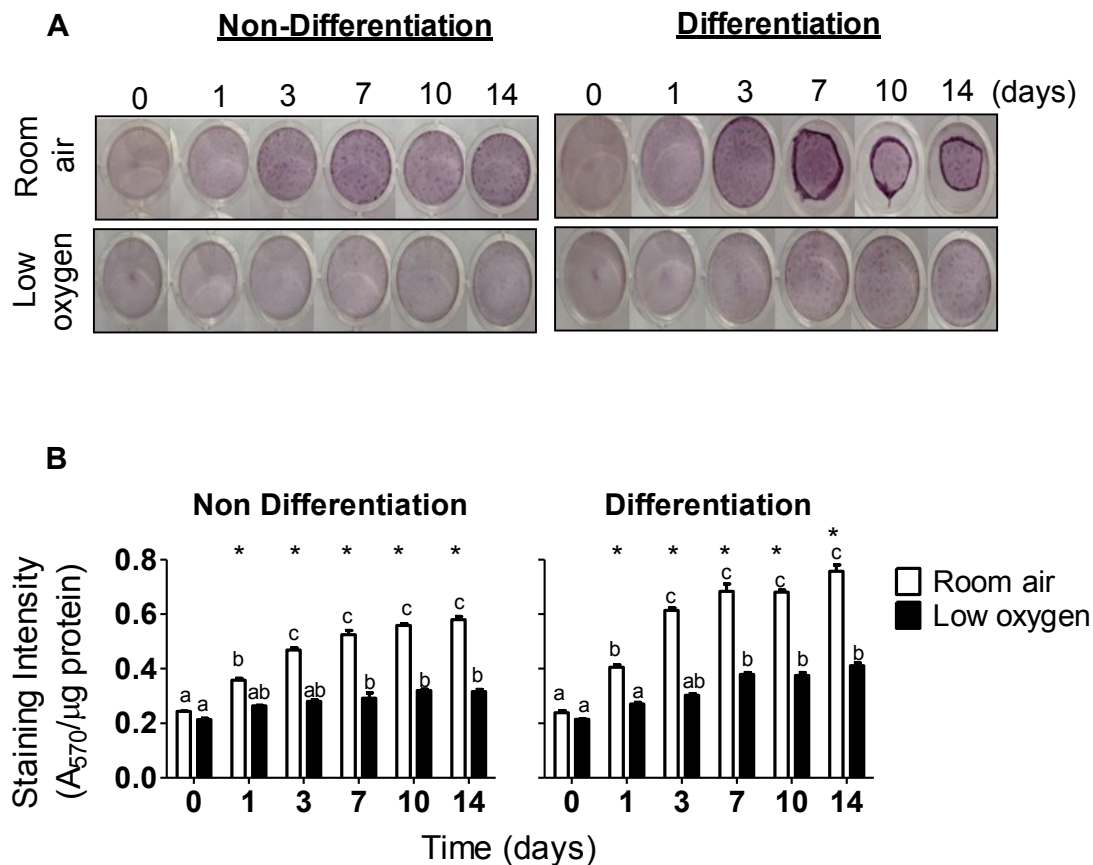
FIGURE 4.1. PMSC differentiation under low oxygen tension. PMSCs were cultured for 14-days in non-differentiation or osteogenic differentiation conditions containing 15% FBS in room air (20% O_2) or low oxygen levels (1% O_2). Treatments were stopped after (1, 3, 7, 10, 14 days) for alizarin red staining to confirm **(A)** PMSC differentiation morphology and quantified in **(B)**. (Two-Way ANOVA, $P < 0.05$, n=4, * = significant difference between room air and low oxygen tension; a= significant difference of differentiation condition in room air, A= significant difference of differentiation condition in low oxygen tension).

Therefore, low oxygen tension inhibited differ PMSC spontaneous differentiation in growth conditions and osteogenic differentiation under differentiation conditions.

To monitor PMSC stemness during differentiation, stem cell markers OCT4 and SOX2 were determined, in addition to the osteogenic differentiation and commitment transcription factor RUNX2. During osteogenic differentiation, OCT4 and SOX2 levels were consistently higher in low oxygen tension compared to room air (Fig. 4.2A and B). OCT4 levels were unchanged upon differentiation in either oxygen tension, whereas, SOX2 levels were moderately decreased. At 14 days, RUNX2 levels were increased upon differentiation in room air but were lower when maintained in low oxygen tension (Fig. 4.2C). Therefore, low oxygen tension reduced PMSC differentiation by maintaining higher level of pluripotency genes (higher OCT4 and SOX2), and reducing differentiation commitment towards the osteogenic lineage (lower RUNX2).

4.3.2. Low oxygen tension preconditioning enhances PMSC osteogenic differentiation

We evaluated whether preconditioning PMSCs in low oxygen tension, which maintain multipotency, can enhance differentiation when exposed to
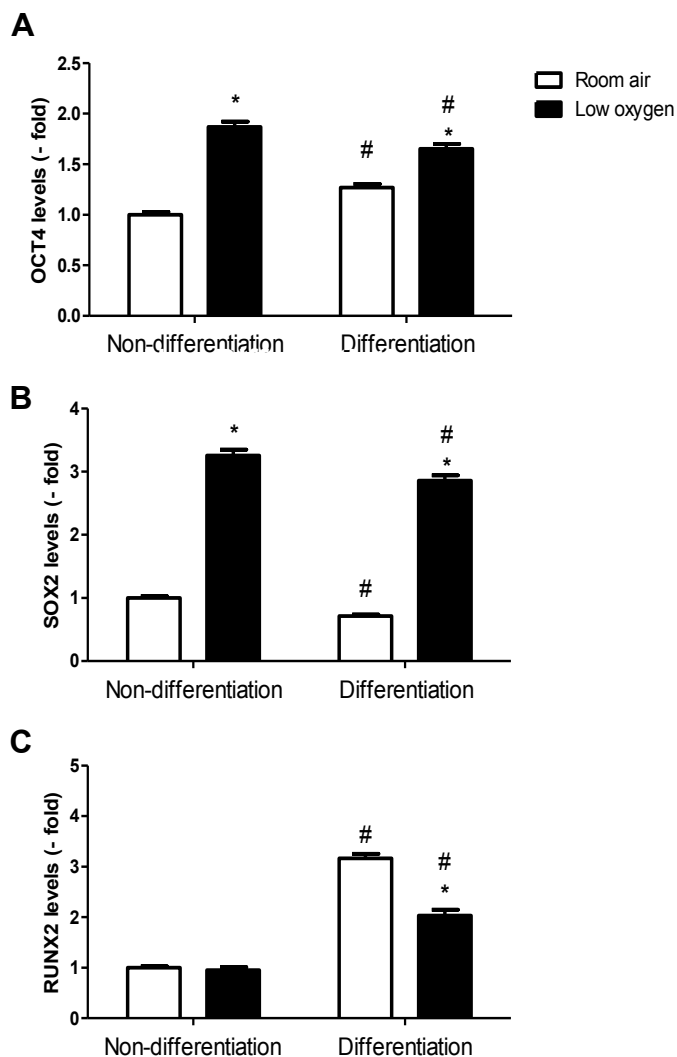
FIGURE 4.2. Effect of low oxygen on PMSC osteogenic differentiation and stemness. PMSCs were cultured for 14-days in non-differentiation or osteogenic differentiation conditions containing 15% FBS in room air or low oxygen levels. Treatments were stopped after 14 days. Protein levels for A) OCT4, B) SOX2, and C) RUNX2 were quantified from Immunoblots. Quantification levels shown were normalized to β-Actin, a protein loading control. (Two-Way ANOVA, $P < 0.05$, n=3, * = significant difference between room air and low oxygen tension, # = significant effect by differentiation condition).
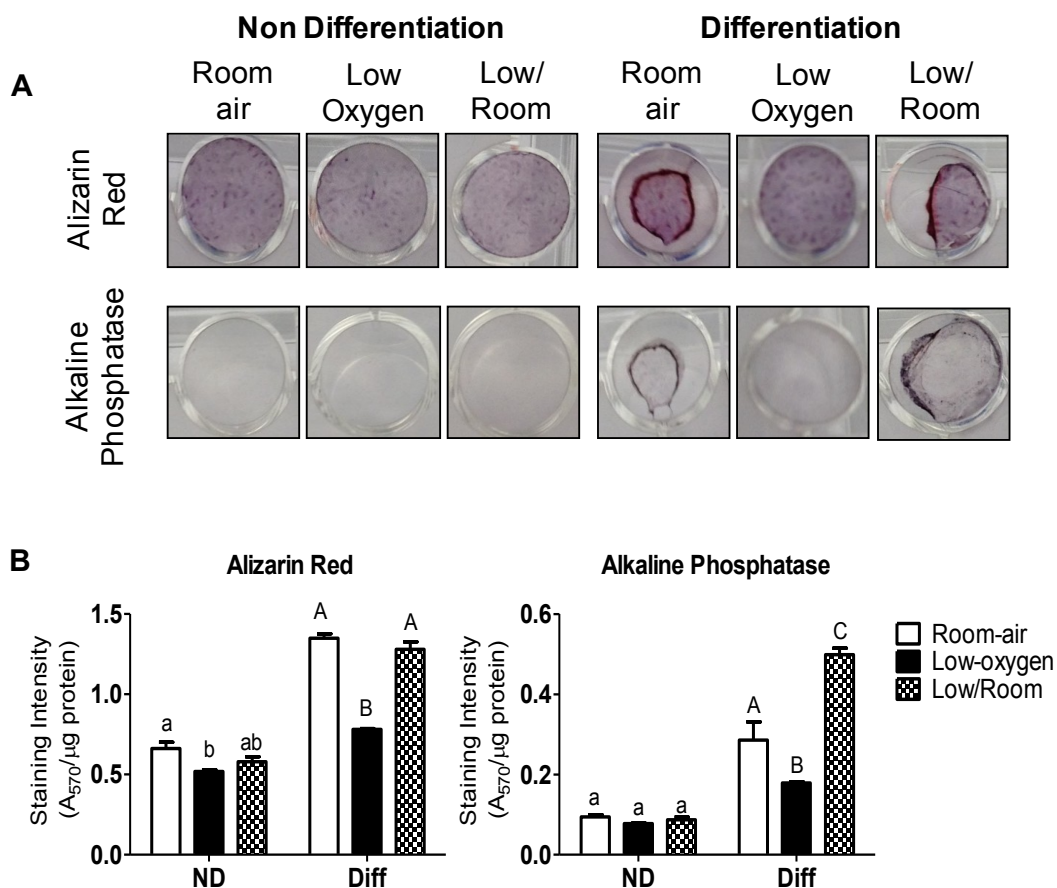
FIGURE 4.3. Effect of low oxygen tension preconditioning on PMSC osteogenic differentiation. PMSCs were cultured in non-differentiation or osteogenic differentiation conditions containing 15% FBS in room air or low oxygen levels for 14-days as in Fig. 4.1. For preconditioning, PMSCs were cultured for 7 days in low oxygen and followed by 7 days in room air (shown in the third panel). Alizarin red or alkaline phosphatase staining was used to detect calcium deposition and enzyme expression changes as shown morphologically in **(A)** and quantified in **(B)**. (Two-Way ANOVA, $P < 0.05$, n=4, a = significant difference by oxygen tension in non-differentiation conditions, A = significant difference by oxygen tension in differentiation conditions).
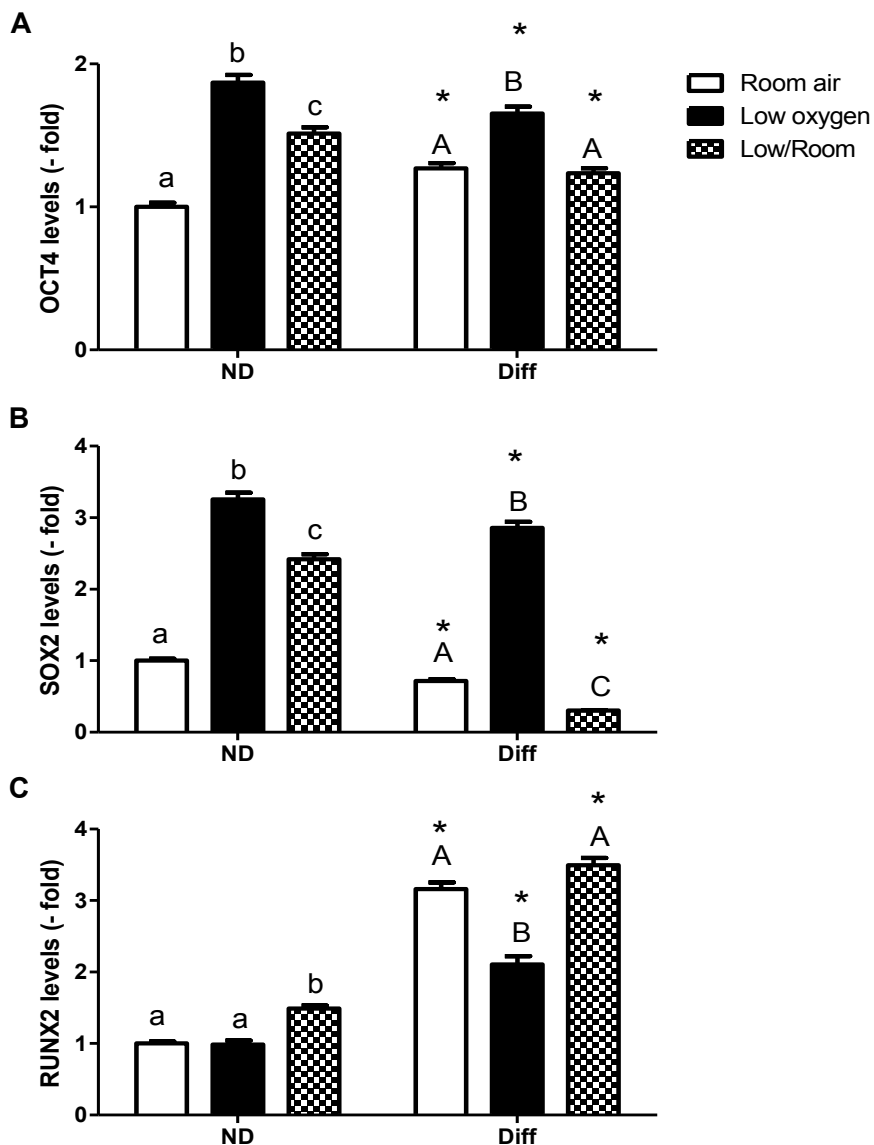
FIGURE 4.4. Effect of low oxygen tension preconditioning on PMSC stemness and osteogenic differentiation. Similarly as in Fig. 4.3, PMSCs were cultured for 14-days in non-differentiation or osteogenic differentiation conditions containing 15% FBS in room air or low oxygen levels. For preconditioning, PMSCs were cultured in low oxygen tension for 7 days followed by 7 days in room air. Immunoblots were used to detect levels of **(A)** OCT4, **(B)** SOX2, and **(C)** RUNX2 levels. Quantification levels shown were normalized to β-Actin, a protein loading control. (Two-Way ANOVA, $P<0.05$, n=3, * = significant difference between differentiation and non-differentiation conditions, a = significant difference by oxygen tension in non-differentiation conditions, A = significant difference by oxygen tension in differentiation conditions).

