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Molecular Mechanisms of Self-Renewal in Marrow Stromal Stem Cells

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UNIVERSITY OF MIAMI

MOLECULAR MECHANISMS OF SELF-RENEWAL IN MARROW STROMAL STEM CELLS

By

Carmen I. Rios

A DISSERTATION

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MOLECULAR MECHANISMS OF SELF-RENEWAL IN MARROW STROMAL STEM CELLS

Carmen I. Rios
Adult stem cells currently represent the most promising cell type for cell therapy approaches in regenerative medicine. To obtain a thorough understanding of their properties is essential for successful stem cell therapy approaches. We previously identified a unique population of human marrow-isolated adult multilineage inducible (MIAMI) cells isolated from males and females 3 to 72 years of age. Although their numbers decrease with age, their molecular profile is sustained, suggesting that these cells self-renew during the lifetime of the individual. Understanding the mechanisms of in vitro self-renewal will allow us to expand these cells to numbers suitable for cell therapy without undermining their biological and physiological properties. Oxygen tension plays a key role in modulating the self-renewal of MIAMI cells. The focus of this study is to delineate the molecular mechanisms by which oxygen tension regulates their self-renewal. We have shown that low oxygen tension may assist in the maintenance of an immature stem cell phenotype and possibly make MIAMI cells capable of self-renewal by activating the canonical Wnt and Notch signaling pathways. Our data show that low (3%) oxygen tension promotes proliferation and increases the expression of the stem cell self-renewal markers Oct4, Sox2,
and Nanog while selectively increasing expression of key mediators in the canonical Wnt and Notch pathways, such as Wnt3a, LRP5, β-catenin, CyclinD1, and the Notch intracellular domain (NICD). In addition, we have shown the low oxygen increased accumulation of the proteins β-catenin, Oct4, CyclinD1 and NICD in the nucleus as well as nuclear localization of hypoxia inducible factor (HIF)-1α, a key transcriptional mediator of the oxygen sensing pathway.

Treatment of MIAMI cells with Wnt3a increased nuclear accumulation of Oct4a and increased proliferation at 3% and 21% oxygen, suggesting it did not mediate the low oxygen effects. In contrast, siLRP5-mediated inhibition of LRP5 decreased Oct4a and Sox2 transcripts and nuclear accumulation of Oct4a, suggesting it may participate in mediating the low oxygen effects. Our data suggest that expanding human stromal cells, such as MIAMI cells, at low oxygen tension contributes to maintaining the cells in a developmentally immature state by modulating the Wnt and Notch signaling pathways.
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CHAPTER 1:
INTRODUCTION TO STEM CELLS
AND THEIR CAPACITY TO SELF-RENEW

**Stem Cells: Embryonic and Post-Natal**

All stem cells—indeed, regardless of their source—have three general properties: they are capable of dividing and self-renewing for extended periods (the stem cell’s ability to replenish its undifferentiated state while undergoing normal, mitotic cell division); they are unspecialized with respect to function; and they can give rise to one or more functionally specialized cell types. As a consequence they should be capable of functionally reconstituting a given tissue *in vivo*. Cells derived from many different sources have been shown to fulfill these criteria, including embryonic stem cells (ESCs) and postnatal stem cells isolated from various tissues.

Postnatal stem cells have been isolated from umbilical cord blood (UCB) (Weiss et al. 2006), amniotic fluid (De Coppi et al. 2007), placental tissue (Matikainen et al. 2005), and numerous tissues of young and old individuals. The latter stem cell populations are generally referred to as adult stem cells. Adult stem cells are found in many tissues and in general can give rise only to differentiated cell types from the tissue of origin and thus are considered multipotent. Some of these include neural stem cells (NSCs) which can undergo self-renewing cell divisions, giving rise to neurons, astrocytes, and oligodendrocytes, the three main types of nerve cells in the adult brain (Gage 2000). Other stem cells like spermatogonial, corneal, and endothelial stem cells also fulfill the criteria of a stem cell, except that they differentiate only into a
single type of differentiated cell. In contrast, ESCs can give rise to cells from the three somatic germ layers (ectoderm, mesoderm, and endoderm) as well as to germ cells (Martin 1981; Thomson et al. 1998) and are considered pluripotent.

ESCs are derived from pluripotent cells in the inner cell mass of the blastocyst. These cells become lineage restricted in vivo at day 7–10 of human development and day 4 of mouse (except in diapause). At this point they commit to a germline or somatic cell fate and subsequently to more tissue-specific cells (Rossant 2008). The presumably unlimited self-renewal capacity of ESCs must be maintained in vitro by specific culture conditions that allow them to self-renew and prevent them from differentiating. It has been recently demonstrated that unlimited self-renewal capacity and pluripotency can be conferred to differentiated cells by the introduction of three or four transcription factors (Okita et al. 2007; Olariu et al. 2007; Wernig et al. 2007). While fibroblasts derived from human ESCs or from human fetus can be reprogrammed to generate induced pluripotent stem (iPS) cells by the introduction of genes encoding for Oct4, Sox2, Klf4, and cMyc, the efficiency of reprogramming of neonatal and adult-derived fibroblasts as well as adult-derived marrow stromal cells was significantly lower and required transduction with telomerase (hTeRT) and SV40 large T antigen (Park et al. 2008). Although iPS cells represent an interesting and useful model for developmental studies, their clinical use is limited by the requirement that several genes must be introduced into somatic cells.

Adult stem cells are able to self-renew and proliferate to different extents in vivo depending on the turnover rate of differentiated cells required for maintaining
tissue homeostasis. Tissues such as the epithelium of skin, gut, and the hematopoietic system, where very large numbers of differentiated progeny need to be generated on a daily basis, self-renew and proliferate extensively throughout the life of the individual. In other tissues with a low rate of differentiated progeny turnover, such as the CNS, or where proliferation of terminally differentiated cells contributes to maintain tissue homeostasis such as in the liver, tissue-specific stem cells serve as a reserve for cellular replacement or repair in case of minor or major injury, respectively. Nevertheless, in most mammalian tissues, with age comes a decline in the ability to replace mature cells.

This thesis focuses primarily on the properties of adult stem cells and their self-renewal and differentiation capacity highlighting key aspects of stem cells derived from the bone marrow.

**Adult Stem Cells**

Stem cells are responsible for tissue formation during embryonic development as well as for repair and maintenance throughout the organism’s life. In order to achieve such feats, stem cells have developed the ability to maintain a critical balance between quiescence, proliferation, and differentiation; while avoiding the irreversible state of senescence. Stem cells are exposed to constant signals within their microenvironment that must be accurately identified and processed. The response to these signals may be latent or may progress the cell to the next stage of maturity, all at the discretion of the cell itself and in response to physiological and environmental cues. How this process is controlled is a subject
of great interest. Better understanding of the molecular mechanisms that mediate each step in the life of the stem cell is gradually being achieved.

A fundamental characteristic of stem cells is their ability to undergo asymmetric division—the production of daughter cells, in which one retains its stem cell identity while the other engages in a differentiation program. Asymmetric cell division is a conserved mechanism for partitioning genetic information-generating daughter cells (progenitors) with unlimited proliferation potential (Roegiers et al. 2004). During differentiation, these progenitor cells give rise to definitive somatic cells. Both stem and progenitor cells are kept on reserve in most tissues of the organism, providing a source of constant replacement of cells lost due to cellular damage or death.

**Hematopoietic Stem Cells**

It is generally accepted that the reserve of stem cells exists within a specialized microenvironment called a “niche” (Schofield 1978). The niche is believed to be lined with more-differentiated cells to create a source of nourishment and protection for the stem cell population within (Calvi et al. 2003) (Figure 1). The niche also serves as a line of communication between the stem cells and areas of need within the organism, such as a damaged artery requiring repair. The importance of the microenvironment for stem cell function was first determined after a mutation in the gene encoding membrane-bound stem cell factor (SCF; also known as KIT ligand) was revealed to cause changes in the hematopoietic stem cell (HSC) niche leading to failure of bone-marrow
Figure 1. Adult stem cell fate determination. The most developmentally primitive stem cells are thought to be localized to specific anatomical sites, or "niches," where their self-renewal is carefully regulated. This regulation must occur throughout the organism's life by supplying stem cells that allow for tissue repair. As stem cells leave the niche, they give rise to intermediate progenitors. These progenitors will continue to differentiate into terminally-differentiated and functional cells in response to an increasingly complex set of trophic and environmental factors.

HSC maintenance in vivo (Barker 1997). However, details on the structure, localization, and molecular basis for niche activity, remain complex and unclear. Current research is under way that delves into the concept of a stem cell niche.
with promising supporting data on the molecules and cell types that are involved after transplantation into lethally irradiated recipients (Wilson et al. 2006).

Hematopoietic stem cells (HSCs) are responsible for producing all mature red and white blood cells and represent the best-characterized adult stem cell population. The most primitive HSC, also termed the long-term repopulating HSC (LTR-HSC), is a relatively quiescent cell during steady-state hematopoiesis, which undergoes expansion and differentiation in its niche within the bone marrow. HSCs’ self-renewal capabilities allow them to achieve a balance between differentiation and non-differentiation while ensuring an adequate population size for the lifetime of the organism (Oakley et al. 2007). The daily replenishment of blood cells is achieved to a large extent by divisions and subsequent discrete differentiation steps of cells that are immediate descendants of the most primitive LTR-HSC, termed short-term repopulating HSC (STR-HSC), a slightly more committed hematopoietic progenitor cell (HPC). Within the bone marrow, signals are received that may be either transient or dominant, triggering a sequence of events that decide the cell’s fate as it migrates out of the niche. The co-mingling of the HSCs with a complex network of vasculature in the bone marrow allows for easy uptake into the blood stream, transporting the cells throughout the organism.