room air. This scenario mimics the *in vivo* differentiation condition. Alizarin red staining was returned to same levels as room air by exposure to room air for the final 7 days of the differentiation course (Fig. 4.3A and 4.3B-left). This was also confirmed by alkaline phosphatase staining (Fig. 4.3B-right). At the molecular level, preconditioning in low oxygen tension maintained higher levels of OCT4 and SOX2 which were reduced upon differentiation in room air (Fig. 4.4A and B). RUNX2 levels were also increased to room air levels upon re-exposure to room air (Fig. 4.4C). This shows that low oxygen tension programs PMSCs and enhance/potentiate osteogenic differentiation when exposed to higher oxygen tension in room air, which is required for completing osteogenic differentiation.

4.3.3. Effect of insulin-like growth factors on PMSC osteogenic differentiation in low oxygen tension

The additional role of IGFs in mediating the differentiation process in low oxygen tension was investigated by adding IGF-I or -II for 14 days under continuous room air or low oxygen tension conditions (Fig. 4.5). There was no increase in alizarin red staining or increase in spontaneous differentiation in either room air or low oxygen tension in absence of IGFs (Fig. 4.5A). However, IGF-I was able to increase earlier spontaneous differentiation in room air. In differentiation conditions, both IGF-I and IGF-II enhanced osteogenic differentiation in room air

A Non Differentiation

B Differentiation

153
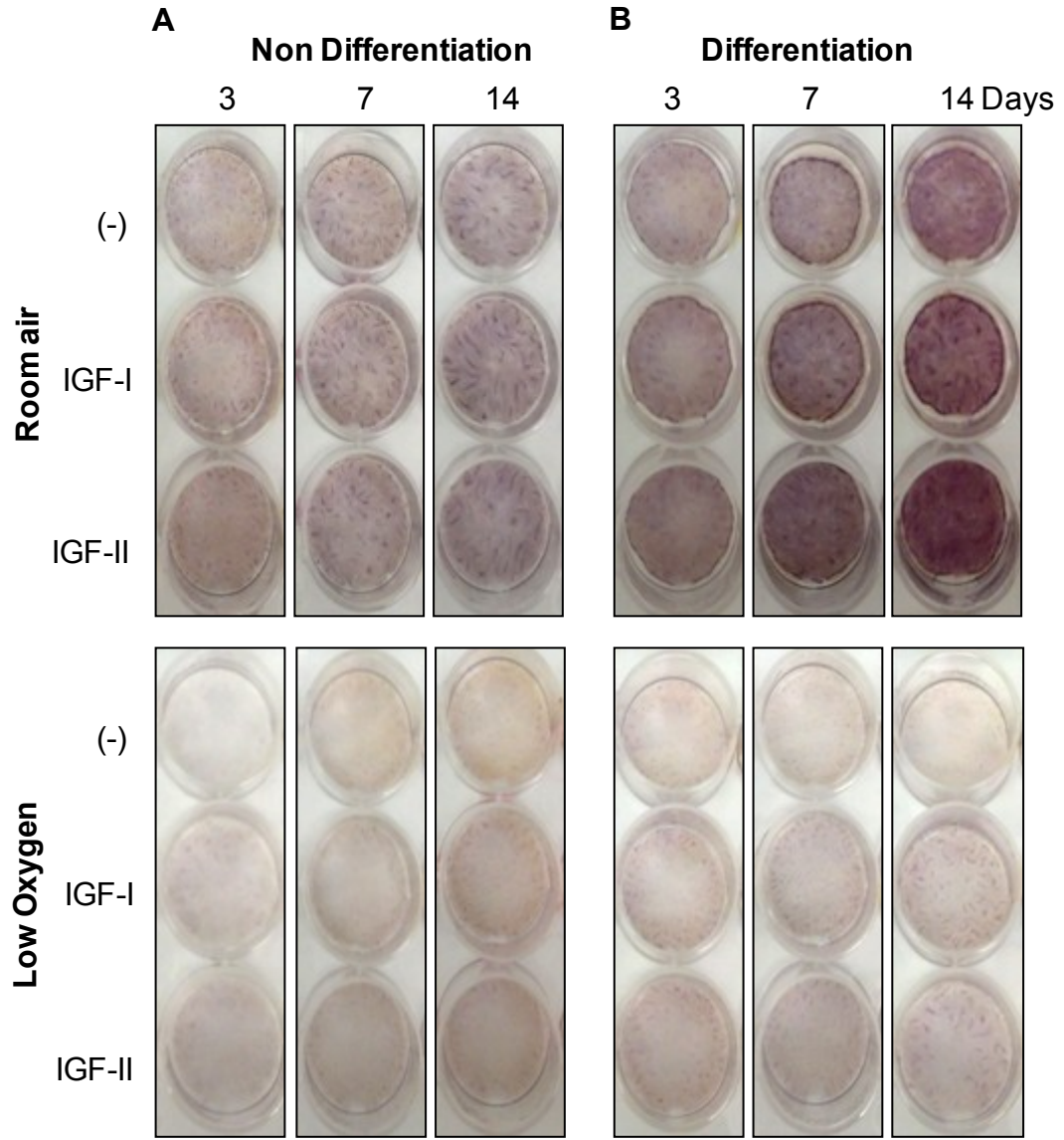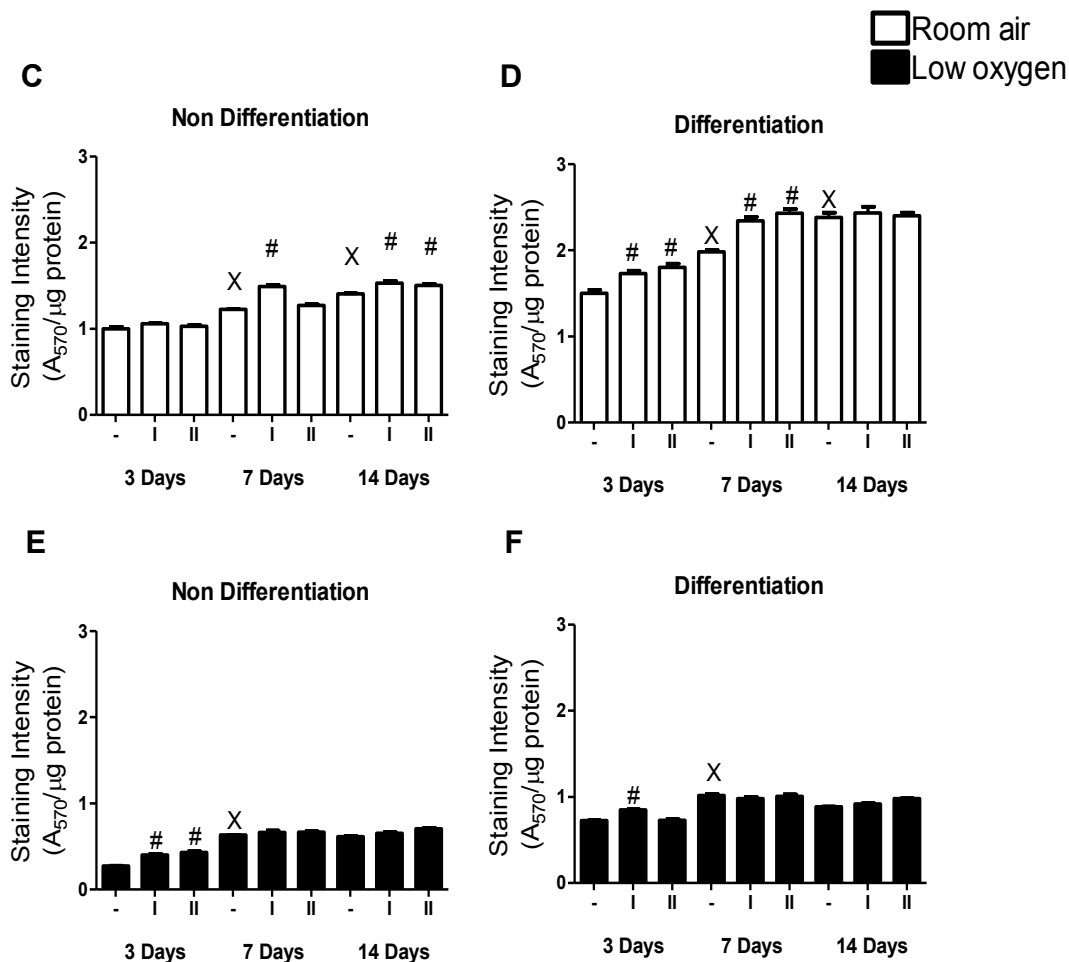
FIGURE 4.5. PMSC Differentiation is regulated by IGFs. PMSCs were cultured in non-differentiation or osteogenic differentiation conditions containing 2% FBS and either 100 ng/mL of IGF-I or IGF-II, in room air or low oxygen levels. Treatments were stopped after 3, 7, and 14 days for **(A-B)** alizarin red staining to confirm PMSC differentiation morphology changes into osteogenic lineage and quantified in **(C-F)**. (Two-Way ANOVA, P<0.05, n=4, X = significant difference between different days of the same IGF condition, # = significant difference by IGF at the same day compared with 0 ng/mL IGF).

as confirmed by the increase in alizarin red staining (Fig. 4.5D). In low oxygen tension, IGF-I and IGF-II enhanced cell condensation (Fig. 4.5B), but did not increase alizarin red staining (Fig. 4.5F). Interestingly, IGF-I or IGF-II in room air enhanced alizarin red staining intensity starting at day 3 and reached maximum by day 7, whereas control (IGF free conditions) required 14 days to reach the same level (Fig. 4.5D). Therefore, IGF-I and -II enhance differentiation at an earlier time point in room air.

At the molecular level, low oxygen tension inhibited differentiation as shown by OCT4 and SOX2 levels (Fig. 4.6A and B). SOX2 was reduced upon differentiation in room air only (Fig. 4.6A and B). The osteogenic initiation transcription factor RUNX2 was increased in differentiation in room air but not in low oxygen tension (Fig. 4.6A and B). OPN, used as a later marker of differentiation for matrix deposition, increased by day 14 only in room air in differentiation conditions (Fig. 4.6A and B). Therefore, differentiation in room air is required to reduce SOX2, and increase RUNX2 and OPN upon PMSC differentiation.

In osteogenic differentiation, IGFs increased OCT4 levels at day 3 and day 7 in room air with no effect was observed at day 14. When differentiation occurred in low oxygen tension, IGFs induced higher levels of OCT4 at all time points (Fig.
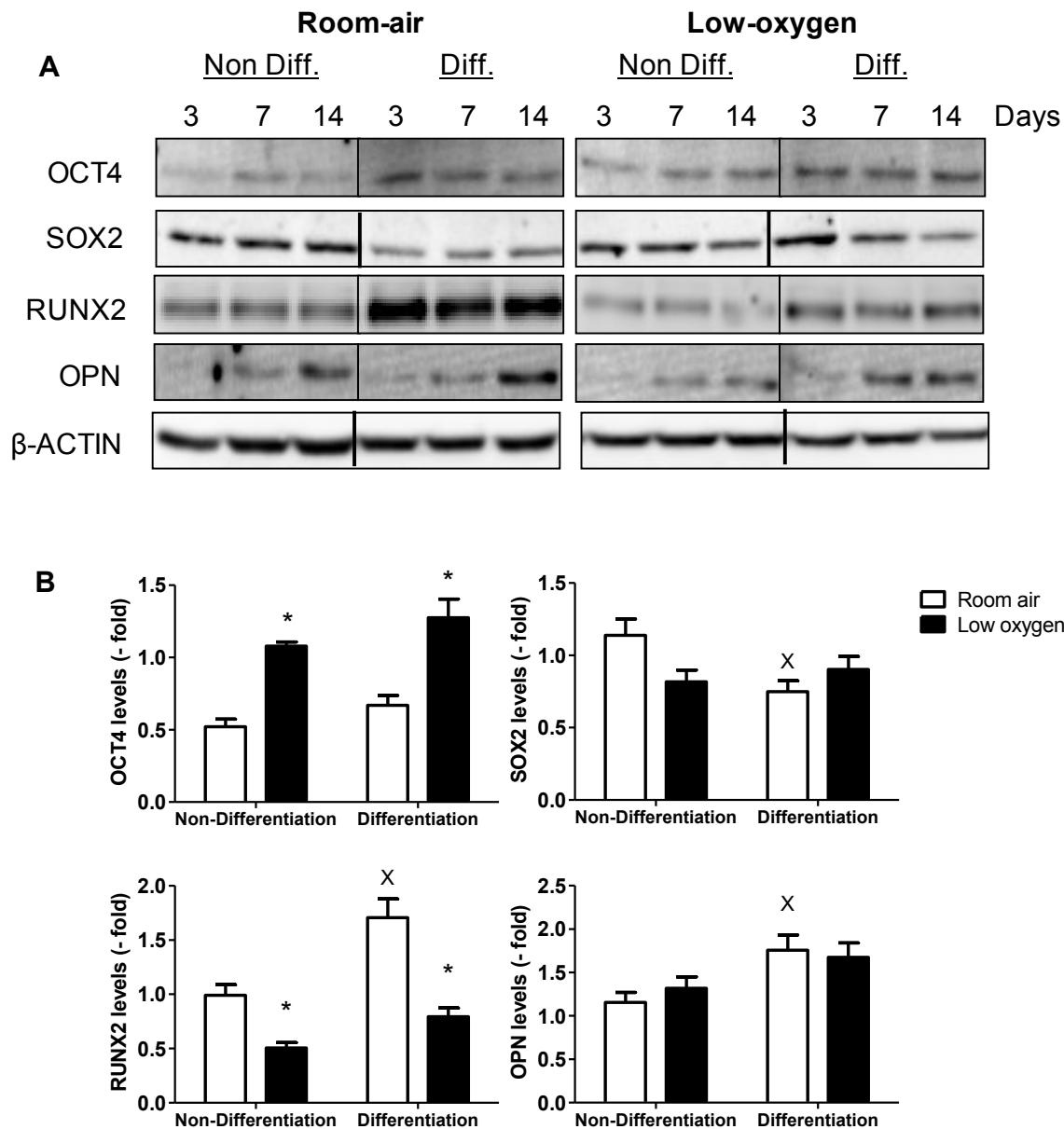
FIGURE 4.6. PMSC differentiation is inhibited by low oxygen tension. Similarly to Fig. 4.5, PMSCs were cultured for 14-days in non-differentiation or osteogenic differentiation conditions containing 2% FBS reduced from 15% FBS as shown in Fig. 4.2, in room air or low oxygen levels. Treatments were stopped after 3, 7, and 14 days. Immunoblotting was used to detect levels of **(A)** OCT4, SOX2, RUNX2, and OPN over the three stop days. **(B)** Quantification levels are shown from day 14 time point for OCT4, SOX2, RUNX2 and OPN, normalized to β-Actin, a protein loading control. (Two-Way ANOVA, $P < 0.05$, n=3, * = significant difference between room air and low oxygen tension, X = significant difference by differentiation condition).

4.7A). Under room air, SOX2 was increased by IGF-I treatment at day 3 but showed no significant changes following IGF treatment at day 7 or day 14. Under low oxygen tension, SOX2 was reduced by IGF-I at day 3 and by IGF-II at day 3 and 7 but with no effect at day 14 (Fig. 4.7B). RUNX2 was increased by IGF-I and IGF-II at the initial stages of differentiation (day 3), and maintained at day 14 (Fig. 4.7C), and low oxygen tension negated this effect. Changes in OPN were similar to RUNX2 (Fig. 4.7D). Therefore, IGF-I can enhance the PMSC differentiation towards the osteogenic lineage, more than IGF-II. This differentiation effect was accelerated by inducing an earlier increase in osteogenic differentiation markers (RUNX2 and OPN) with a reduction in pluripotency markers (SOX2).

4.3.4. Effect of insulin-like growth factors on the role of IGF-IR and IR on PMSC osteogenic differentiation in low oxygen tension

IGF-I and IGF-II signaling is mediated via the interaction with the IGF-IR or the IR to promote proliferation and differentiation. In room air, IR is increased in differentiating PMSCs and upregulated by IGF-I; IGF-IR was not affected by IGFs (Fig. 4.8A and C). In low oxygen tension, the IGF-IR was increased by differentiation conditions and reduced by IGFs. No change of the IR was observed in presence of IGFs in low oxygen tension (Fig. 4.8A and B).
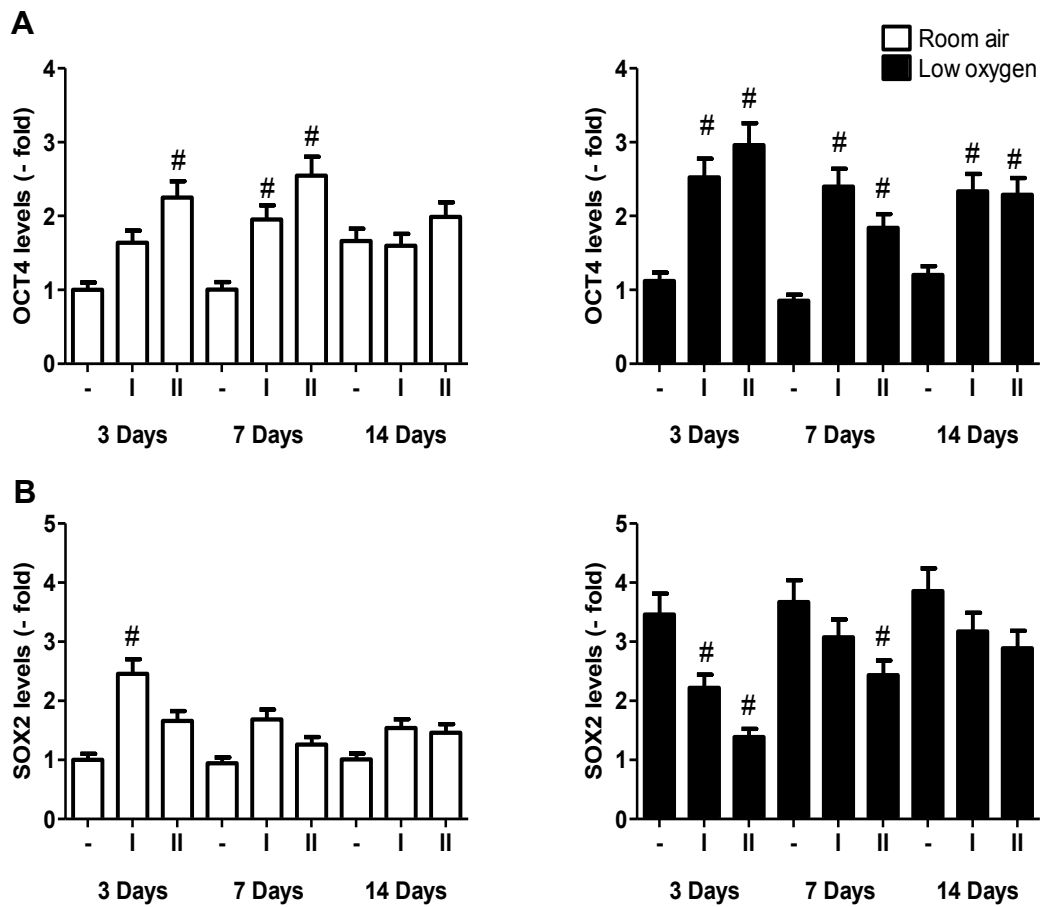
FIGURE 4.7. PMSC multipotency and differentiation is regulated under low oxygen tension by IGFs. Similarly to Fig. 4.6, PMSCs were cultured for 14-days in non-differentiation or osteogenic differentiation conditions containing 2% FBS containing 100 ng/mL of IGF-I or IGF-II in room air or low oxygen levels. Treatments were stopped after 3, 7, and 14 days. Immunoblotting was used to detect levels of **(A)** OCT4, **(B)** SOX2, **(C)** RUNX2, and **(D)** OPN changes induced by IGFs over the three stop days. Quantification levels were normalized to β-ACTIN, a protein loading control. (Two-Way ANOVA, $P<0.05$, n=3, * = significant difference by time in no IGF conditions, X = significant difference by IGF-I or IGF-II in differentiation conditions).