**Multipotent Mesenchymal Stromal Cells**

The bone marrow niche for HSCs is thought to be composed of stromal cells, which support the HSCs while maintaining their stem cell characteristics, either through direct contact with the HSCs and/or by contact with soluble factors.
within the niche that signal for quiescence, maintenance, or expansion. There is strong indication that Mesenchymal Stem Cells (MSCs) and cells descending from MSCs are critical components of the HSC niche (Moore and Lemischka, 2006; Kiel and Morrison, 2008). The proximity of MSCs to HSCs and their production of soluble factors supports the notion that they most likely are involved in HSC maintenance. MSCs isolated from bone marrow produce a complex combination of growth factors and chemokines: VEGF-C and VEGF-A, CXCL12, M-CSF, Flt-3 ligand, BMP-2, IL-3, GM-CSF, and SCF to name a few that have been shown to directly support HSC long-term (LTC) colony output (Sorrentino, Ferracin, et al, 2008). Within other tissues, stromal cells are linked to HSC maintenance by the release of trophic factors. Primary stromal cells within the fetal liver, for example, support the expansion of HSCs by their production of angiopoietin-like 3, IGF2, SCF and thrombopoietin (Chou and Lodish, 2010). Within the heart, MSCs release angiopoietin-1, VEGF and its receptor, Flk-1, erythropoietin. Interestingly, hypoxic preconditioning increased expression of these angiogenic factors in the heart leading to enhanced capacity to repair damaged heart tissue due to myocardial infarct (Hu and Yu, et al, 2008). Although not yet completely clear, further understanding of the cellular and chemical architecture within the niche is vital to understanding stem cell behavior (Wagner et al. 2008). Human multipotent mesenchymal stromal cells (hMSCs) make up a non-hematopoietic, heterogeneous population of uncommitted and lineage-committed adult stem cells that have the ability to adhere to plastic, express the surface antigens CD73, CD90, and CD105, and are capable of
differentiating into osteoblasts, adipocytes, and chondrocytes (Colter et al. 2001; Dominici et al. 2006). Thus, these two types of marrow stem cells may share a common niche or may have similar niches that regulate their self-renewal, proliferation, differentiation, senescence, or cell death. hMSCs were first described by A. J. Friedenstein as fibroblast-like cells residing in the bone marrow of vertebrate animals, including humans (Friedenstein et al. 1970). Adult stem cells assure lifelong regeneration of adult tissues (e.g., blood cells, bone, fat, cartilage, vasculature, muscle, etc.). If the rejuvenating effect of self-renewal in stem cells were perfect, senescent cells (cells that have lost their ability to grow and divide) could be replaced indefinitely. In the context of aging, stem cell self-renewal is important for two reasons. On the one hand, stem cells are the ideal source of regenerating aging tissue. Second, reactivation of stem cells, if properly controlled, could be used in the treatment of degenerative diseases (Ho et al. 2005).

Recent studies have shown the multi-potentiality of hMSCs. Experiments in which bone marrow was used in transplantation demonstrated that stromal cells can serve as long-lasting precursors for bone, cartilage, and lung (Pereira et al. 1995). Ferrari et al. showed that bone marrow–derived myogenic progenitors can migrate into degenerating muscle and give rise to fully differentiated muscle fibers (Ferrari et al. 1998). After isolation from the marrow, hMSCs attach to the surface of culture dishes and form colonies, and their numbers can be expanded ex vivo (Bruder et al. 1997; D'Ippolito et al. 1999). The population of undifferentiated hMSCs can be expanded using different culture conditions and
directed to differentiate into specific phenotypic cell lineages (Bruder et al. 1997; D'Ippolito et al. 1999). The potential of hMSCs lies in their plasticity and ability to repair damaged tissue by giving rise to fully differentiated and functional cells within such tissue (Ferrari et al. 1998). Interestingly, differences have been reported in the number of hMSCs with \textit{in vitro} osteogenic potential as a function of age (D'Ippolito et al. 1999). While the number of hMSCs with osteogenic potential was high in donors younger than 15 years old, a decrease was found in donors over age 40. However, the responsiveness of hMSCs to osteogenic stimulation remained intact and unaffected by the age of the donor (D'Ippolito et al. 1999).

Human MSCs have been isolated and cultured by different labs in somewhat different conditions (Minguell et al. 2001), which has led to the use of different names to refer to this cell population. Methodologies have been developed to isolate, expand, and characterize these cells obtained after gradient centrifugation of human bone marrow cells (Haynesworth et al. 1992; Majumdar et al. 1998). hMSCs are morphologically heterogeneous in culture, and phenotypic differences have been observed using flow cytometry and real-time PCR (Majumdar et al. 1998). hMSC-like cells have been identified in other tissues, including umbilical cord blood (UCB, Minguell et al. 2001), peripheral blood (Huss 2000), adipose tissue (Zuk et al. 2001), and skeletal muscle and dermis (Young et al. 2005).

In recent years, several groups have isolated developmentally primitive cells from BM, UCB, or fetal and adult tissues, which can be extensively cultured in
vitro and have the ability to generate cells of multiple germinal layers. These include the multilineage adult progenitor cells (MAPCs) (Jiang et al. 2002), human BM-derived multipotent stem cells (hBMSCs) (Yoon et al. 2005), unrestricted somatic stem cells (USSCs) (Kogler et al. 2004), fetal somatic stem cells (FSSCs) (Kues et al. 2005), amniotic fluid-derived stem (AFS) cells (De Coppi et al. 2007), human fetal liver multipotent progenitor cells (hFLMPCs) (Dan et al. 2006), and multipotent adult stem cells (MASCs) (Beltrami et al. 2007). Some of these cell populations, including MIAMI cells, MAPC, hBMSC, AFS, and MASC, may be able to proliferate without telomere shortening. Many of them are reported to express the ES cell–specific transcription factors Oct4 and Nanog. However, some of these cells are found at relatively low levels and it is not clear if they play determining roles during the normal tissue repair process.

The point of origin of these primitive cells indeed dictates their support requirements. In 1997, Connie Eaves et al showed that human cord blood and adult marrow cells have different cytokine requirements for progenitor maintenance and function. This group showed that relatively high concentrations of a growth factor cocktail containing flt3-ligand, Steel factor, interleukin 3 (IL-3), IL-6 and G-CSF stimulated 20- to 30-fold expansions of human marrow LTC-IC (Long Term Culture – Initiating Cell population) and only a 4-fold expansion of human CD34TD38- cord blood cells LTC-IC (Eaves and Miller, 1997).

It is necessary, in order to understand how stem cells are regulated on the molecular level, to purify stem cells from their respective tissues in ex vivo culture systems that allow the cells to behave as they would in vivo. Here, we would like
to recognize a unique subpopulation of hMSCs obtained in a Dexter-like long-term culture where whole bone marrow was plated in order to maintain a level of physiological integrity. The Dexter culture system was originally designed to study the bone marrow microenvironment in the context of hematopoiesis (Dexter and Testa, 1977). In our modified growth format, adherent cells were isolated and characterized by the expression of unique distinctive markers, including those of pluripotent embryonic stem cells (Oct4, Rex1, and SSEA4), distinguishing them from marrow-derived cells previously described. These cells were named marrow-isolated adult multilineage inducible (MIAMI) cells, based on their extensive proliferative capacity while maintaining telomere length and potential to generate mature cells derived from all three embryonic germ layers (D'Ippolito et al.). MIAMI cells have been isolated as early-passage cells capable of extensive expansion in vitro from males and females, 3 to 72 years old, maintaining a remarkably consistent molecular profile independent of age and gender (D'Ippolito et al. 1999). This consistent molecular profile is achieved using culture conditions that aim to mimic the niche where these cells are predicted to reside in vivo, which include a low oxygen tension environment. The oxygen tension (pO₂) in bone marrow ranges from 1 to 7%, which prompted an examination into the role of pO₂ in regulating the capacity of MIAMI cells both to self-renew and maintain their pluripotentiality (stemness) or to progress toward osteoblastic differentiation.
**Oxygen in the Bone Marrow Environment**

Oxygen tension is a key determinant of stem cell proliferation and differentiation. While ambient air is 78% N₂, 21% O₂ and small quantities of carbon dioxide, argon, and helium, physiologic oxygen tension is much lower and varies among tissues, ranging from 1% in cartilage and bone marrow to 10 to 13% in the arteries (Chow et al. 2001). Routinely, marrow stem cells isolated from bone marrow are cultured in incubators where the partial pressure of the atmospheric oxygen (pO₂) at sea level is 159 mmHg corresponding to ~21% O₂. Chow and colleagues used mathematical modeling to show the importance of the bone marrow architecture in relation to pO₂ levels, finding bone marrow pO₂ low, between 1% and 7% (Chow et al. 2001). The first studies using methodology that allowed for the cultivation of mammalian cells at a number of discreet pO₂ values opened the door to the refinement of cellular metabolomics (Kilburn et al. 1969). Since then, evidence has accumulated that continues to implicate oxygen tension as an effector of not only cell growth in general, but osteoblastic proliferation and differentiation (Salim et al. 2004; D'Ippolito et al. 2006). However, at this time the role of oxygen as a metabolic regulator was not yet understood.

It is now known that oxygen plays a physiological role in the bone marrow microenvironment, particularly as a regulator of the balance between stemness and differentiation. Salim and colleagues used mesenchymal stem cells to show that disruption to the vasculature that occurs after injury to the bone creates a hypoxic gradient within a wound dipping oxygen levels to <0.02% O₂. Their
results reveal short and long-term inhibition of key osteogenic differentiation markers such as RUNX2 on both mRNA and protein levels and BMP2 expression as well (Salim et al. 2004).

In a study using hematopoietic stem and progenitor cells from human cord blood, cells were evaluated under 5% and 20% oxygen tension. Reduced oxygen tension resulted in an increase of total cell numbers by as much as 5-fold and a substantial increase in both the number and frequency of colony-forming cells. Progenitor cell numbers were as much as 10-fold higher. Cell counts and flow cytometry analysis revealed a total cell production and the production of immature cells from murine bone marrow were significantly enhanced under low oxygen with perfusion. A 24-fold higher number of murine stem and progenitor cells were observed in the low oxygen perfusion system after three weeks compared to culture under ambient oxygen at 20%. Thus, low oxygen, more accurately approximates the in vivo environment enhancing the growth and maintenance of human stromal and progenitor cells in vitro (Koller et al. 1992).

The known effects of oxygen on stem cell self-renewal may be indicative of more than a regulatory mechanism, but a spatial one as well. The human bone marrow is not only the center of hematopoiesis, but is a tissue of complex architecture. Understanding this architecture and the molecular regulators that act within this space could help address key aspects of controlling self-renewal. Physiologically relevant modeling provides an alternative means to analyzing this complex tissue. Most models produced for this purpose are variations based on the homogeneous Kroghian model of the bone marrow that considers only one
cell type. Chow and colleagues used such modeling as a basis for their evaluation of the placement of hematopoietic progenitor cells within the extravascular bone marrow space. They determined that these cells are not randomly distributed throughout the marrow space, rather, committed precursors are located in close proximity to oxygen-rich blood vessels, while less mature progenitors preferentially reside in areas of lower oxygen tension (Chow et al. 2001). Together with the knowledge that stem cells proliferate at low oxygen tension, such a model may suggest that the human stem cell population is likely to proliferate without limit when located in a specific area - the “stem cell niche”. A cell in such an environment would not differentiate, whereas daughter cells, moving outside the influence of the niche, would do so (Schofield 1978). Oxygen may be the key factor regulating this stem cell niche.

**Molecular Regulation**

The influence of oxygen on the modulation of signaling pathways, particularly, pathways that influence self-renewal, proliferation and differentiation of hMSCs has not received much attention. However, MIAMI cells have clearly shown that hMSCs are indeed impacted by low pO₂. MIAMI cells grown under pO₂ conditions (1, 3, 5, 10, and 21% oxygen) show different rates of proliferation. The proliferation rate of cells exposed to 3% oxygen increases, resulting in cell numbers more than threefold higher than those of cells exposed to air. In cells grown under osteoblastic differentiation conditions, the expression of the osteoblastic markers osteocalcin, bone sialoprotein, osterix, and Runx2 and alkaline phosphatase activity were upregulated when incubated in air; however,
expression was blocked at low (3%) pO$_2$. In light of these results, a very feasible physiological scenario might be one in which primitive MIAMI cells self-renew while localized to areas of low pO$_2$ in the bone marrow, but tend to differentiate toward osteoblasts when they are located closer to blood vessels and exposed to higher pO$_2$. Therefore, the maintenance of developmentally primitive human cells in vitro at low pO$_2$ would be more physiological and favor stemness over differentiation (D'Ippolito et al. 1999).

Understanding the interplay between oxygen and stem cell maintenance can be achieved by determining the molecular mechanisms by which immature hMSCs, like the MIAMI cells keep expression of self-renewal markers up while keeping markers of differentiation down. Several signaling transduction pathways have been shown to be involved in stem cell self-renewal including the Notch and Wnt pathways as well as repressor element (RE)-1 silencing transcription factor (REST) (Singh et al. 2008; Buckley et al. 2009; Jorgensen et al. 2009).

The highly conserved Notch signaling pathway has been shown to function as a means of regulating stem cell self-renewal. Artavanis-Tsakonas and colleagues reported that a signal exchange between neighboring cells through Notch receptors can amplify and consolidate molecular changes that lead to an unfolding of specific developmental programs that ultimately determine cell fate (Artavanis-Tsakonas et al. 1999). Notch, a single-pass transmembrane receptor, is cleaved upon activation of the pathway, releasing an intracellular fragment (notch intracellular domain) that migrates to the nucleus where it regulates the
expression of target genes. The NICD has been shown to recruit HIF-1α, a universal monitor of oxygen homeostasis, to a Notch-responsive promoter,

**Figure 2 Schematic Diagram of Crosstalk between Notch and Hypoxia (Gustafsson, 2005).** This model depicts two modes of hypoxic response: (1) canonical hypoxic signaling leads to activation of downstream genes; VEGF, PGK1, and Epo through HIF1α/HIF-1β and (2) maintenance of stem cell state through interaction with the Notch signaling pathway where HIF-1α and Notch ICD form a point of convergence between the two signaling mechanisms, leading to stabilization of Notch ICD, recruitment of HIF-1α to Notch-responsive promoters, and activation of Notch downstream genes Hey and Hes (Gustafsson et al. 2005).

elevating expression of Notch downstream targets (Gustafsson et al. 2005) (Figure 2). Gustafsson and colleagues reported that hypoxia blocks the differentiation of neuronal and myogenic progenitors in a Notch-dependent manner (Gustafsson et al. 2005). Hypoxia appears to block this differentiation
program by upregulating the stem cell transcription factor Oct4. Covello and colleagues used a genetic "knock-in" strategy to demonstrate that targeted replacement of the oxygen-regulated transcription factor HIF-1α with its alternative isoform HIF-2α results in expanded expression of HIF-2α-specific target genes including Oct4 (Covello et al. 2006). In this same study, the Covello group showed that inhibition of Oct4 with Oct4 shRNA also reduced elevated β-catenin levels in Hif-2α KI/KI tumors (Covello et al. 2006). β-catenin is a major mediator of another self-renewal pathway, the canonical Wnt signaling pathway, also involved in self-renewal.

The Wnt signaling pathway is regulated by genes that encode a family of secreted growth factors shown to play a critical role in embryonic development and adult homeostasis. It is involved in multiple differentiation events; yet aberrant expression of the Wnt pathway has been implicated as causing over 90% of colorectal cancers where its upregulation facilitates proliferation through non-ligand mutations (Giles et al. 2003). However, the pivotal role of Wnt signaling in cellular homeostasis has stirred great interest in its possible regulation of stem cell maintenance as well. Likewise, the Wnt pathway’s regulation of multiple downstream targets such as cell cycle regulator Cyclin D1 (Benhaj et al. 2006) and pluripotency marker Oct4 (Hochedlinger et al. 2005) has drawn our attention to this pathway making it a major focal point of this study. Canonical Wnt mediators are listed in Table 1.

In a ground-breaking paper from 1983, Nusse and Varmus revealed the first of the Wnt genes to be discovered, Wnt 1 (Nusse et al. 1982). Although Wnt 1 was
named an oncogene causing mouse mammalian tumors; the 18 other Wnt genes
to be discovered would reveal that the Wnt pathway plays a role in much more
than cancer, but also normal cell proliferation, differentiation, cell survival,
polarity during embryogenesis, and cellular movement.

The canonical Wnt signaling pathway (Figure 3) acts through the nuclear
localization of the protein β-catenin. Once inside the nucleus, β-catenin binds
and co-activates transcription factors, TCF/LEF (T cell factor/lymphoid enhancer
factor) that in turn lead to the expression of Wnt-responsive genes (Bienz et al.
2003). Cytosolic levels of β-catenin are kept in check through its phosphorylation
by casein kinase I (CKI) and glycogen synthase kinase 3 (GSK-3) within a large
protein complex containing APC (adenomatous polyposis coli gene product),
thus named the APC complex and subsequent degradation at the proteosome
(Bienz et al. 2003). Changes in any part of this pathway could lead to
constitutive β-catenin entry into the nucleus, thus causing aberrant signaling, as
seen in many cancers.

The activation of the pathway begins by the association of a soluble Wnt
ligand with two Wnt receptors, LRP5/6 (LDL receptor-related protein) and one of
10 Frizzled (Fz) family members. The outcome of gene expression varies
depending on the combination of Wnt ligand and LRP receptors. LRP5/6 recruits
protein Axin away from the APC complex and concentrates it to the plasma
membrane; rendering the complex unable to phosphorylate β-catenin (Cliffe et al.
2003).
Figure 3 Overview of the canonical Wnt signaling pathway (He, 2003). (A) Without a Wnt ligand, β-catenin is contained by a degradation complex containing the scaffold protein Axin, the tumour suppressor gene product APC, and the kinases CKI and GSK3β, among others. Sequential phosphorylation causes β-catenin to be ubiquitinated by the β-TrCP–E3-ligase complex and subsequently degraded by the proteasome. (B) In the presence of a Wnt ligand, one of the Wnts associates with Fz and LRP5/6 co-receptors. This causes translocation of Axin to the plasma membrane through direct interaction with LRP5/6 and Dsh/Fz. Translocation results in Axin degradation and/or dissociation of the degradation complex and release of non-phosphorylated β-catenin causing its accumulation in the cytoplasm. β-catenin is now free to migrate into the nucleus where by association with TCF/LEF factors it promotes transcription of downstream Wnt target genes (He 2003).

To add further to the complexity of Wnt signaling, another player, Disheveled (Dsh), a ubiquitous cytoplasmic protein, has been shown to effect levels of downstream β-catenin as well. Dsh interacts with Fzds as well as Axin leading to
the reconfiguration of the APC complex that normally keeps levels of β-catenin in check (Logan et al. 2004).

It has been found that cross-regulation of events occurring in development are orchestrated through Wnt and Notch acting together. Martinez-Arias and colleagues reported that loss of Notch resulted in Disheveled-independent Wnt signaling (Martinez Arias et al. 2002). However, the mechanism by which these interactions occur between these two pathways is still unknown.

The balance between self-renewal and the differentiation process is well described for development of the central nervous system. Neural stem cells located strategically within the developing nervous system divide symmetrically to maintain the progenitor cell population. Meanwhile, progenitors divide asymmetrically to produce cells destined to become neurons (Temple 2001). Interestingly, Wnts have been implicated in neuronal development as well. Wnt1 mutants display loss of midbrain/hindbrain boundary cell pools (McMahon et al. 2001).

### Table 1: Key mediators of the canonical Wnt signaling pathway

<table>
<thead>
<tr>
<th>Receptors/Co-receptors</th>
<th>Antagonists</th>
<th>Intracellular components</th>
<th>Transcription factors</th>
<th>Wnt Target Genes w/Tcf binding sites; other targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frizzleds (Fzds) 10 members identified LRP5 &amp; 6</td>
<td>Dickkopfs (Dkks) Secreted Frizzled-Related Proteins (sFRP)</td>
<td>β-catenin, GSK3β APC complex Dsh</td>
<td>Lef1/Tcf family</td>
<td>CyclinD1, DKK1, FoxN1, Sox9, Frizzled7, fibronectin, connexin 43, NRSF/REST; Oct4, Sox2, Nanog.</td>
</tr>
</tbody>
</table>
and Wnt3a mutants lack hippocampus formation (Lee et al. 2000); whereas over-expression of Wnt genes cause proliferation of neural progenitors (Dickinson et al. 1994). The means by which the Wnt pathway may be regulating genes involved in maintaining the neural stem cell population is not clear. An interesting association however, is NRSF/REST (neuron restrictive silencer factor/repressor element 1 transcription factor); a direct target of the canonical Wnt pathway (Nishihara et al. 2003). NRSF/REST negatively regulates neuronal genes containing the neuron restrictive silencer element (NRSE) (Schoenherr et al. 1996). More recently, Nishihara and colleagues have found a direct link between the canonical Wnt pathway and NRSF/REST. They reported in 2003 that NRSF possesses a binding site for TCF (the known co-activator of β-catenin in canonical Wnt signaling) (Nishihara et al. 2003).

In addition to serving as a transcriptional repressor of several neuronal genes, the DNA-binding protein NRSF/REST is abundantly expressed in both human and mouse pluripotent embryonic stem cells (ESCs) (Chen et al. 1998; Gupta et al. 2009). NRSF/REST has been found to play various roles in cells from oncogenicity to tumor suppression depending on cellular context. However, it has been shown that NRSF/REST may be a component of the transcriptional network involved in maintaining stem cell self-renewal (Gupta et al. 2009) (Figure 4).
Figure 4 Model of NRSF/REST gene regulatory network (Mortazavi, 2006). (A) The model shows protein NRSF /REST, coREST and other corepressors that may prevent transcription of hundreds of target genes. Some target genes include neuronal splicing factors, transcription factors, microRNAs, and stem cell differentiation genes. (B) This portion of the model shows how the onset of neurogenic signals to terminally differentiate cells may be received while subsequently causing the NRSF protein to be degraded. This degradation leads to derepression of target genes by activators. NRSE- associated miR-153 may down-regulate NRSF and coREST mRNA thus maintaining derepression (Mortazavi et al. 2006).
Maintenance of self-renewal has been proposed to be in part controlled by preventing the expression of miRNA21, a microRNA thought to inhibit the expression of stem cell self-renewal markers Oct4, Nanog, and Sox2, thus setting the stage for cells to differentiate (Singh et al. 2008). However, evidence opposing this proposed mechanism has also been reported (Buckley et al. 2009; Jorgensen et al. 2009). Nevertheless, NRSF/REST expression is needed to block at least neuronal differentiation (Gupta et al. 2009).