To specify the role of downstream signaling of ERK1/2 and AKT in mediating PMSC differentiation, U0126 and LY294002 were used to inhibit the signaling via the MEK1/2 and PI3K, respectively. In non-differentiation conditions, the addition of U0126 and LY294002 every 48 hours reduced phosphorylation of ERK1/2 (Fig. S4.2A) and AKT (Fig. S4.2B), respectively. After 14 days, the alizarin red staining was reduced by LY294002 more than with U0126 (Fig. 4.9A and B). Also, after 14 days of differentiation, LY294002 increased p-ERK1/2 levels in low oxygen tension (Fig. S4.3A). AKT phosphorylation was inhibited by LY294002, and even more in differentiation condition in low oxygen tension conditions (Fig. S4.3B). Incubation with LY294002 or U0126 for 14 days did not change PMSC viability (Fig. S4.4); however, differentiation was inhibited (Fig. 10). U0126 increased RUNX2 under differentiation conditions in low oxygen tension, while OPN was decreased in room air (Fig. 10C and D). LY294002 reduced OCT4, SOX2, RUNX2 and OPN in differentiation conditions but more in low oxygen tension (Fig. 10A - D). Therefore, PI3K signaling is required for PMSCs to maintain multipotency in non-differentiation condition and to mediate differentiation in osteogenic differentiation conditions. MEK1/2 inhibited RUNX2 levels in low oxygen tension. Therefore, both signaling pathways are important for PMSC differentiation depending on oxygen tension, with PI3K signaling being of greater importance to mediate PMSC differentiation towards the osteogenic lineage.
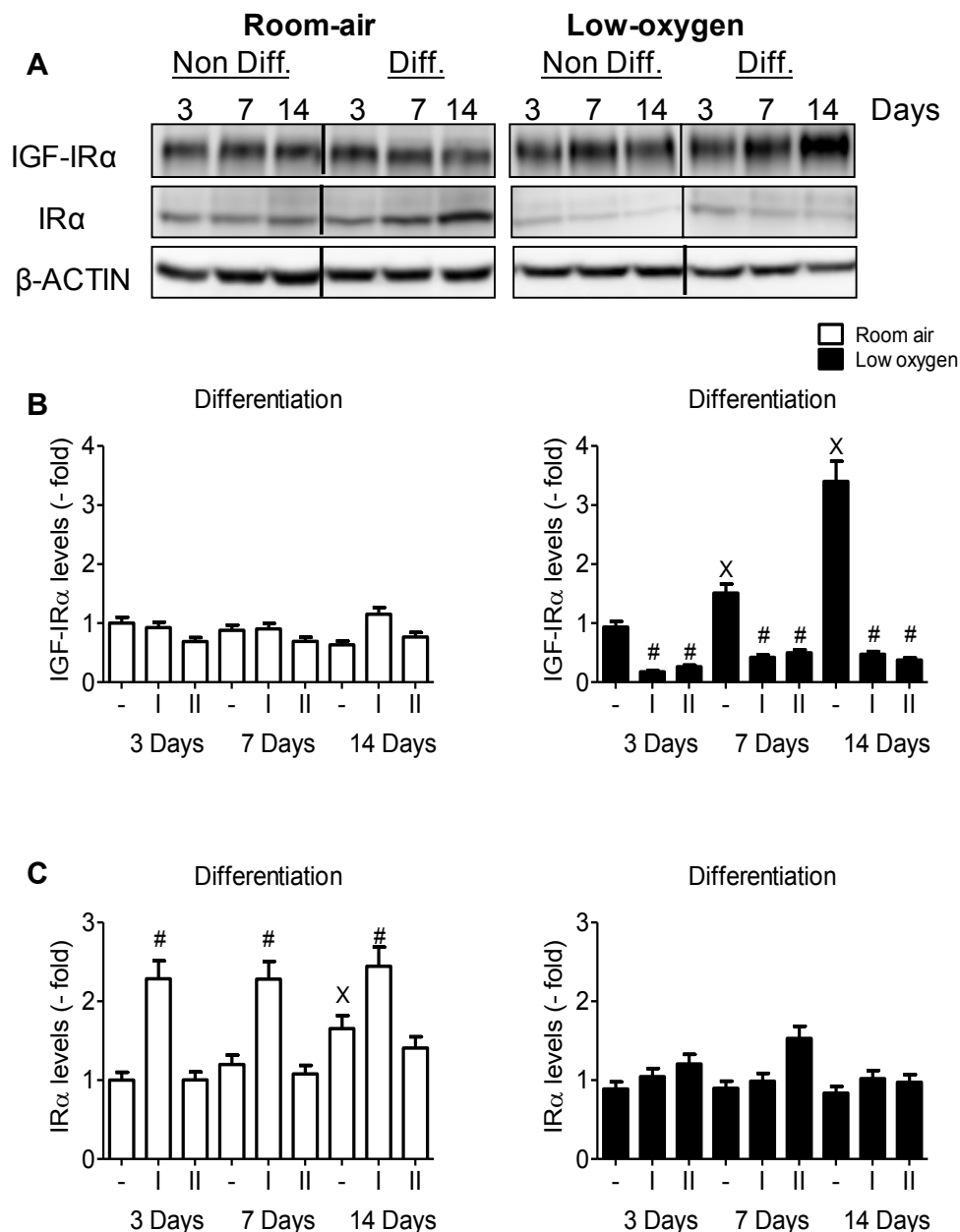
FIGURE 4.8. IGF-IR and IR levels in differentiating PMSCS is regulated by oxygen tension and IGFs. PMSCs were cultured for 14-days in non-differentiation or osteogenic differentiation conditions containing 2% FBS with 100 ng/mL of IGF-I or IGF-II in room air or low oxygen levels. Treatments were stopped after 3, 7, and 14 days. Immunoblots were used to detect levels of **(A)** IGF-IR and IR in absence of IGFs over time. Quantification of IGF effects on **(B)** IGF-IR and **(C)** IR over the three days is shown. Levels were normalized to β-ACctin, a protein loading control. (Two-Way ANOVA, $P < 0.05$, n=3, X = significant difference by time in no IGF conditions, # = significant difference by IGF-I or IGF-II in differentiation conditions).

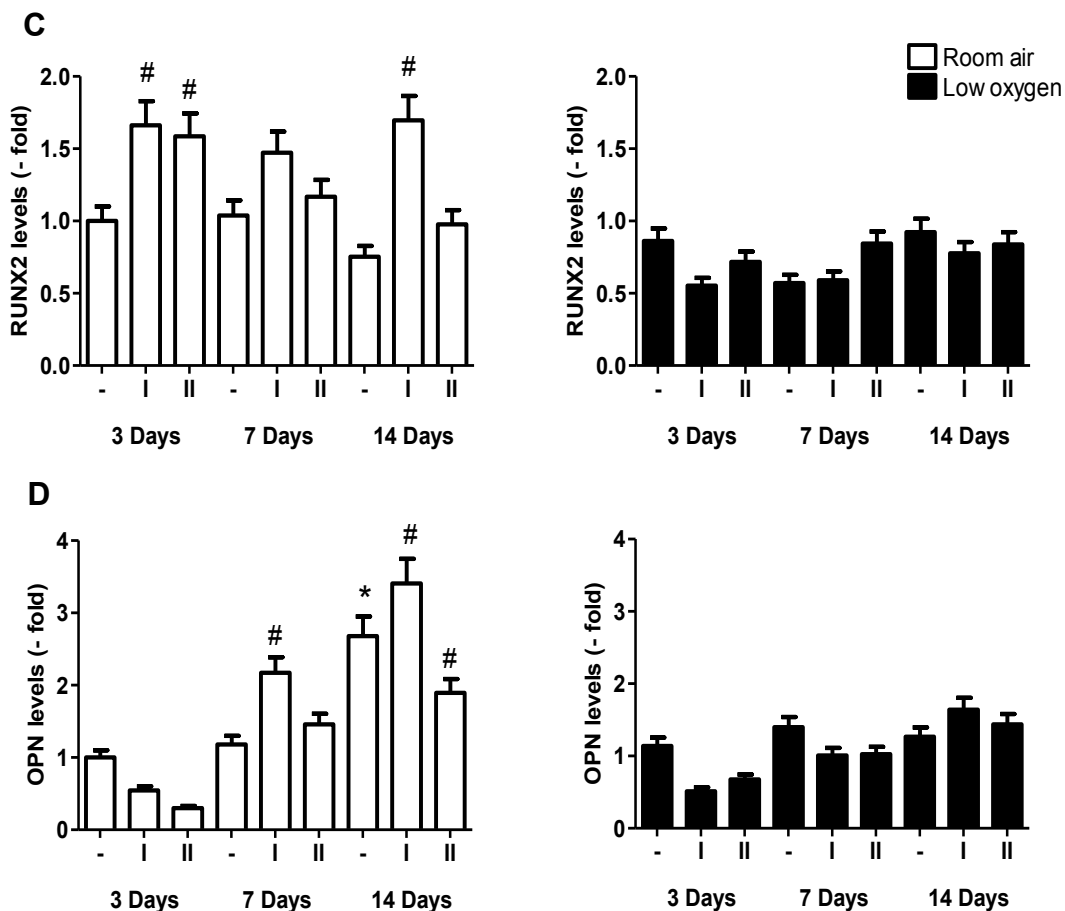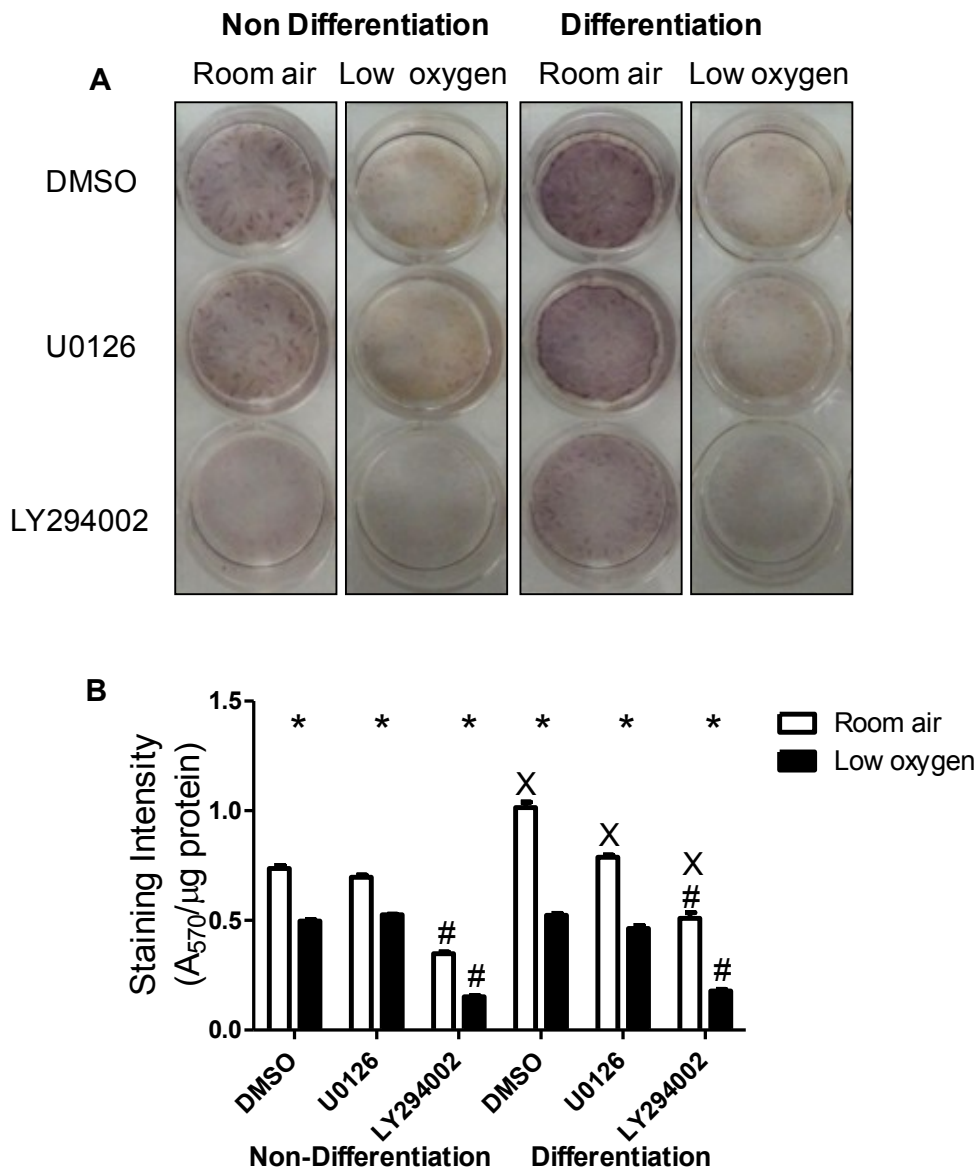**FIGURE 4.9. PMSC differentiation is mediated via MEK1/2 and PI3K signaling under low oxygen tension.** PMSCs were cultured for 14-days in non-differentiation or osteogenic differentiation conditions containing 2% FBS in room air or low oxygen levels. During the 14 days, a continuous exposure to (5 µM) U0126 or (10 µM) LY294002 containing media was changed every 48-hrs. Treatments were stopped at 14 days and stained with alizarin red to confirm **(A)** PMSC differentiation morphology changes into osteogenic lineage and quantified in **(B)**. (Two-Way ANOVA, $P<0.05$, n=4, * = significant difference between room air and low oxygen tension, X = significant difference between differentiation and non-differentiation conditions, # = significant difference by inhibitor as compared to DMSO).

4.4.   DISCUSSION

For the successful use of stem cells for cell based therapies, optimization of stem cell survival and potency *in vitro* and preventing cell death *in vivo* after injection are major considerations (41). In addition, differentiation towards a progenitor cell population without residual undifferentiated cells is required (42). In this study, we used low oxygen tension and IGFs to manipulate the PMSC microenvironment to understand their interactions to improve PMSC differentiation towards the osteogenic lineage. We found that low oxygen tension delayed or inhibited PMSC differentiation and reduced the effects of IGF-induced earlier osteogenic differentiation. *In vitro*, osteogenic differentiation follows a three phase process: a differentiation phase (days 0 – 5), a matrix formation phase (days 5 – 12) and a mineralization phase (days 12 – 19) (27). In this study, we investigated the role of IGFs in combination of low oxygen tension in the commitment and matrix formation phases of PMSC differentiation (days 0 – 14). We demonstrated that RUNX2 is expressed in early differentiation where low oxygen tension impeded its increased levels. OPN, as a late osteogenic differentiation marker, was also reduced by low oxygen tension. IGFs enhanced PMSC differentiation only in room air (a greater effect of IGF-I than IGF-II) where low oxygen tension abolished this process. Similarly, this has been shown previously that IGF-I increases the early differentiation marker RUNX2, and the late markers Type-I collagen and alkaline phosphatase (35).
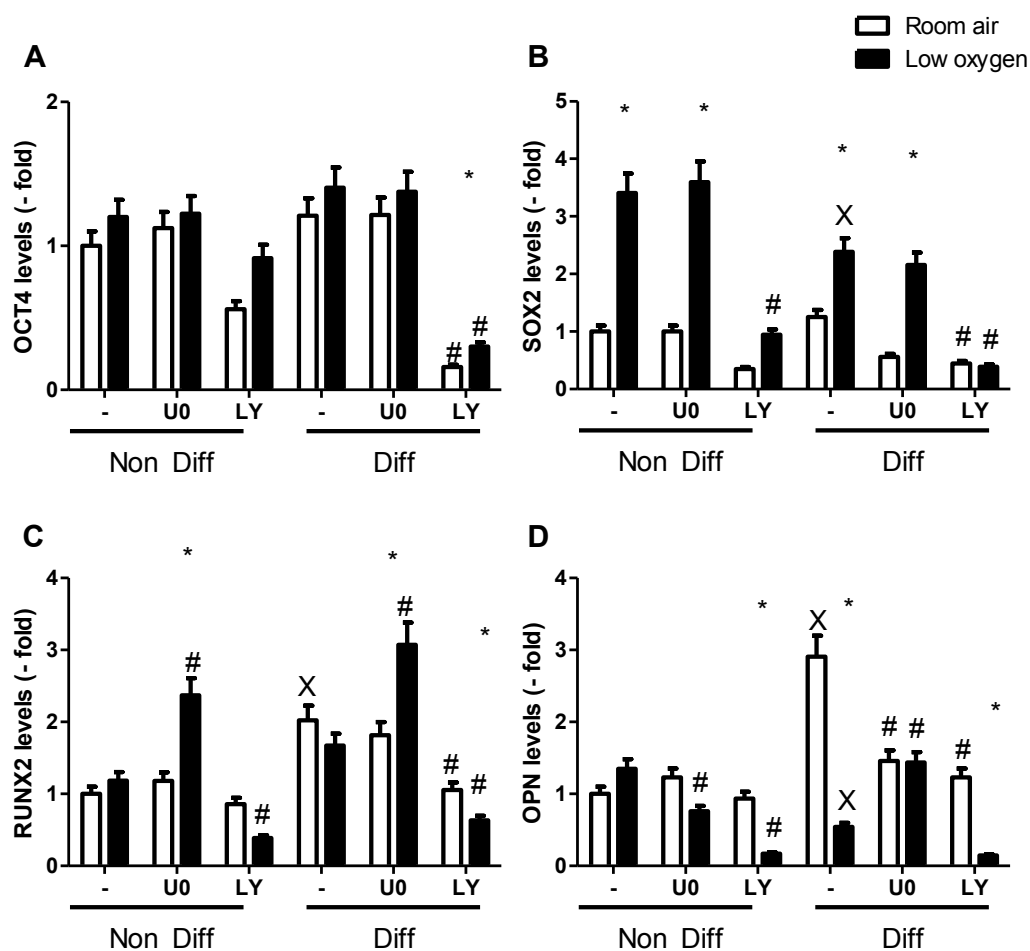
FIGURE 4.10. PMSC multipotency and differentiation is regulated by MEK1/2 and PI3K and is dependent on low oxygen tension. PMSCs were cultured for 14-days in non-differentiation or osteogenic differentiation conditions containing 2% FBS in room air or low oxygen levels. During the 14 days, a continuous exposure to (5 µM) U0126 or (10 µM) LY294002 containing media was changed every 48-hrs. Treatments were stopped at 14 days and immunoblots were used to detect levels of **(A)** OCT4, **(B)** SOX2, **(C)** RUNX2, and **(D)** OPN changes induced by signaling inhibition. Quantification levels were normalized to β-Actin, a protein loading control. (Two-Way ANOVA, P<0.05, n=3, * = significant difference between room air and low oxygen tension, X = significant difference between differentiation and non-differentiation conditions, # = significant difference by inhibitor as compared to DMSO).

The role of the transcriptional network of OCT4, SOX2 and NANOG in maintaining pluripotency and suppressing differentiation was elucidated recently in mouse and human embryonic stem cells. In mESCs, mesodermal lineage specification is determined by OCT4 and SOX2 expression levels: a balanced expression maintains pluripotency, upregulation of OCT4 relative to SOX2 induces mesendodermal lineage specification, and downregulation of OCT4 relative to SOX2 induces neural ectodermal lineage specification (43, 44). In human ESCs, OCT4 maintains an embryonic stem cells state and represses extraembryonic differentiation, while SOX2 is required to suppress the differentiation towards the mesendodermal and NANOG suppresses the ectodermal linage (45). In PMSCs, osteogenic differentiation increased the levels of OCT4 and decreased the levels of SOX2, as would be expected in a mesodermal lineage differentiation. Low oxygen tension increased both OCT4 and SOX2 levels, thereby maintaining a more multipotent state even under differentiation conditions (46). Recently, OCT4 was shown to be required for *in vivo* and *in vitro* differentiation processes as it participates in the downregulation of the pluripotency program and that OCT4 deficient cells are unable to differentiate (47). In this study, we observed that differentiating PMSCs increased OCT4 levels prior to differentiation.

Oxygen tension is increasingly recognized as an important factor of the stem cell niche for proliferation, migration, metabolism and differentiation. In bone fractures, oxygen level can go as low as 0.1% (~0.76 mm Hg) (27, 48). Also,

oxygen tension at the fracture site of the femoral head can be as low as 17.3-19.9 mm Hg, and even lower, at 1 cm away from fracture site (12.5-12.8 mm Hg) (49). Therefore, preconditioning of PMSCs in low oxygen tension, before application into bone fracture, can improve survival of PMSCs and therefore healing of the tissue in the injury site of scarce oxygen tension just as hypothesized previously (25). This was confirmed as reported previously that low oxygen tension preconditioning of MSCs enhances successful engraftment to infarcted myocardium, reduced cell death and increased angiogenesis (50).

The inhibition of differentiation by low oxygen tension could be due to the metabolic switch requirement for osteogenic differentiation, which relies on the upregulation of mitochondrial function and aerobic respiration seen in room air (28). Low oxygen tension can hinder this process by reducing energy production (ATP) by glycolysis alone, which is not sufficient for the high energy demanding differentiation processes required for matrix deposition and mineralization. In our study, we observed that re-exposure of differentiating PMSCs to higher oxygen tension in room air led to a more robust PMSC differentiation.

The role of the IGF-IR in osteoblast differentiation (36) and in *in vivo* bone development (37, 51) is well recognized. However, the role of the insulin receptor is unknown. The IR has two isoforms IR-A and IR-B and it was demonstrated that IR-B is abundantly expressed in differentiating MSCs and mature osteoblasts

than IR-A which was abundantly expressed in proliferating cells (52). In this study, we showed that the IR might play a role in the differentiation process of PMSCs opposite to the expression of IGF-IR. This finding suggests that differentiating osteoblasts expresses higher IR levels to accommodate the higher energy demand in differentiating condition and utilizing more glucose for metabolism, especially via IR-B signaling which is enhanced by IGF-I.

It was shown previously that signaling through IGF-IR in MSCs during differentiation is regulated by PI3K/AKT, and not MAPK, signaling (in a positive feedback loop) to inhibit apoptosis of osteoblasts (51). In this study, we showed that the inhibition of PI3K caused a significant reduction in pluripotency markers (OCT4 and SOX2) and osteogenic markers (RUNX2 and OPN), suggesting that PI3K is required not only to maintain multipotency but also to regulate PMSC differentiation. Interestingly, MEK/ERK signaling may also play a role in low oxygen tension by reducing the levels of RUNX2 and OPN as they were elevated by inhibiting MEK1/2, suggesting a possible role for MEK1/2 to suppress osteogenic differentiation.

In conclusion, PMSCs successfully differentiate towards the osteogenic lineage, as an alternative source for MSC other than the more popular bone marrow MSC for tissue regeneration therapy. PMSC preconditioning in low oxygen tension and the use of IGFs (mainly IGF-I) to stimulate PMSCs is promising strategy to

generate osteogenic progenitor cells for tissue regeneration therapy for bone deficiencies. However, whether this strategy will enhance successful engraftment for the regenerative therapy in OI or bone fractures will require studying in animal models.

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Appendix 3
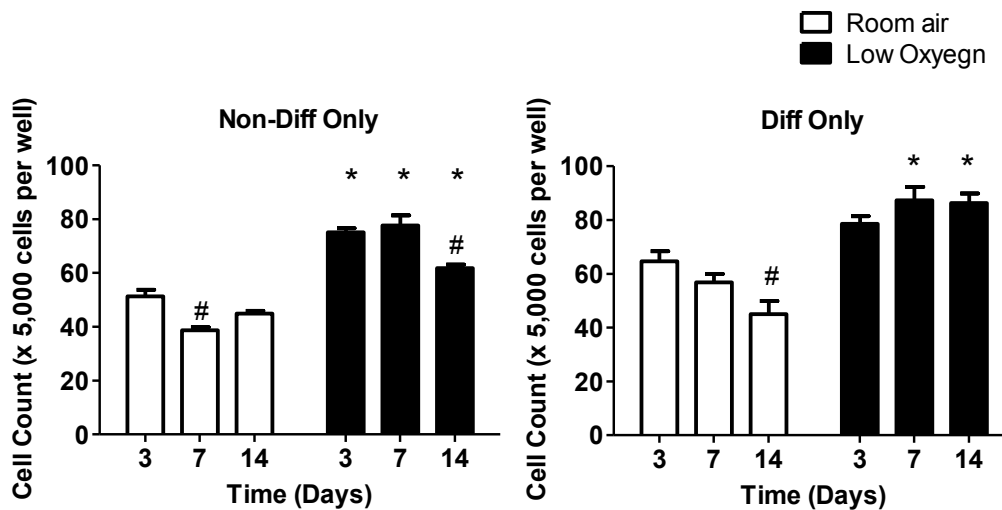
FIGURE S4.1. PMSC proliferation is more active in low oxygen tension regardless of the differentiation conditions. PMSCs were cultured for in non-differentiation or osteogenic differentiation conditions containing 2% FBS in room air or low oxygen levels. Treatments were stopped after 3, 7, and 14 days for cell counting using a hemocytometer. (Two-Way ANOVA, $P<0.05$, n=6, * = significant difference between room air and low oxygen tension, # = significant difference between day 3 and day 7 and 14).