To increase our understanding of the mechanisms by which low oxygen may be stimulating the process of self-renewal in MIAMI cells, we examined in greater detail the effects of low oxygen on mRNA and protein of self-renewal transcription factors and the expression of genes involved in mediating the signaling pathways described above; Wnt, Notch, and NSRF/REST. Here we show involvement of each of these pathways acting in a low oxygen environment to maintain self-renewal in MIAMI cells. If and how cross-talk is occurring in this system remains to be determined, however, two models proposed in this report suggest a means by which this may occur.
CHAPTER 2:  
MATERIALS AND METHODS

Isolation and Culture of Bone-Marrow-derived human Mesenchymal Stem Cells (hMSCs)

Whole bone marrow cells were plated on fibronectin-coated (10-ng/ml) T-75 tissue culture flasks (Nunclon, Rochester, NY) in the following media preparation: filtered DMEM-Low Glucose (LG)/3% FBS (Hyclone Laboratories, Logan, UT), with 100 U/ml penicillin (Gibco-BRL, Grand Island, NY), 1 mg/ml streptomycin (Gibco-BRL) and grown at 3% oxygen tension, 37°C. After 7 days, non-adherent cells were removed. Single-cell-derived colonies (MIAMI cells) were isolated and expanded under niche-like conditions in the following manner. Expansion of cells was on fibronectin-coated T-75 tissue culture flasks in DMEM-LG/3% FBS, 100 U/ml penicillin, and 1 mg/ml streptomycin for 7 days with half-media changes every 2 days. Cells were harvested with 0.25% Trypsin-EDTA solution (Gibco) for 5 minutes at 37°C then reseeded at a plating density of 100 cells/cm² as counted with a hemocytometer and put at either 1%, 3% or 21% oxygen tension at 37°C for subsequent experiments. Cells receiving exogenous treatments with recombinant mouse Wnt3a underwent a 24 hour period of serum starvation prior to 5ng/ml of recombinant mouse Wnt3a (R&D Systems; Minneapolis, MN) in the media. Cells used for cell growth assays were trypsin-harvested at various timepoints. A 1:1 suspension was prepared with 0.4% trypan blue for cell death exclusion. Live cells were counted by hemocytometer on a Nikon inverted light microscope.
**RNA Isolation**

For RNA isolation, total RNA was extracted using the RNAqueous-4PCR Kit (Ambion; Austin, TX) according to manufacturer’s instructions. Briefly, $10^2$ to $10^7$ cells were disrupted with 100 µL of manufacturer’s lysis/binding solution and vortexed until solution is homogeneous. Lysis was followed by the addition of 100 µL 64% ethanol. Lysate/ethanol solution was drawn through the provided filter cartridge, washed, then centrifuged at RCF 10,000–15,000 × g ($10,000–14,000$ rpm) for ~15sec–1min. Flow-through was discarded after all washes. Finally, RNA was eluted with 2 sequential applications of pre-heated manufacturer’s elution solution followed by centrifugation at RCF 10,000–15,000 × g. Trace amounts of DNA were removed with DNase I treatment. 1-µL RNAse-free DNase-I was applied to every 100-µL RNA in elution solution and incubated at 37°C for 30 minutes. DNase was removed by application of DNase Inactivation Reagent from manufacturer, centrifugation at 10,000 × g for 1 minute; DNase Inactivation Reagent was pelleted. RNA solution was carefully removed and placed in a new tube. RNA was concentrated by adding 0.1 volume 5M ammonium acetate and 0.02 volumes of linear acrylamide and vortexed. 2 volumes of 100% ethanol was added, mixed, and incubated at ≤ -20°C overnight. RNA was pelleted by centrifugation at RCF ≥ 10,000 × g, with removal of supernatant. RNA was resuspended in 20 µL elution solution provided by manufacturer. 2 µL of RNA was quantified on a NanoDrop spectrophotometer (ThermoScientific; Wilmington, DE). Only RNA with A260/A280 of approximately 1.8 µL was used for cDNA preparation.
**Quantitative real-time PCR (qRT-PCR)**

RNA was reversed transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA). Briefly, 1µg of RNA was mixed with dNTP’s, reaction buffer, and random primers. Reactions were run on a Stratagene Mx5000P thermocycler set to specified conditions; 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 seconds. 1µg of cDNA was amplified with 10 pmol of 5’ and 3’ primers in RT-qPCR buffer SYBR Green (Stratagene; Cedar Creek, TX). Amplifications were performed with Stratagene 96-well plate reader for 40 cycles (typically: 94°C/30 seconds; 55°C/45 seconds; 72°C/60 seconds) after an initial denaturation at 94°C. PCR products were quantitatively analyzed using the Stratagene software where the cycle threshold (C\text{\text{T}}) values were normalized to housekeeping gene elongation factor alpha or elongation factor alpha and ribosomal protein –large subunit 13a (Curtis 2010). A list of primer used for RT-qPCR follow in Table 2.

**ELISA**

Wnt3a concentrations were measured in conditioned media obtained from MIAMI cells cultured in 3% and 21% oxygen tension using an original ELISA preparation from materials described here. A general protocol was followed as a guideline (R&D Systems; Minneapolis, MN). A sandwich ELISA was prepared by coating a 96 well plate with capture antibody anti-Wnt3a (R&D Systems; Minneapolis, MN) at a 0.5µg/ml dilution with PBS. Conditioned media from MIAMI cells grown at 3% and 21% oxygen was added to the capture antibody preparation. Standard, fresh media was used as a negative control. Media was
<table>
<thead>
<tr>
<th>Gene (Accession Number)</th>
<th>Primer Pair Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axin 1 (NM_003502)</td>
<td>Fwd: 5'CCTCGGAGCAAGTTTCACC 3'</td>
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<tr>
<td></td>
<td>Rev: 5'CAGTGACTCAGCCCACTTC 3'</td>
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<tr>
<td>Cyclin D1 (NM_053056.2)</td>
<td>Fwd: 5'GTGCTGCGAAGTGGAACG 3'</td>
<td>173</td>
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<td></td>
<td>Rev: 5'ATCCAGGTGGCCAGATCCT 3'</td>
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<td>DKK1 (NM_012242)</td>
<td>Fwd: 5'CAGGGCGTCAATCTGCT 3'</td>
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<td>Rev: 5'GCTTTCAGTGATGTTCCTCA 3'</td>
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<td>EF1α (NM_001402.5)</td>
<td>Fwd: 5'AGGTGATTATATCCTGAACCATCC 3'</td>
<td>235</td>
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<td></td>
<td>Rev: 5'AAAGGTGATATATGCTAGGAACG 3'</td>
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<td>Frizzled1 (NM_003505)</td>
<td>Fwd: 5' CCAGAACACGTCCGACAAAG 3'</td>
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<td></td>
<td>Rev: 5'ACCTTGGTGCTGGCTACAAG 3'</td>
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<td>Frizzled 2 (NM_001466)</td>
<td>Fwd: 5' CCAGACCAGCATGCGCAAAAC 3'</td>
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<td>Rev: 5'GGGCCACACTGAAACGGAAC 3'</td>
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<td>Frizzled 4 (NM_012193)</td>
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<td></td>
<td>Rev: 5'GCCAAAAACCAAGTGGTCA3'</td>
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<tr>
<td>Frizzled 5 (NM_003468)</td>
<td>Fwd: 5' GGAACGGCTTGCTATCTG 3'</td>
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<td></td>
<td>Rev: 5'TGTACCAGTGAGATGTG3'</td>
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<td>Frizzled 6 (NM_003506)</td>
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<td>Rev: 5'TCCGTCACATTCAAGTCTCTCA 3'</td>
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<td>hTeRT (NM_198253.2, NM_198255.2)</td>
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<td>Rev: 5'CCAGACACTCTTTGGCTAG 3'</td>
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<td>LRP 5 (NM_002335.2)</td>
<td>Fwd: 5' GCCGACCTTACCGACATCG 3'</td>
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<td>Rev: 5'GGTCCAGTAGGTTCTCGG 3'</td>
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<td>Nanog (NM_024865.2)</td>
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<td></td>
<td>Rev: 5'GAGGCATCTCAGCAGAAGAC 3'</td>
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<td>Oct 4a (NM_002701.4)</td>
<td>Fwd: 5'GGCCAGATGTGGTTGCTGAATAA 3'</td>
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<td></td>
<td>Rev: 5'GGCAGATGGTTGCTGGCTGG 3'</td>
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<tr>
<td>Oct 4a/b (NM_001173531.1)</td>
<td>Fwd: 5' GATGGTGTGGCAGTGTTCTC3'</td>
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<tr>
<td></td>
<td>Rev: 5'TGTGCATACTGCGTTGCTG 3'</td>
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</tr>
<tr>
<td>REST/NRSF (NM_005612.3)</td>
<td>Fwd: 5' AAGCTCCCTCTCCGAAAGAT 3'</td>
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</tr>
<tr>
<td></td>
<td>Rev: 5'GATGCCTCTGTGGCTACTTTCC 3'</td>
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<tr>
<td>Rex1 (NM_174900.3)</td>
<td>Fwd: 5' AACGGGCAAAGACAAACAC 3'</td>
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<td></td>
<td>Rev: 5'GCTGACAGGTTCTATCTGGC 3'</td>
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<tr>
<td>Sox2 (NM_003106.2)</td>
<td>Fwd: 5' GCAGTACTCCACCCTAGAC 3'</td>
<td>160</td>
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<td></td>
<td>Rev: 5'AGGAAGAGGTTAACCACAG 3'</td>
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<tr>
<td>Wnt 3a (NM_033131)</td>
<td>Fwd: 5'CAAACTCTGAGTCTCGCTGAC 3'</td>
<td>119</td>
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<tr>
<td></td>
<td>Rev: 5'CTTGGGCGCAAAGCATCCTC 3'</td>
<td></td>
</tr>
<tr>
<td>Wnt 7a (NM_004625.3)</td>
<td>Fwd: 5'TGTGACCTTCTCTACTGACG 3'</td>
<td>107</td>
</tr>
</tbody>
</table>
carefully washed from plate and biotinylated anti-Wnt3a (0.5\(\mu\)g/ml) was added as the capture antibody to a 96-well plate. A horseradish peroxidase conjugated streptavidin solution was used as the secondary (1:200) this produces a measurable end product. The reaction was stopped with 2M sulfuric acid and the intensity of color produced was proportional to the concentration of Wnt3a concentration present in each sample. Optical density was measured at 450nm on a microplate reader (BioRad; Hercules, CA).