Appendix 3
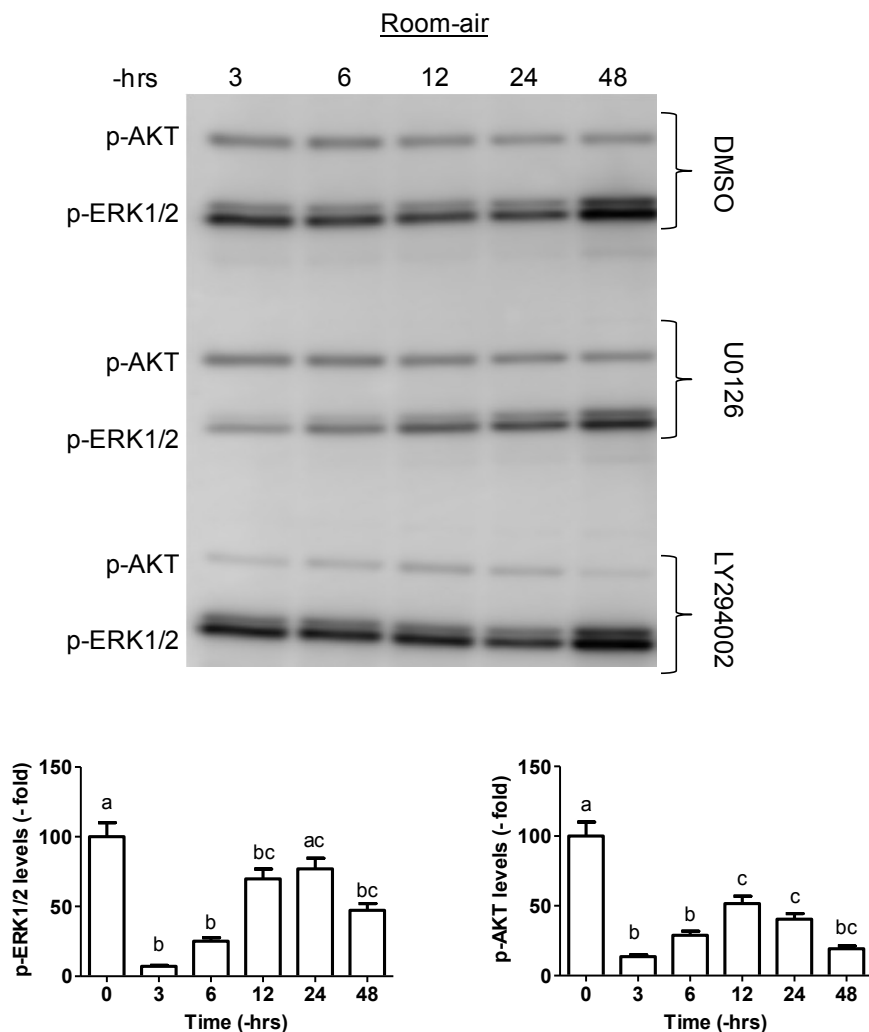
FIGURE S4.2. Inhibition of MEK1/2 and PI3K inhibits the phosphorylation of ERK1/2 and AKT. PMSCs were treated over 48-hrs period the time between media changes during a regular PMSC differentiation course. Treatment was stopped after 3, 6, 12, 24, and 48 hrs after inhibition by adding (5 μM) U0126 or (10 μM) LY294002 in presence of 2% FBS. Levels of p-ERK and p-AKT as shown in the immunoblots were normalized to total kinase levels and β-Actin, a protein loading control. (Two-Way ANOVA, P<0.05, n=4, a = significant difference by the presence of the inhibitor over time).

Appendix 3
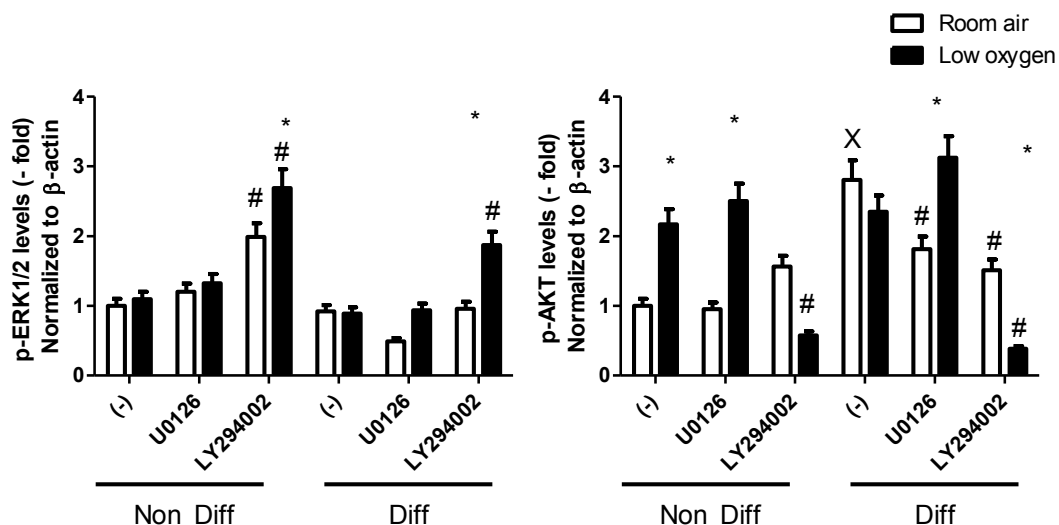
FIGURE S4.3. Inhibition of MEK1/2 and PI3K affects ERK1/2 and AKT signaling in PMSCs. PMSCs were cultured for 14-days in non-differentiation or osteogenic differentiation conditions containing 2% FBS in room air or low oxygen levels. During the 14 days, a continuous exposure to (5 µM) U0126 or (10 µM) LY294002 containing media was changed every 48-hrs. Treatments were stopped at 14 days and immunoblots were used to detect levels of ERK1/2 and AKT changes induced by signaling inhibition. Quantification levels were normalized to total kinase level and β-Actin, a protein loading control. (Two-Way ANOVA, P<0.05, n=3, * = significant difference between room air and low oxygen tension, # = significant difference by adding the inhibitor, X = significant difference between differentiation and non-differentiation conditions).

CHAPTER FIVE

DISCUSSION

5.1. Summary and Perspectives

This study provides an insight into the understanding PMSC fate determination by IGFs and low oxygen tension. We found that low oxygen tension, a physiologic oxygen tension in stem cell niches *in vivo*, can regulate PMSC fate (multipotency or differentiation) in the presence of IGFs. First, low oxygen tension was found to enhance the IGF-I facilitated proliferation and multipotency response depending on PMSC gestational age (Chapter One). Second, low oxygen tension was found to enhance IGF-II expression and maintain PMSC multipotency by signaling *via* both the IGF-IR and the IR (Chapter Two). Lastly, low oxygen tension was found to inhibit the IGF-I stimulated osteogenic differentiation and maintain PMSC in a higher stemness state (Chapter Three).

In this study, maintaining stem cells in low oxygen tension and improved the stemness possibly by mimicking the *in vivo* conditions. The manipulation of the IGF receptors (IGF-IR, IR and HR) signaling by IGFs in a concentration and time-dependent manner was important to understand the stem cell behaviour. IGF-II, more than IGF-I, maintained a higher multipotency state in low oxygen tension, whereas IGF-I, more than IGF-II, improved the differentiated state of PMSCs in higher oxygen tension (room air). Also, low oxygen tension maintained a higher multipotent state even in differentiation-promoting culture conditions. Therefore, these results have implications for (1) defining the placental microenvironment during development that maintains PMSCs *in vivo* in a multipotent state; and (2)

identifying the molecular mechanisms underlying these microenvironmental changes *in vitro* in PMSCs to maintain multipotency or differentiation. We demonstrated in this study that during placental development, low oxygen tension, gestational age and IGF-axis signaling, can define the *in vivo* conditions that determine stem cell fate (multipotency or differentiation).

5.1.1. The microenvironment is a major determinant of stem cell fate changes *in vivo* and *in vitro*

5.1.1.1. Gestational Age

Placental mesenchymal stem cells (PMSCs) used in this study were isolated from chorionic villi of preterm (11–13 wks) and late third trimester (37–39 wks) human placentae. *In vivo*, during chorionic villi formation, fetal mesenchyme migrates into primary villi at early gestation between 5th and 7th weeks, until the 12th week of gestation when terminal villi are formed, and become the majority of villous type at term gestation (1, 2). Chorionic villi have the highest abundance of mesenchymal cells (undifferentiated and differentiated) that decreases with gestation; they are still found in term placentae but only 1% of total tissue by volume (3). This was described as a thinning that occurs to the stromal layer, hence a decreased number of mesenchymal tissue cell populations per section of chorionic villi (4). Placental gestational age can therefore be a determining factor in the abundance of PMSC isolated and colony formation capacity as they originate from the mesenchymal stromal layer of the chorionic villi. As

demonstrated by increased expression of stem cell markers, the higher proliferation capacity and earlier osteogenic differentiation changes, preterm PMSCs may have different characteristics and a more primitive state than term PMSCs. Although, both populations of PMSCs can proliferate in response to IGF-I and low oxygen tension and can differentiate into all lineages obtained by PMSCs. This can be naturally occurring *in vivo* by the changing microenvironment (gradual increase in oxygen tension and growth factor levels) of the chorionic villi with gestation to program stem cells; therefore PMSCs show distinct response to the same stimulation condition of IGFs and low oxygen tension *in vitro*. Thus, advancing in gestation can lead to some of the differences in the PMSC multipotency state and differentiation potential that should be considered when isolating PMSCs from the placenta.

Low oxygen tension was shown to narrow the differences between the two types of PMSCs that were seen in room air of which term PMSCs could regain higher stemness and an increased proliferation level comparable to preterm PMSC. Hence, maintaining PMSCs in low oxygen tension in self-renewal supportive conditions is permissive to maintain stem cell markers, regardless of developmental stage of PMSCs. Also, preterm PMSCs were more responsive to IGF-I and IGF-II stimulation than term PMSCs. However, as shown previously, first trimester placenta provides a valuable source of MSCs which have greater expansion potential than adult bone marrow MSCs and are an excellent source for therapeutic application (5). Likewise, term human placenta is an abundant

source of chorionic villi MSCs and to have a high therapeutic potential as these cells can differentiate into the three germ layer cells (6). However, this is the first study to put into perspective the comparison of preterm and term PMSC multipotency control. Overall, the human placenta at different gestation is an excellent source of MSCs that may have different capacities due to the different microenvironment *in vivo*.

5.1.1.2. Low Oxygen Tension

The changing extracellular microenvironment is a major factor to regulate stem cell proliferation and differentiation in many developing tissues, including the placenta. Oxygen tension doubles between the first and second trimesters from about 18 mm Hg to 40 mm Hg, and then gradually increases to approximately 60 mm Hg and maintained up to the third trimester (7, 8). This oxygen gradient is created starting at the first trimester of pregnancy (higher at the endometrium compared with fetal stromal layer) by the undeveloped fetoplacental circulation, resulting in low oxygen tension conditions, which is necessary for early development (9). In fact, blood flow from the spiral arteries into the intervillous space is prevented by the endovascular trophoblasts until the 12th week of gestation, to protect the early embryo from the excessively high oxygen levels (10). Therefore, preterm PMSCs naturally reside in low oxygen tension conditions compared to the later gestation PMSCs, and hence may adapt better to low oxygen tension conditions *in vitro* than term PMSCs.

The hypoxia inducible factor (HIF) system is activated more in early pregnancy. It is an upstream activator of VEGF, which is highly expressed in early gestation and decreased by third trimester, a stage when the placenta is well vascularized and less hypoxic (9). HIF-1α and HIF-2α expression are increased during the first trimester, peaking between 7-10 wks of gestation and declining thereafter with development having the lowest levels at term gestation (11). This HIF signaling initiates the development of the fetoplacental circulation by increasing proliferation of trophoblast cells and stromal mesenchymal cells and forming new capillaries, in the oxygen deprived stromal layer. This is triggered by growth factors, such as b-FGF, VEGF and PlGF (12). In this microenvironment, IGF-II and not IGF-I, is a major signaling factor targeted directly by HIF-1 regulation to induce an increased transcription (13, 14), leading to increased IGF-II levels in early gestation. In turn, IGF-I and IGF-II stimulation can stabilize HIF-1α and HIF-2α (11). Therefore, low oxygen tension and IGFs may have a crosstalk with a positive feedback mechanism. In our PMSCs, low oxygen tension upregulated IGF-II expression at the mRNA level even in presence of IGF-I or IGF-II, mirroring the *in vivo* microenvironment of the placenta.