**Western Blot**

Protein samples were collected from trypsinized cells using NP-40 lysis buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1% NP-40, nonyl phenoxypolyethoxylethanol, 5mM EDTA). For cytoplasmic and nuclear protein extraction, proteins were separated using the NE-PER Nuclear and Cytoplasmic Protein Kit (Thermo Fisher Scientific; Rockford, IL) per manufacturer’s instructions summarized briefly; 2 x 10⁶ cells were placed in a microcentrifuge tube and pelleted by centrifugation at 500 x g for 3 minutes. Ice-cold cytoplasmic extraction reagents were added to the pellet and cytoplasmic protein was removed after a series of rigorous vortexing and centrifugations at approximately 16,000 x g. Nuclear extraction reagent was added to remaining pellet and nuclear protein was removed after a series of rigorous vortexing and centrifugations at approximately 16,000 x g. All proteins stored at -80°C until used.

Proteins were quantified using the Bradford Protein Assay (Bio-Rad; Hercules, CA). Equal concentrations (50-100 \(\mu\)g) of proteins were separated on 10% to
12% SDS-polyacrylamide gels and electroblotted onto PVDF membranes (electrophoresis and transfer apparatus from Bio-Rad; Hercules, CA) for 60 to 90 minutes at 100V. Blots were blocked in 0.1% BSA in TBS or 5% non-fat milk in TBS for at least 1 hour at room temperature. Blots were incubated in primary antibodies diluted in either 0.1% BSA or 5% non-fat milk) overnight at 4°C and secondary antibodies conjugated with horseradish peroxidase (diluted in either 0.1% BSA or 5% non-fat milk) for 45 minutes at room temperature. See Table 3 for a list of antibodies and dilutions used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-catenin</td>
<td>Cell Signaling Technology; Beverly, MA</td>
<td>WB: 1/1000</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Santa Cruz Biotech., Inc.; Santa Cruz, CA.</td>
<td>WB: 1/200</td>
</tr>
<tr>
<td>Nanog</td>
<td>Abcam; Cambridge, MA</td>
<td>WB: 1/500</td>
</tr>
<tr>
<td>NICD</td>
<td>Abcam; Cambridge, MA</td>
<td>WB: 1/500</td>
</tr>
<tr>
<td>Oct 3/4</td>
<td>Santa Cruz Biotech., Inc.; Santa Cruz, CA.</td>
<td>IF: 1/100</td>
</tr>
<tr>
<td>Oct 4</td>
<td>Abcam; Cambridge, MA</td>
<td>WB: 1µg/ml</td>
</tr>
<tr>
<td>NRSF/REST</td>
<td>Santa Cruz Biotech., Inc.; Santa Cruz, CA.</td>
<td>WB: 1/200</td>
</tr>
<tr>
<td>Sox 2</td>
<td>Abcam; Cambridge, MA</td>
<td>WB: 2uµg/ml</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Santa Cruz Biotech., Inc.; Santa Cruz, CA.</td>
<td>WB: 1/1000</td>
</tr>
</tbody>
</table>
Blots were incubated with ECL chemiluminescence solution (GE Life Sciences; Piscataway, NJ) per manufacturer’s instructions for band detection. After separation, final levels of proteins were normalized to α-tubulin band and/or Coomassie-stained blots of total protein per lane by measuring area and pixel values from digitized images. Quantitation of blots was performed on ImageJ software from NIH (http://rsbweb.nih.gov/ij/) . Fold change was calculated by dividing the densitometry value of the treated sample by the densitometry value of the control (untreated sample). The densitometry value is the Relative Intensity that is derived from the ratio of the mean and pixel values of the target band to the normalization band or lane of total protein.

**Immunofluorescence**

Cells were grown according to expansion protocol on glass coverslips treated with fibronectin in 6-well-plates (Nunclon). Cells were fixed with 4% paraformaldehyde at 4°C for 10 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. Blocking and diluent solution consisted of phosphate-buffered saline (PBS), 1% BSA (Sigma, St. Louis, MO). Fixed cells were blocked for 30 minutes, incubated sequentially 1 hour with the primary antibody to Oct4 (see Table 1) followed by 1 hour incubation of the fluorescein- or rhodamine-conjugated secondary antigoat IgG antibody. PBS containing 0.3% BSA was used for washes between each step. Specific immunostaining was demonstrated in control experiments in which cells were exposed to primary isotypic antibodies and then incubated with conjugated antibodies. DAPI mounting media (Vector Laboratories, Burlingame, CA) was used for visualization of the nucleus. Cells were visualized on a Nikon fluorescence microscope.
**Immunoprecipitation**

Protein A/G Plus Agarose Beads (Santa Cruz Biotech., Inc.; Santa Cruz, CA) were aliquoted at volumes of 25 µl with 2 µg of primary antibody to TCF4 in a Eppendorf tube and incubated for 1 hour at room temperature on a rotator. Protein A/G Agarose Beads were washed three times with 1 ml PBS, then centrifuged at 3,000 x g for 2 minutes at 4°C. Supernatant was discarded after each wash. 200-400 µg of protein sample was added and brought up to a final volume of 200 µl with PBS. Protein/bead solution was incubated overnight at 4°C on a rotator. The next day, the protein/bead solution was washed three times with PBS. The solutions underwent centrifugation at 3,000 x g for 2 minutes at 4°C. The supernatants were saved and proteins were separated using polyacrylamide gel electrophoresis (PAGE). Samples run without primary antibody or with isotypic non-immune (normal rabbit) serum served as negative controls. Blots were incubated in primary antibodies diluted in either 0.1% BSA or 5% non-fat milk) overnight at 4°C and secondary antibodies conjugated with horseradish peroxidase (diluted in either 0.1% BSA or 5% non-fat milk) for 45 minutes at room temperature. Blots were incubated with ECL chemiluminescence solution (GE Life Sciences) per manufacturer’s instructions for band detection. After separation, final levels of proteins were normalized to Coomassie-stained blots for total protein.

**siRNA Transfections**

MIAMI cells were transfected one day after seeding with 50, 100, or 200 nM siRNA ON-TARGET plus SMART pool or siCONTROL Non-Targeting pool
(Dharmacon, Chicago, IL). Sequence pool for siRNA Lrp5 (CGUCAAAGCCAUCGACUAUUU, CGUCAUGGGUGGUGUCUAUUU, GGACGGACCUCGGAGGAUUU, GUACAGGCCCUCUCAUCUAUUUUU) or sequence pool for siRNA Lrp6 (GCAGAUUCAGACGAAUUUUU, CAGAUGAACUGGAUGUAUUU, CCACAGAGCGACUCAGUAUUU, GCUCAACCGUGAAGUUAUU) and siControl non-targeting pool (a combination of four siRNA sequences that are not targeted to Lrp5 or 6 and found to have little off-target effects). Transfections were performed with the Nucleofector electroporator (Amaza; Walkersville, MD). 1 x 10^6 to 2 x 10^6 were trypsinized and collected for each transfection procedure. Nucleofector Solution was used as our transfection reagent according to the Amaza protocol for the Nucleofector Kit. Immediately following transfections, cells were placed drop-wise into incubated 6-well-plates containing MIAMI expansion media. 24 hours after the transfections, all media was removed and fresh MIAMI expansion media was applied. Cells were left undisturbed for 3 days, trypsinized and collected for a second round of transfection using the same procedure stated above. Cells were collected 3 days after the second transfection for RNA and protein analysis. To determine transfection efficiency, MIAMI cells were transfected with 1 μg GFP plasmid - green fluorescent protein - pEGFP-C1 (BD Clontech; Mountain View, CA) and visualized on a Nikon fluorescence microscope. For transfection with TOP/FOPFLASH plasmids MIAMI cells were transfected by electroporation as described above with 3 μg of either type of plasmid. After 24 hours, the cells were collected for luciferase assays as described below.
**Luciferase Assay**

Activation of the Wnt pathway was determined using the TCF/Lef reporter gene TOPFLASH (Upstate; Lake Placid, NY) and the non-responsive mutated control FOPFLASH (Upstate). Cells were transfected according to manufacturer’s protocol with TransIT-TKO chemical transfection reagent (Mirus; Madison, WI). 4ug of the reporter construct plasmid was placed in media with the TKO transfection reagent for 20 minutes before addition to cells. The reporter plasmid used to measure luciferase expression contained six TCF response element constructs cloned upstream of a cytomegalovirus (CMV) promoter. Cell preparation was left for 24 hours at either 3% or 21% oxygen tension. Subsequently, the media was removed and fresh expansion media was added to the cells and they were returned to the appropriate oxygen tension for another 24 hours. Transactivation of the luciferase reporter gene was measured using the Dual-Luciferase Reporter Assay System (Promega; Madison, WI). Briefly, cells were collected by addition of luciferase reporter assay buffer and centrifugation at 15,000 x g for 1 minute at 4°C. Luciferase expression was measured using the dual luciferase activity reagent on a luminometer. 100 µL of Luciferase Assay Reagent was placed in luminometer tubes. 20 µL of cell lysate was added to the assay reagent and sample was read on the luminometer. Reporter-driven luciferase activity was normalized to total protein content using the Bradford Protein Assay (Biorad; Herucles, CA).
**Statistical Analysis**

Only data sets containing $N \geq 3$ independent experiments with 2-3 samples per condition in each experiment were used for statistical analysis. A One-way ANOVA (Analysis of Variance tests for significant differences between means of two or more samples) followed by Tukey’s post-hoc analysis (a series of mean comparisons that are done after ANOVA) was used to calculate statistical significance between conditions using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, www.graphpad.com. All error bars represent standard deviations. Statistical nomenclature used throughout this report is defined as follows:

<table>
<thead>
<tr>
<th>P value</th>
<th>Wording</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.001</td>
<td>Extremely significant</td>
<td>***</td>
</tr>
<tr>
<td>0.001 to 0.01</td>
<td>Very significant</td>
<td>**</td>
</tr>
<tr>
<td>0.01 to 0.05</td>
<td>Significant</td>
<td>*</td>
</tr>
<tr>
<td>&gt;0.05</td>
<td>Not significant</td>
<td>ns</td>
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</tbody>
</table>

(P values > 0.05 may be specified in some figures)
CHAPTER 3:  
RESULTS

Oxygen Tension and the Canonical Wnt Pathway

Previously, we reported that low oxygen tension (3% pO\textsubscript{2}) caused an upregulation of mRNAs for total Oct4, Rex-1, human telomerase reverse transcriptase (hTeRT), and Hypoxia Inducible Factor-1α (HIF-1α). Upregulation of these mRNAs occurs even under osteogenic culture conditions in comparison to cells grown at 21% oxygen (D'Ippolito et al. 2006). This unique molecular profile is indicative of a developmentally immature cell type that maintains the capability of differentiating in vitro (D'Ippolito et al. 2006). Based on our previous findings, we determined whether low oxygen tension may also mediate the expression of other known embryonic stem cell markers in primitive MIAMI cells, such as Nanog, Sox2, and Oct4a - the active Oct4 splice variant localized to the nucleus (Atlasi et al. 2008). These three transcription factors; Oct4, Sox2, Nanog are thought to be the center of transcriptional regulation in embryonic stem cells due to their expression profile and functional role in early development (Avilion et al. 2003; Chambers et al. 2003; Lee et al. 2004).

As anticipated, we found that MIAMI cells display an increase in mRNA transcripts for the three core transcription factors; Oct4 (a/b and a), Sox2, and Nanog under low oxygen tension. In addition, we observed an upregulation of mRNA transcripts for Lrp5, a key co-receptor and modulator of the Wnt signaling pathway implicated in the maintenance of mouse embryonic stem cell (mESC) pluripotency. Also increased at low oxygen tension were mRNA transcripts for
hTeRT; human Telomerase Reverse Transcriptase, a ribonucleoprotein that extends telomere ends by addition of a telomere sequence TTAGGG, a critical enzyme for maintaining appropriate telomere lengths needed for stem cell self-renewal (Figure 5A). Messenger RNA transcription levels of other Wnt mediators were measured and found to be positively influenced by a decrease in oxygen tension. In particular, the soluble ligand Wnt3a showed an upregulation of its mRNA transcript as well as a known downstream target of the canonical Wnt pathway, Cyclin D1; a member of a family of cell cycle regulators and present in most proliferating cells (Figure 5C). These data implicate the canonical Wnt signaling pathway as playing a role in oxygen-mediated self-renewal in MIAMI cells.

We have also shown that the primitive MIAMI cell profile is sustained under long-term culture conditions (7 days) when grown under low-oxygen conditions (1, 3, 5, and 10% oxygen) compared to air (21% oxygen). The proliferation rate of cells exposed to 3% oxygen (3 days) increased, resulting in cell numbers more than threefold higher than those of cells exposed to air (at 7 days) (D'Ippolito et al. 2006). MIAMI cells used for the experiments in Figures 5A were grown for 7 days with exposure to 3% oxygen tension (expansion conditions). In order to elucidate in more detail the temporal nature of embryonic stem cell marker expression in primitive MIAMI cells at low oxygen tension, we decided to evaluate gene expression of Oct4a (active isoform), Nanog, Rex-1, and hTeRT at a hourly time-points after acute hypoxia treatment (incubating the cells at 3% pO2 after maintaining the cells at 21% pO2). An upward trend was observed at the
lower time-points (1-2 hours) in each of the markers measured, while gene expression appears to return to baseline levels after 2 hours (not shown). Protein levels of total Oct4 (Oct4a/b), Nanog, and Sox-2 were also analyzed and observed to be increased after 1 hour exposure to acute hypoxia as shown in a representative blot from more than 3 experiments (Figure 5B).

**Figure 5: Hypoxia increases mRNA levels of Wnt mediators and self-renewal transcription factors.** A) Graph depicts fold change of gene expression of Wnt mediators and self-renewal markers (Nanog, hTeRT, Sox2, Oct4a, Oct4a/b, LRP5) in MIAMI cells grown for 7 days at low oxygen compared to cells kept at 21% oxygen for 7 days. B) Western Blot of MIAMI cell proteins from 1 hour at 3% oxygen. Specific antibodies to Nanog (top panel), Sox2 (second panel), Oct4a/b (third panel) were used to detect proteins. α-tubulin (bottom panel) shown here as a loading control. C) Summary of Wnt and self-renewal markers measured by RT-qPCR with corresponding Wnt pathway showing placement of key mediators.

**Figure 5A**

![Figure 5A](image-url)
Figure 5B

Western Blot Analysis of Transcription Factors Oct4, Sox2 and Nanog

Figure 5C

Fold Increase of Wnt Mediators and Down Stream Targets in MIAMI cells
3%O₂/21%O₂

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The effects of low oxygen on proliferation with simultaneous inhibition of osteoblastic markers (D'Ippolito et al. 2006) is similar to Wnt3a-mediated activation of canonical Wnt signaling in MSCs. As shown by Boland and colleagues, Wnt3a exposure inhibits MSC osteogenic differentiation, marked by decreased matrix mineralization and reduced alkaline phosphatase activity. Boland and colleagues showed that Wnt3a treatment of osteogenically differentiated MSCs could also suppress gene expression of osteoblastic markers. They further showed that the effect of exogenous Wnt3a was accompanied by an increase in cell number resulting from an increase in proliferation and a decrease in apoptosis, particularly during expansion of undifferentiated MSCs (Boland et al. 2004).

While we have shown that low oxygen tension promotes self-renewal in MIAMI cells, the effect of Wnt3a on the expression of primitive stem cells markers, such as; Oct4, Sox2, and Nanog, has not yet been determined. Thus, we decided to perform these analyses by specifically examining the role of Wnt3a, since it has been shown to be the archetypical activator of canonical Wnt signaling in human marrow stromal cells (Boland et al. 2004; de Boer et al. 2004) and we have observed that its expression is induced by low oxygen tension. Thus, low oxygen may increase Wnt3a secretion which in turn may act as a paracrine/autocrine factor to stimulate canonical Wnt signaling.

The concentration of Wnt3a produced and secreted in MIAMI cell conditioned media was measured by ELISA (Figure 6A). The Wnt3a concentration was found to be between 9 and 10 ng/mL during expansion conditions at 3% and
21% oxygen tension. Approximately 2 ng/mL was detected in media alone; most likely introduced by FBS in the media preparation (Figure 6B). These data differ from our real time quantitative PCR results (Figure 5C) at low oxygen tension; where a 20-fold increase in Wnt3a mRNA transcripts was measured. However, the ELISA did not detect a difference between Wnt3a production from cells grown at 3% and 21% oxygen tension. The equal release of Wnt3a at low and high oxygen indicates that Wnt3a is not the rate-limiting step in canonical Wnt regulation of self-renewal in MIAMI cells at least at a long time point of 7 days. It is possible that cells release Wnt3a differentially at shorter time points. This issue still needs to be addressed.

Since the Wnts are soluble ligands and MIAMI cells were found to release Wnt3a, we thought to increase their exposure to the ligand even further and look for the subsequent effects. We treated MIAMI cells with exogenous recombinant mouse (rm) Wnt3a (R&D Systems; Minneapolis, MN). Mouse Wnt3a shares 96% amino acid identity to the human version of Wnt3a. We performed cell growth assays to determine effects on proliferation and viability once its use was optimized in our cells. MIAMI cells were treated with 0, 2.5, 5, or 10 ng/mL of rmWnt3a for 24 hours after a 24-hour period of serum starvation to determine an appropriate concentration. MIAMI cells responded to all levels of rmWnt3a. The expected increase in cell growth at low oxygen was observed. However, a more robust response to rmWnt3a was observed at 3% compared to 21% oxygen; possibly because the cells are primed and ready to respond to Wnt signal activation under low oxygen conditions; i.e., increased levels of receptors at low
oxygen (Figure 6C). We selected 5ng/mL as our optimal concentration for subsequent experiments as this concentration allowed for a 1.8-fold increase in cell growth in cells treated with rmWnt3a at 21% oxygen and a 1.4-fold increase in cells treated with rmWnt3a at 3% oxygen (Figure 6D). Interestingly, the Wnt3a effect on enhancing cell growth was seen at both 3% and 21% oxygen tensions. These data indicate that Wnt3a may act independently of the hypoxic effect and not mediate the low oxygen stimulation of proliferation and self-renewal in MIAMI cells. This is further substantiated by other comparisons made in Figure 6D; where cells grown at 3% and 21% oxygen without rmWnt3a grew in a similar manner as cells grown at 3% and 21% oxygen with rmWnt3a.

**Figure 6. MIAMI cells secrete Wnt3a and respond to exogenous Wnt3a treatment.** A) Diagram depicting ELISA preparation to detect Wnt3a protein in MIAMI conditioned media. B) Result of ELISA from MIAMI cell conditioned media taken from cells grown for 7 days at 3% and 21% oxygen. A media-only sample was run to determine baseline levels of Wnt3a. C) Optimization of rmWnt3a for exogenous treatments. Cells were expanded for 7 days at either 3% or 21% oxygen then synchronized by serum starvation for 24 hours. Cells received 0, 2.5, 5, or 10 ng/mL of rmWnt3a for 24 hours before harvesting for cell counts on a hemocytometer. D) Total cell numbers of cells seeded at 100 cells/cm² and grown at 3% or 21% oxygen with or without rmWnt3a treatment for 7 days.
To confirm the ability of MIAMI cells to activate canonical Wnt signaling at low oxygen tension, we used a TCF reporter plasmid TOPFLASH and its mutant counterpart, FOPFLASH (Upstate; Lake Placid, NY). TOPFLASH contains six
TCF binding sites (Figure 7A). The reporter plasmid tests for the transcriptional activity that occurs upon TCF binding by nuclear β-catenin. Luciferase activation allows for a quantitative measure of this activity. We transfected MIAMI cells by electroporation with 4µg of either TOPFLASH or FOPFLASH plasmids and cells were then grown to less than 50% confluency at 3% and 21% oxygen. Selected cells received treatment with 20mM LiCl for 1 hour, an agonist of the Wnt signaling pathway, just prior to harvesting. Canonical Wnt activity was detected by correlation to relative luciferase units measured (Figure 7B). Cells grown at 3% pO₂ showed an increased response (1.35-fold), while cells that received LiCl treatment grown at 3% pO₂ showed the greatest response (1.74-fold) (Figure 7B).

MIAMI cells were transfected with 2µg of pmaxGFP, a green fluorescent protein derived from the copepod Pontellina sp. that can be used as a positive control to monitor transfection efficiency. When cells are transfected with a GFP-containing plasmid along with another DNA plasmid, whose protein product does not glow, the success and extent of transfection can be monitored by using GFP as a reporter. pmaxGFP-transfected MIAMI cells remained viable and expressed GFP for up to 2 weeks; shown here at 24 hours and six days post-transfection (Figure 7C). MIAMI cells transfected with TOPFLASH reporter plasmid and treated with rmWnt3a for 1 to 3 hours showed stimulation of the canonical Wnt pathway as measured by luciferase activity. Cells displayed an increase of luciferase activity after 3 hours of treatment (Figure 7D) which is in agreement with others who have observed this trend (Qiang et al 2003). The use of LiCl, a
GSKβ inhibitor and activator of the canonical Wnt pathway, elicits a cellular response and activates luciferase as well. Here we used LiCl treatment in a set of pilot experiments to determine initial evidence of Wnt activity in MIAMI cells. We selected an extended time frame (24 hours) under the presumption that we would certainly capture any evidence of activity (Figure 7B). LiCl experiments were conducted before rmWnt3a was commercially available. Subsequent experiments were conducted with rmWnt3a in order to use a Wnt activating treatment that was more clearly defined.