5.1.1.3. Insulin-like Growth Factor System

The PMSCs we studied are derived from chorionic villi and they reside in the perivascular region of the fetal compartment where the exchange of nutrients,

oxygen and waste between the mother and the fetus. Therefore, they are in contact with factors from both the fetal and maternal circulations, and hormones and factors synthesized by the syncytiotrophoblasts, cytotrophoblasts and the chorionic mesoderm. IGF-I and IGF-II are synthesized in the placenta by the chorionic mesoderm (where PMSCs reside), where IGF levels do not relatively change during gestation across the three trimesters (15). In the fetus, IGFs are one of the most abundant growth factors in the circulation that are just downstream signaling of the GH axis at the level of organs, tissues and cells (16). In the placenta, a pituitary GH variant, the placental lactogen/chorionic somatomammotropin, functions in a paracrine fashion to upregulate IGF expression and sustain an increased growth in the placenta (17). In the fetus, the liver is the major source of circulating IGFs, whereas in the placenta, as in many extra-hepatic tissues, IGFs are expressed in a paracrine/autocrine fashion regulated in part by placental GH (16, 18). It has been shown that IGF-I and IGF-II paracrine/autocrine signaling were required for first trimester placental primary fibroblast increased survival, proliferation, and migration and decreased apoptosis (19). Our PMSCs express their own IGFs, both IGF-I and IGF-II, which promoted localized paracrine/autocrine cellular growth as was shown in serum free growth conditions.

The ability of IGF-I and IGF-II to maintain PMSC multipotency or differentiation

The importance of IGF function in the human placenta stems from their role in mediating increased growth, reduced apoptosis, and induced differentiation of the different cells of the chorionic villi (20, 21). Placental development is dependent on IGF and IGF receptor expression, however, the relative role of IGF-I and IGF-II can vary depending on spatial and temporal expression during pregnancy (22). The cord blood IGF-I concentration, and not IGF-II, is correlated with birth weight fetal growth restriction in humans, whereas the placenta is more correlated with IGF-II (23). This is similar to the mouse in which *Igf2* knockout severely reduced placental growth (24). In the human placenta, IGF-II mRNA is expressed in the villous mesenchymal core, where PMSCs reside, at all three trimesters (22). IGF-II plays a role in migration, proliferation, and decrease apoptosis of extravillous- and cyto-trophoblasts in the placenta (11), whereas IGF-I is implicated in later gestation, and is involved in cytotrophoblast differentiation. In PMSCs, we showed that both IGF-I and IGF-II induced a concentration and a time response increased proliferation in low oxygen tension and that IGF-I enhanced spontaneous and osteogenic directed differentiation.

IGF Receptor Expression

In the placenta, both IGF-IR and the IR are expressed mainly in the microvillous membrane of the proliferating cytotrophoblasts in the first trimester, with a switch

to an expression in the basal membrane facing fetal circulation at term gestation (15). However, there is no information on the localization of the IGF-IR or IR in PMSCs in the placenta during different gestational ages. In PMSCs, IGF-IR and IR are highly expressed in preterm PMSCs that decline with gestational age. This higher expression level of both receptors correlates with the higher IGF-II levels expressed at the low oxygen tension conditions at early gestation. The role of the IR in placental growth was suggested by the dual knockout mouse for *Igf2* and *Igf1r* that was more severely growth restricted than *Igf1r* knockout alone, indicating that IGF-II can signal through another receptor to mediate placental growth (24); this can also be the case in human placenta. IR signaling (*via* IR-A) was confirmed to mediate placental growth via IGF-II signaling in early gestation (25); the IGF-IR is expressed abundantly by the middle of the first trimester— becoming of higher affinity to IGFs with advanced gestation (26). This consequently spares the most abundant IGF-II signaling for the IR-A rather than IGF-IR during early gestation. We have observed the dual expression of IGF-IR and IR in PMSCs, which can favour the formation of hybrid receptors IGF-IR/IR that preferentially binds to IGFs rather than insulin. In the human placenta, 55% of the IGF-IR is assembled in the hybrid form to specifically bind IGF-I than insulin (27). IR-A and IGF-IR/IR-A (HR-A) are upregulated in various cancers (breast, thyroid, myosarcoma, osteosarcoma, cervical and prostate) to mediate more aggressive malignant cancer forms (28). Therefore, HR-A can be involved to maintain a more naïve state of cancer stem cell development. A similar biological function may be present in PMSCs *in vivo*.

PMSC fate is likely determined by the differential signaling due to receptor affinity, choice of receptor (IGR-IR, IR, hybrid IGF-IR/IR) and the combination of downstream receptor signaling cascades. At the receptor level, low oxygen tension increased IR-A as well as IGF-IR expression even in presence of high levels of IGFs. IGF-I and IGF-II may utilize different signaling molecules as IGF-IR has greater affinity to IGF-I than IGF-II, and IR-A has greater affinity to IGF-II than IGF-I (29). Therefore, the increased expression of pluripotency triad transcription factors by IGF-II maybe transduced via IR-A. Also, in PMSCs, IR has played a role in increasing proliferation in low oxygen tension in addition to IGF-IR. This could be because IR-A, which unlike IR-B, can induce a mitogenic signal similar to IGF-IR suggesting that the increased abundance of IR or HR-A in low oxygen tension can induce proliferation. Therefore, PMSCs cultured in low oxygen tension can utilize different signaling cascades to enhance PMSC proliferation and multipotency.

IR was also increased in differentiating PMSCs towards the osteogenic lineage in room air but not in low oxygen tension. A recent report showed that IR increases with a higher ratio of IR-B to IR-A in differentiating osteoblasts (30). Unlike IR-A, which mediates mitogenic actions involved in increased cell proliferation, atherosclerosis and cancer, IR-B is responsible for metabolic action to facilitate metabolism, cell differentiation and increased longevity (28). Hence, IR-A is required for stem cell self-renewal while IR-B is required following differentiation. In osteogenesis, a metabolic switch is required for a complete osteogenic

differentiation to upregulate mitochondrial count and cellular aerobic respiration, which is obscured by low oxygen tension (31). Therefore, an upregulation of IR-B may increase insulin signaling for glucose uptake which is required as an energy source in aerobic respiration for increased ATP production.

Kinase Signaling

We demonstrated that ERK1/2 and AKT are important downstream mediators of IGF signaling in stimulating proliferation and multipotency of PMSCs *in vitro* that may be applicable *in vivo*. ERK1/2 is important in mediating cell proliferation and growth, whereas AKT is important in cell survival and metabolism. These two signaling cascades are important for regulating PMSC proliferation and maintaining multipotency. How these two signaling cascades interact in regulating stem cell multipotency at the molecular level has not been delineated. A recent identification of 14 phosphorylation sites on OCT4, away from the DNA binding site, revealed three putative phosphorylation sites for ERK2 at S111, T118, and S355 (32) which may provide a direct link to IGF signaling in stem cells; however, the role of OCT4 phosphorylation is not yet clear. Also, ERK2 has been shown to interact directly with chromatin at the SOX2 promoter regions to maintain its transcription (33); in addition, ELK1 (ERK2 downstream substrate) can interact with the repressive complex polycomb group protein complex 2 (PRC2) to repress differentiation genes and therefore can maintain ESC pluripotency (33). On the other hand, AKT has been shown to interact directly

with EZH2, a component of PRC2, to phosphorylate it on Ser-21 (34). Thus, IGFs can stimulate phosphorylation of EZH2 via AKT pathway and reduce the capacity of PRC2 to trimethylate histone H3K27 in promoter regions of OCT4 and promote the de-repression of the OCT4 promoter transcription and maintain multipotency (unpublished data).

During differentiation, MEK1/2 is important for osteoblast differentiation and skeletal development, as it is required for RUNX2 phosphorylation and transcriptional activity (35, 36). Also, the PI3K plays an important role in osteogenic differentiation (37). However, the role of PI3K and MEK in facilitating and inhibiting MSC differentiation is still controversial (37). In a previous study, PI3K was shown to be essential in mediating osteoblast differentiation of MSCs, while MEK repressed MSC differentiation towards the osteogenic lineage (38). In addition, IGF-I expression in MSCs increased osteogenic differentiation for bone repair, which was mediated via IRS1-PI3K (39). In our PMSCs, both PI3K and MEK were required for osteogenic differentiation in room air, and their inhibition repressed complete differentiation. The role of PI3K was greater than MEK in mediating differentiation in both low oxygen tensions and room air, while in low oxygen tension MEK showed a role in repressing differentiation. Therefore, low oxygen tension can change the role of signaling kinases in mediating PMSC differentiation. However, these signaling differences still need investigation to determine the mechanisms of how PI3K and MEK can mediate the differentiation process of PMSCs in low oxygen tension.

5.1.1.4. PMSC Fate Changes

Low oxygen tension improved the stemness level of PMSCs (increased OCT4, SOX2 and NANOG expression) and increased proliferation. The enhanced stem cell gene expression can be mediated via HIF-2 direct interaction with all three pluripotency genes to increase transcription (40, 41). OCT4, SOX2 and NANOG gene expression was upregulated by longer stimulation time by IGFs but decreased by increased IGF concentration. In our study, we mainly focused on OCT4, the master regulator of pluripotency. OCT4 mutation/loss is embryonic lethal (42), whereas ectopic overexpression causes dedifferentiation in somatic cells (43). In MSCs, ectopic expression of OCT4 increased proliferation and maintained cell morphology during prolonged passaging and inhibited spontaneous differentiation (44). In PMSCs, IGF-I and IGF-II increased OCT4 expression (more with IGF-II), confirming that IGFs can enhance stemness in combination with low oxygen tension.

MSCs differentiate into the mesodermal lineage and also can differentiate towards endodermal and ectodermal lineages; however, this phenomenon of transdifferentiation is still controversial *in vivo* (45). We have found that PMSCs can differentiate towards the mesodermal lineage (adipose, osteogenic, endothelial, myogenic), and ectodermal cells (neuron-like cells expressing Nestin). MSCs transdifferentiation occurs due to MSC heterogeneity, which is referred to a mixture of progenitor cells at different stages of commitment to

multiple lineages (45). *In vivo*, MSC plasticity is needed to adapt to varying stressors and microenvironmental demands to repair tissue damage (46). Therefore, MSCs express genes from at least two germ layers and pluripotency triad genes demonstrating a "multi-differentiation" potential awaiting the fate restriction signal (47). Therefore, differentiation of MSCs is not a simple on/off switch but is regulated through transcription of specific genes depending on the microenvironment. Osteogenic differentiation is a classical mesodermal lineage originated from the MSC in the bone marrow. Therefore, osteogenic differentiation of PMSCs, which does not normally occurs *in vivo*, demonstrates PMSC plasticity.

In the placenta, PMSCs can differentiate into four mesodermal lineage cells: macrophage-like mesenchymal cells, haemangioblastic cords, pericytes and stromal fibroblasts (48), where IGFs and low oxygen tension can play an important role (49, 50). In this study, we show that PMSCs can differentiate into mesodermal osteoblasts expressing RUNX2, osteopontin, and alkaline phosphatase. Moreover, differentiated osteoblasts deposit bone matrix and stain positive for calcium deposition. During this process, room air was a major facilitator of differentiation, while low oxygen tension repressed this effect. Low oxygen tension increased OCT4 and SOX2 and lowered RUNX2 and osteopontin. IGFs facilitated an earlier commitment towards osteogenic differentiation with higher levels of OCT4 and SOX2. However, IGF-I was more potent than IGF-II to enhance osteogenic differentiation.

The ratio between OCT4 and SOX2 is important for mesodermal differentiation with lower SOX2 (51) and higher OCT4 (52, 53). In PMSCs, OCT4 levels did not decrease post differentiation and was enhanced by IGFs; however, SOX2 levels were decreased confirming mesodermal differentiation. In fact, OCT4 overexpression in MSCs enhanced adipogenic and chondrogenic differentiation by increasing differentiation markers and promoting the complete differentiation morphology (44). Therefore, OCT4 might be required for osteogenic differentiation. SOX2 overexpression inhibits osteoblast differentiation by promoting higher proliferation (54). Therefore, the reduction in SOX2 levels in osteogenic differentiation can also confirm PMSC differentiation. The coexpression of RUNX2 and osteopontin is a marker of progenitor osteoblasts formation (55). We found that an increased RUNX2 expression occurs early in osteogenic differentiation, while osteopontin levels were increased by the end of differentiation period confirming osteogenic commitment.