**Figure 7. Wnt Reporter Plasmid TOPFLASH is activated by MIAMI cells.**
A) TOPFLASH 5.5 kb construct for luciferase reporter assays. B) MIAMI cells expanded at 3% or 21% oxygen for 7 days, treated with Wnt agonist, LiCl. Relative luciferase units reflect degree of TOPFLASH activity. C) MIAMI cells with pmaxGFP after 24 hours (top left), phase contrast (bottom left); after 6 days (top right), phase contrast (bottom right); 10x. D) TOPFLASH activity in MIAMI cells at 21% oxygen for 7 days with 5ng/mL rmWnt3a for 1, 2, or 3 hours.
Activation of Wnt signaling results in stabilization and nuclear translocation of β-catenin protein. Thus, nuclear localization of β-catenin has become the primary means to detect active canonical Wnt signaling. In 2001, Eberhart and Argarni analyzed the subcellular localization of β-catenin immunohistochemically in human fetal and post-natal tissues such as capillary endothelium and mesenchyme surrounding renal tubules. They identified activation of Wnt
signaling to be specific to these tissues and suggest that the Wnt pathway plays a clear role during fetal development based on the observation of β-catenin in the nucleus (Eberhart et al. 2001). In a like manner, we resolved to assess by nuclear localization of β-catenin, if there is active Wnt signaling in MIAMI cells. We analyzed two cell types for comparison; 293T cells (human embryonic kidney cells) and MIAMI cells (Figure 8A). Figure 8A, a representative blot from more than 3 independent experiments, shows levels of serine and threonine phosphorylation of β-catenin in relation to total β-catenin. As described earlier, β-catenin is phosphorylated at serine and threonine residues then degraded at the proteosome in order to regulate levels of the protein within the cytosol. However, with canonical Wnt signaling, β-catenin is not phosphorylated at serine and threonine residues and degraded; rather it accumulates in the cytoplasm and migrates to the nucleus. In cells where canonical Wnt is active, less serine/threonine-phosphorylated β-catenin is found in relation to total β-catenin. 293T cells were grown at 21% oxygen and MIAMI cells were grown at 3% and 21% oxygen for 7 days. 293T cells received treatment with Wnt pathway agonist, LiCl (20mM). As expected, 293T cells receiving LiCl treatment had less serine/threonine phosphorylated β-catenin than without (Figure 8A, lanes 1 & 2). MIAMI cells grown at 3% and 21% oxygen appear to have approximately the same levels of serine/threonine-phosphorylated β-catenin. However, in comparison to total β-catenin the 3% oxygen samples have less serine/threonine-phosphorylated β-catenin present (Figure 8A, lanes 3 & 4).
In other experiments, protein lysates were taken from MIAMI cells grown at 3% and 21% oxygen for 7 days with exogenous rmWnt3a treatment (5ng/mL). Shown in a representative blot from more than 3 experiments, cells grown at 21% oxygen showed a 1.4 fold increase in total β-catenin in the cytoplasm (Figure 8B, lanes 1 & 3). Nuclear lysates from cells grown at 21% oxygen showed a 2.3 fold increase in total β-catenin with rmWnt3a (Figure 8B, lanes 5 & 7). In cells grown at low oxygen without rmWnt3a treatment, a 2.1 fold increase was observed (Figure 8B, lanes 5 & 6). These data show that exogenous Wnt3a treatment in 21% MIAMI cells is similar to the effect of cells grown at 3% oxygen specifically regarding translocation of β-catenin into the nucleus.

Once β-catenin migrates to the nucleus; it binds a member of the high mobility group (HMG) DNA binding protein family of transcription factors, T cell Factor 4, (TCF4) to activate downstream Wnt targets. We investigated this association in MIAMI cells by performing immunoprecipitation and Western Blot analyses (Figure 8C). We pulled down protein with protein A/G beads that had been bound with antibody against TCF4. Subsequently, we probed the blots with antibody to β-catenin. In samples taken from MIAMI cells grown at 3% and 21% oxygen tension, we found that the 3% - low oxygen samples display more than 2-fold greater association between β-catenin and TCF4 (Figure 8C) compared to the 21% samples as shown in this representative blot of more than 3 experiments.

Once we observed activation of the canonical Wnt pathway, we looked for downstream activation of canonical Wnt pathway targets. We found that cell
cycle protein and direct Wnt target, Cyclin D1, was up by 1.36-fold in the cytoplasm - an effect of 3% oxygen tension and by 1.28-fold in the nucleus with rmWnt3a treatment. Oxygen alone had its greatest effect in the nucleus where Cyclin D1 increased more than 80-fold in (Figure 8D). Interestingly, there is virtually no difference in the nuclear samples at 21% and 3% oxygen with rmWnt3a; indicating that oxygen has greater influence on nuclear levels of Cyclin D1 than rmWnt3a. We also looked for Oct4 protein; another direct target of the canonical Wnt pathway (Figure 8E). Using the densitometry method described in the Methods/Western Blot section of this document, we measured an 8.6-fold increase of nuclear Oct4 in the 21% oxygen samples treated with rmWnt3a, and a 6.2-fold increase in nuclear Oct4 was measured in the 3% oxygen samples treated with rmWnt3a. These data show that Oct4 is indeed relocating to the nucleus under low oxygen conditions, however it does not clearly define the role of rmWnt3a under these conditions. Although it is possible that rmWnt3a may influence the entry of Oct4 into the nucleus, it is also possible that rmWnt3a may facilitate the degradation of the cytoplasmic Oct4 protein which would result in a greater nuclear to cytoplasmic concentration of Oct4. Additionally, an 8.6- versus 6.2-fold difference between rmWnt3a-treated 21% and 3% samples, is about 1.38-fold (8.6/6.2), which is the fold difference observed between 3% and 21% oxygen in the absence of rmWnt3a. This further supports a small but consistent increase in nuclear Oct 4 in response to low oxygen. However, before this point could be addressed, we set out to establish the isoform of Oct4 we were detecting by Western Blot. The sub-cellular localization of Oct4 protein in our
cells could identify the isoform present under low oxygen tension. Understanding what portion of this protein translocates into the nucleus, where its known activity occurs, could provide insight as to the level of Oct4 activity in the MIAMI cells.

Nuclear localization is a known factor in discriminating between the two isoforms of Oct4 (Oct4a and Oct4b). Oct4a has been shown by immunocytochemistry to be present in the nuclei of pluripotent stem cells and absent from non-pluripotent somatic cells (Atlasi et al. 2008). In this manner, we used immunofluorescence (IF) where we detected Oct4 (Figure 8F) in an attempt to confirm our Oct4 Western Blot results (Figure 8E). However, we detected what appears to be a much stronger nuclear signal with fluorescent dye, fluorescein isothiocyanate (FITC) at 3% oxygen (Figure 8F) compared to the nuclear localization of Oct4 measured by Western Blot (Figure 8E). This apparent discrepancy could be explained by one or several factors. First, the antibody that worked best for the Western Blot application was not from the same manufacturer as the one used for IF. It is also possible that the way in which proteins are processed for each of these applications (Western and IF) causes subtle differences in the antigen binding sites. Yet another explanation could be that while Western samples are all processed on a single blot, IF samples are processed on separate slides, which could introduce a new level of variability. Thus, the IF result serves solely as a confirmation of Oct4a nuclear accumulation to our Western Blot analysis, rather than a comparative quantitative assessment. Similar discrepancies have been documented in the literature by other groups using the same applications and conclusions have been made that IF elicits
more variability in results (Porsch, 2004 and Jensenius, 2004). In light of this information, we conclude that low oxygen increases nuclear accumulation of Oct4a by at least 1.3-fold. However, based on the IF data, this effect could be of a higher magnitude.

Canonical Wnt activity requires receptors, LRP5/6. Badders and colleagues showed that basal mammary cells express Lrp5 and co-express Lrp6 in a similar fashion and that the absence of Lrp5 specifically depletes adult regenerative stem cell activity to less than 1% (Badders et al. 2009). Increased expression of LRP5 has been shown to be sufficient for the activation of canonical Wnt.

**Figure 8. Nuclear localization of β-catenin, CyclinD1, and Oct4.**
A) Serine-threonine phosphorylated β-catenin in 293T cells with LiCl (lane 1) and without (Co = control, lane 2). MIAMI cells at 21% (lane 3) and 3% oxygen (lane 4). B) Cytoplasmic β-catenin (lanes 1-4); rmWnt3a treatment (+) or without (-) and 3% and 21% oxygen. Nuclear protein fractions (lanes 5-8). Coomassie stain is loading control. Numbers below blot represent β-catenin to total protein ratio. Fold change is ratio, treated:untreated sample. C) Immunoprecipitation of TCF4 and β-catenin in 200µg protein samples; 21% oxygen (lane1), 3% oxygen (lane2). Isotypic negative controls (normal rabbit serum); 21% samples (lane3), 3% (lane4). D) CyclinD1 cytoplasmic protein (lanes1-4); with rmWnt3a treatment (+) or without (-) and 3% and 21% oxygen. Nuclear protein (lanes5-8). Coomassie stain is loading control. Numbers below blot represent ratios as in 8B. E) Oct4 cytoplasmic protein (lanes 1-4); rmWnt3a treatment (+) or without (-) and 3% and 21% oxygen. Nuclear protein (lanes5-8). F) IF of Oct4-FITC in MIAMI cells at 3% and 21% oxygen in panel 1 (top and bottom). Nuclei stained with DAPI in panel 2 (top and bottom). Merged image is Oct4-FITC/DAPI. All 20X.
Figure 8B

Pulldown with TCF4, probed with β-catenin > 2-fold

Figure 8C

Pull-down with TCF4, probed with β-catenin
Figure 8D

Figure 8E
signaling pathway (Zhang et al. 2009). We have also detected similar protein levels of LRP5 and LRP6 in 3% and 21% MIAMI cells (Figure 9A). It is possible that changes to the protein level of these receptors occurs outside the time-point designated here. In light of our ELISA data that show equal secretion of Wnt3a at low and high oxygen tension (Figure 6B) and our Lrp5 mRNA data which shows an increase in mRNA transcript levels (Figure 5A), we decided to consider the possibility that Lrp5 could be the rate-limiting step in the oxygen-stimulated Wnt activity we observed in our cells. We started this process by knocking down expression of Lrp5 with sequence specific-siRNA. First, we optimized the concentration of siLrp5 for use in MIAMI cells with electroporation rather than continue with the chemical transfection, as the chemical transfection reagents caused excessive adherence of the cells to the flasks; which created a problem for cell growth assays to be conducted. We tested 0, 50, 100, 200 nM/mL and found that 100nM produced the most consistent results by dropping Lrp5 and Oct4a transcript levels (Figure 9B). Initial experiments performed showed a

Figure 8F

![Image of Figure 8F](image-url)
substantial reduction in mRNA levels of self-renewal and Wnt genes; Lrp5, Oct4a, Sox2, Nanog, Cyclin D1, and hTeRT in comparison to the negative siRNA control (Figure 9C). However, subsequent experiments reflect the variability in transcript levels for these genes although Sox2 and Lrp5 were found to be significantly reduced by siLrp5 knockdown (Figure 9D).