5.2. Overall Conclusions

In this study, we showed that the human placenta is a valuable source of mesenchymal stem cells that can be expanded and differentiated *in vitro* by manipulating the surrounding microenvironment. Low oxygen tension is the major regulator of stemness by enhancing PMSC proliferation and pluripotency gene expression. As summarized in Figure 5.1, although PMSCs from different

gestations respond differently to IGF stimulation, low oxygen tension enhances PMSC proliferation and pluripotency gene expression levels. Low oxygen tension activates ERK1/2 signaling, while IGFs activates AKT signaling of which both are required to maintain PMSC proliferation and multipotency. PMSC fate changes were differently mediated by IGFs: IGF-I induced differentiation and IGF-II maintained higher PMSC stemness. Low oxygen tension decreases spontaneous differentiation and inhibits PMSC osteogenic differentiation but can enhance differentiation if re-exposed to room air. Therefore, to maintain PMSCs in multipotent state, PMSCs from different gestations should be cultured under IGF-II and low oxygen tension conditions, whereas, to differentiate PMSCs to progenitor cells, PMSCs should be cultured under IGF-I and room air conditions.

Overall, mimicking the *in vivo* microenvironment of placental mesenchymal stem cells can improve the *in vitro* culture conditions for tissue regeneration therapy. And also, priming PMSCs by IGFs and preconditioning by low oxygen tension are important steps to improve PMSC growth, multipotency, and differentiation that can improve PMSC survival and differentiation potential for tissue regeneration.
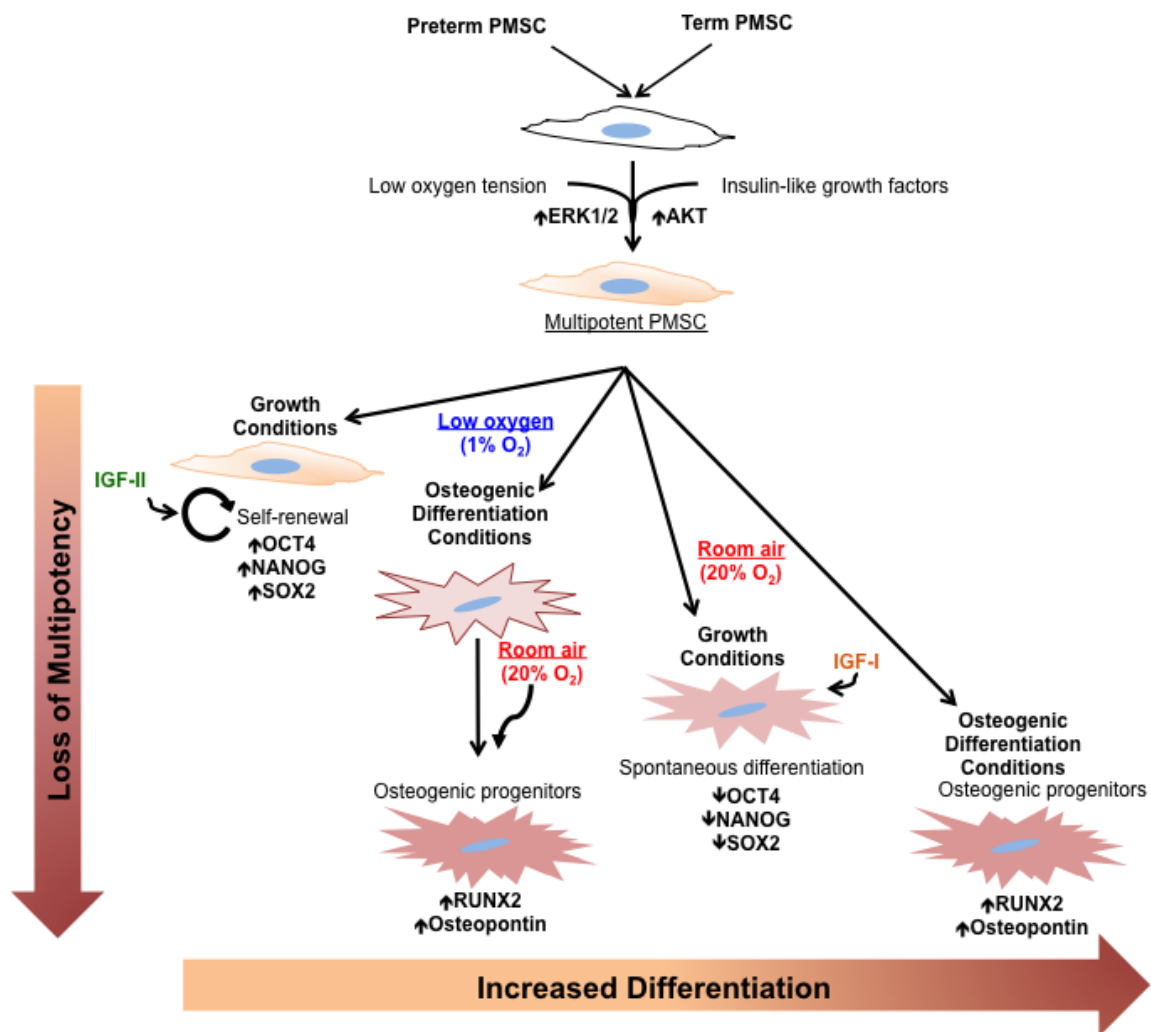
Figure 5.1. stem cell fate in low oxygen tension and insulin-like growth factor conditions. PMSCs are isolated from preterm and term gestations. To maintain PMSCs in a multipotent state, low oxygen tension can activate endogenously the ERK1/2 pathway while IGFs activate the AKT signaling pathways. Both signaling pathways are indispensible for PMSC proliferation and pluripotency gene expression. PMSC spontaneously differentiate towards the osteogenic lineage if grown in room air conditions, whereas, self-renewal is maintained if PMSCs are maintained under low oxygen tension conditions. IGF-I is shown to drive PMSC differentiation, while IGF-II can maintain PMSC multipotency and higher stemness state. Low oxygen tension inhibits PMSC osteogenic differentiation by maintaining higher pluripotency gene levels and lower osteogenic differentiation proteins and transcription factors.

5.3. Limitations and Future Studies

A comparative study of MSCs from chorionic villi of different gestations and umbilical cord blood with bone marrow and embryonic stem cells can improve our understanding of the hierarchy of multipotency of stem cells from different placental sources. Information about IGF-I or -II concentration in the human placenta during different gestational ages is lacking; however, we inferred from the concentration of either ligand in the umbilical cord blood to be 100 ng/mL and 300 ng/mL of IGF-I or -II, respectively. We used 10, 50, up to 100 ng/mL of either IGF-I or -II; as the highest reached by IGF-I as measured by the umbilical cord blood to equalize the molecular concentration of both IGFs; however, we may need to increase IGF-II concentration to 300 ng/mL, mimicking the *in vivo* conditions, to see more physiological effect at term gestation placenta development. Also, the combination of both IGFs in one treatment may delineate different effects on PMSC multipotency or differentiation as both IGFs coexist *in vivo*.

For further experimentation, it is important to synchronize cells by serum deprivation before the treatment by IGFs. Serum deprivation was not used here as we judged that in a physiological condition *in vivo* no withdrawal of all growth factors and cytokines is possible all at once. Monitoring the cell surface markers CD73, CD105, CD117 expression during IGF stimulation will give an indication of how multipotency changes during stimulation time in proliferation or

differentiation conditions. An inhibition by siRNA or an ectopic expression of OCT4 would delineate the role of OCT4 in response of IGFs in self-renewal and differentiation conditions. OCT4 localization is important for its function; therefore nuclear vs. cytoplasmic fractionation and immunocytochemistry experiments would be essential to show the dynamics of OCT4 during differentiation. This becomes of importance when the high expression of OCT4 in differentiation conditions is due localization and not function.

The use of a phospho-kinase array could specify the interacting adaptor and signaling protein with the IGF-IR upon stimulation by IGFs under the two different oxygen tensions. Knockdown studies of IGF-IR or IR can improve the understanding of receptor role in mediating PMSC differentiation and the prolonged proliferation effect in IGF and oxygen tension changes. Specific inhibitors for ERK1/2 and/or AKT could specify the role of these two kinases in IGF mediated proliferation and differentiation effects as MEK1/2 and PI3K have more targets than just ERK1/2 and AKT. Also, using dominant negative kinases can help identify the role ERK1/2 and AKT in the differentiation process. As IGF-IR and IR-A/-B expression change during differentiation, it is important to use a real-time PCR to identify the receptor level changes and determine whether expression changes are mediated by IGFs and low oxygen tension.

Extending the differentiation protocol to 21 or 28 days can improve and complete the osteogenic differentiation and perhaps lower the OCT4 expression to minimal levels. This can confirm full mineralization correlating with higher levels of osteocalcin and loss of RUNX2 and osteopontin in the mature osteoblast phenotype. In line with RUNX2 phosphorylation, we need to understand the phosphorylation changes induced by low oxygen tension and IGFs upon differentiation stimulation.

Besides osteogenic differentiation, we can study the effect of low oxygen tension on chondrogenic differentiation, which is known as an avascular tissue and requires low oxygen tension. Also, as PMSCs naturally develop into endothelial cells, differentiating PMSCs into endothelial cells will improve our understanding of the natural development of chorionic villi during gestation.

Changes in PMSC fate triggered by the microenvironmental factors can cause reprogramming effects that can change the epigenetic profile of initial genes. It is of interest to understand the epigenetic changes by IGFs and low oxygen tension to maintain multipotency or mediate differentiation. DNA methylation analysis by bisulfite sequencing or enrichment of variant histones by ChIP analysis, we can identify the state of OCT4, SOX2 and NANOG promoter regions and understand the changes upon differentiation towards the osteogenic lineage at RUNX2 promoter.

The optimal use of PMSCs is for tissue regeneration therapies. Priming PMSCs with IGFs and preconditioning with low oxygen tension should be used into animal models to prove the beneficial effects of these two conditions on PMSC engraftment and survival. Such models can include pancreatic injury or brain ischemia. Also, with osteogenic differentiation abilities of PMSCs, we can study the benefits of either using fully multipotent PMSCs or osteoblast progenitor cell for engraftment into animal model of osteogenesis imperfecta; and whether low oxygen tension and IGFs can improve bone gain and total growth.

5.4. Significance

The manipulation of PMSCs by IGFs and low oxygen tension can help understand stem cell development *in vivo* and define the *in* vitro cell culture conditions for optimal use in tissue regeneration therapies.

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Appendix 8

Use of Human Participants - Ethics Approval Notice

Research
Western

Principal Investigator: Dr. Victor Han
File Number: 101556
Review Level: Delegated
Approved Local Adult Participants: 50
Approved Local Minor Participants: 0
Protocol Title: The Role of Insulin-Like Growth Factors in Human Stem Cells Development and Motility (REB #12154)
Department & Institution: Schulich School of Medicine and Dentistry\Anatomy & Cell Biology,Western University
Sponsor:
Ethics Approval Date: June 05, 2012 **Expiry Date:** June 30, 2016
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Revised Study End Date		

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

r to Contact for Further Information

X Janice Sutherland (jsutherl@uwo.ca)	Grace Kelly (grace.kelly@uwo.ca)	Shantel Walcott (swalcot@uwo.ca)

This is an official document. Please retain the original in your files.

The University *of* Western Ontario
Office of Research Ethics

AMER YOUSSEF – CURRICULUM VITAE

EDUCATION

Doctor of Philosophy, Biochemistry (2014)
University of Western Ontario, Children's Health Research Institute

Bachelor of Medical Sciences (Honours), Biochemistry and Cell Biology (2008)
University of Western Ontario

Certificate of General Arts and Science (2004)
Fanshawe College

SCHOLARSHIPS

Graduate Scholarship (2010 – 2011)
Department of Paediatrics, University of Western Ontario

AWARDS

Best Poster/Basic Science – Paediatrics Research Day (2012)
Travel Award, Children's Health Research Institute (2010 and 2012)
Dean's List Honour Student (2004 – 2008)

EXPERIENCE

Graduate Research Thesis, (2008 – Present)
Department of Biochemistry, University of Western Ontario

Teaching Assistant, (2008 – 2012)
Department of Biochemistry, University of Western Ontario

BMSc. Research Thesis, (2007 – 2008)
Department of Biochemistry, University of Western Ontario

PUBLICATIONS

Journal Articles

Youssef, A., Iosef, C., and Han V.K.M. (2014). Low Oxygen Tension and Insulin-like Growth Factor-I (IGF-I) Promote Proliferation and Multipotency of Placental Mesenchymal Stem Cells (PMSCs) from Different Gestations via Distinct Signaling Pathways. *Endocrinology*. (Accepted)