We used Western Blot analysis to determine post-siLrp5 expression of LRP5 and Oct4a/b proteins. We found a complete depletion in LRP5 protein (Figure 9E, top blot, lane 4) in comparison to the siRNA negative control (Figure 9F, top blot, lane 3) although transcription was reduced to approximately 60%. This result may seem paradoxical, however, the protein that presumably is made from the remaining 40% mRNA may be degraded or simply undetectable. siRNA may induce translational arrest of the remaining 40% LRP5 mRNA resulting in a decrease of LRP5 protein levels as well. The reduced mRNA may alert the cell that the demand for LRP5 protein has dropped. Under normal conditions, the LRP5 protein could serve in a positive feedback loop keeping its gene expression elevated under low oxygen conditions. Unexpectedly, we found that Oct4 protein expression appeared to not be affected after LRP5 knockdown (Figure 9E, center blot, lane 4). We deduce that Oct4 protein turnover may require more time than allotted by our experiments. Cells were initially transfected with siLrp5, left for 3 days to grow, then collected and transfected once again. Protein lysates were collected after this second transfection with siLRP5.

Next, we used immunofluorescence to determine the localization of the Oct4 protein previously observed by Western Blot in Figure 9E. We observed
nuclear localization of Oct4 in siControl cells (Figure 9F, bottom left) consistent with untreated cells grown at 3% pO₂ in Figure 8F. In siLrp5-treated cells, Oct4 protein did not accumulate in the nucleus or cytoplasm (Figure 9F, bottom right); an indication that Oct4a-specific nuclear levels decrease in the absence of LRP5. A diffuse area of Oct4 protein outside the nucleus was observed sporadically in cells transfected with siLrp5 compared to the negative siControl (Figure 9F, top row) with an overall reduced Oct4 signal as seen in untreated cells grown at 21% pO₂ in Figure 8F. Important to note is the inconsistency in Oct4 levels between the Western Blot in Figure 9E and the IF in Figure 9F. Because the antibody that worked best for our Western Blotting experiments was not from the same manufacturer as the one used for IF, we are not able to make conclusive comparisons. However, what can be taken from the data is the suggestion that Oct4 protein levels are maintained with siLRP5 (Figure 9E), while nuclear accumulation of Oct4a clearly decreases (Figure 9F), thus lending to the possibility of signaling alterations.

**Figure 9. siLRP5 reduces self-renewal mRNA but not protein.** A) Western Blot of LRP5 (left, top panel) and LRP6 (right, top panel) from cells grown for 7 days at 3% and 21%; Coomassie-stain is loading control (bottom panels). B) siLrp5 optimization using electroporation; top panel - mRNA transcript level of Lrp5; bottom panel is Oct4a mRNA transcript level C) mRNA transcript levels of Lrp5, Oct4, Sox2, Nanog, hTeRT in siLrp5-treated cells 3 days after transfection D) RT-qPCR determined mRNA levels of genes; Lrp5, Oct4a, Sox2, Nanog, Cyclin D1, and hTeRT. All genes normalized to ELFα; 21% oxygen samples set to 1. E) Western Blot of LRP5 (top panel) and Oct 4 (center panel) from siLrp5-treated cells at 3% oxygen; Lane 1: (Æ) Untransfected cells, Lane 2: (e-) Electroporated cells with no siRNA, Lane 3: (siCo) Electroporated cells with siControl, Lane 4: (siLrp5) Electroporated cells with siLRP5; Tubulin (bottom panel) is loading control. F) IF of cells treated with siControl or siLrp5 with anti-Oct4-FITC; DAPI stained nucleus; photos merged as indicated.
Figure 9D

Wnt and Self-Renewal mRNA with 100nM siLRP5 Knockdown

Figure 9E

LRP5  Oct4  Tubulin

0  e-  siCo  siLrp5

0.815  0.899

Oct 4
Figure 9F

siControl  siLRP5

Oct4/DAPI

DAPI

Oct4
Other Pathways Can Lead β-catenin to the Nucleus

Already established is that the β-catenin protein is an integral component of the canonical Wnt signaling pathway. However, β-catenin is a part of the adheren junction pathway that is required for the regulation cell growth and the adhesion between the cells of epithelial layers. It is an anchor of the actin cytoskeleton and is thought to transmit signals of contact inhibition in epithelial sheets. (Kraus et al. 1994). β-catenin has also been linked to regeneration of the liver in an Hepatocyte Growth Factor (HGF)-induced fashion. HGF and its receptor c-Met send β-catenin to the nucleus allowing it to trigger the expression of genes that are also targets of the canonical Wnt pathway. However, the phosphorylation state of β-catenin involved in HGF-mediated signaling occurs at tyrosine residues, namely, tyrosine-654; not at serine threonine residues known to be phosphorylated in canonical Wnt signaling (Lilien et al. 2005). The pathway is depicted in Figure 10A.

Since MIAMI cells secrete HGF and express the cMET tyrosine kinase receptor we were interested in examining if this pathway is involved in low oxygen-induced β-catenin nuclear translocation. In order to rule out the possibility that the β-catenin we have detected to be in the nucleus in MIAMI cells at low pO₂ is not the tyrosine-phosphorylated form, we looked for its presence in our cells. Using immunofluorescence and antibodies specific to tyrosine-654-phosphorylated β-catenin we were able to clearly visualize that although tyrosine-654-phosphorylated β-catenin is present at low levels in MIAMI cells at low pO₂, it is not localized to the nucleus (Figure 10B).
Yet another means of β-catenin nuclear localization can occur through the Pim family of proteins. Pims are serine/threonine kinases that phosphorylate and inactivate GSK3β, a key player in the APC complex of the canonical Wnt pathway (Mochizuki et al. 1997; Pain et al. 2005). Phosphorylation of GSK3β renders it inactive and unable to phosphorylate β-catenin at its serine/threonine residues. Serine/threonine phosphorylation by GSK3β sends β-catenin to the proteosome for degradation. Thus, by way of the Pim family of kinases, β-catenin could be sent to the nucleus due to its lack of phosphorylation, allowing it to transactivate downstream targets as seen with canonical Wnt signaling. Essentially, this would be a way to by-pass the ligand-dependent stimulation of the Wnt pathway while eliciting the same outcome as canonical Wnt signaling.

To determine if Pim tyrosine kinases could be upregulated by low pO₂, leading to GSK3β phosphorylation/inactivation causing β-catenin to localize to the nucleus in this way, we measured gene expression of the three known Pim isoforms (Pim 1, 2, and 3) as a function of oxygen tension. We observed a significant drop in the expression of the Pim 1 isoform in cells grown at 3% pO₂ compared to control cells grown at 21% pO₂. The Pim 2 isoform increased its expression at low oxygen tension and the Pim 3 isoform remained near the 21% pO₂ level (Figure 10C). These data suggest that Pim 2 may indeed play some role in the self-renewal activity of MIAMI cells at 3% pO₂. Further studies that investigate the downstream effects of over-expression and inhibition of Pim 2 would need to be conducted before any final conclusions can be drawn. However, those studies are beyond the scope of the current analysis.
Figure 10. Alternative routes of β-catenin translocation to the nucleus. A) Hepatocyte growth factor (HGF) binds to tyrosine kinase receptor, c-Met which then phosphorylates β-catenin at tyrosine 654. Tyrosine-phosphorylated β-catenin is sent to the nucleus where it binds TCF and transactivates downstream gene targets in a similar manner to canonical Wnt signaling. B) Tyrosine-654-phosphorylated β-catenin is visualized in MIAMI cells at 3% oxygen in the top panel with specific antibodies to Tyrosine-654-phosphorylated β-catenin and at 21% oxygen (center panel) in comparison to a negative isotypic control (bottom panel). All images merged with nuclear DAPI stain and taken at 20X C) mRNA expression of Pim isoforms using qRT-PCR in MIAMI cells grown at 3% and 21% oxygen. Samples normalized to housekeeping gene, ELF-α.
Oxygen Tension, NOTCH, and REST

As described in the introduction of this thesis, the Notch and REST pathways have been shown to be involved in the self-renewal of embryonic stem cells. Also, Notch has been linked to hypoxic inducible factor, HIF-1α. To determine whether or not these pathways are playing a role in the self-renewal of MIAMI cells at low pO₂, we measured the expression of key mediators along these pathways.

After initial experiments hinted at upregulation of genes in our cell system, we decided to look at the protein expression of the notch intracellular domain (NICD). NICD is cleaved and sent to the nucleus upon activation of the Notch pathway. It has been found to associate with HIF-1α once in the nucleus. We observed that NICD does indeed localize to the nucleus in MIAMI cells (Figure
11A). This increase in NICD is coordinated with an increase in HIF-1α in the nucleus in the same cells. Densitometric analysis indicates a 3.2-fold increase of NICD and a 3.8-fold increase of HIF-1α under hypoxic condition when normalized to appropriate controls.

We also investigated any involvement of the REST pathway by measuring gene expression of NRSF/REST and its co-activator, coREST and REST protein. Here, we found that both REST and coREST mRNA transcripts are upregulated at low pO₂ in MIAMI cells (Figure 11B). The REST protein is also upregulated by low oxygen. Figure 11C shows the presence of the active form of REST that localizes to the nucleus; adding further to the complexity of interactions involved in mediating the self-renewal mechanisms induced by low oxygen tension.

**Figure 11. Notch and REST in MIAMI Cells.** A) NICD detected by Western Blot of cells grown at 21% and 3% oxygen in the cytoplasm (cyto), lanes 1-2 and nucleus (nuc), lanes 3-4. Top panel is HIF-1α protein, center panel is NICD protein and bottom is the loading control, tubulin. Side table shows fold changes in NICD and HIF-1α protein. B) RT-qPCR of REST and coREST gene expression from cells grown at 3% and 21% oxygen for 7 days. C) REST detected by Western Blot of cells grown at 21% and 3% oxygen in the nucleus (nuc), lanes 1-2 and cytoplasm (cyto), lanes 3-4. Top panel is REST protein, bottom panel is the loading control, tubulin.

**Figure 11A**

<table>
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Figure 11B

REST/coREST Expression in MIAMI Cells with Hypoxia

Figure 11C

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Oxygen >

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Expression of Immature Markers in MIAMI Cells at Low Oxygen Tension

The work presented in this thesis describes the critical evaluation of the molecular mechanisms, primarily involving the canonical Wnt pathway, mediating the self-renewal of human stromal MIAMI cells at low oxygen tension in vitro. While the markers associated with a developmentally immature phenotype Oct4a, Nanog, Sox2, hTeRT and SSEA4 are characteristic of human ESCs, they are not expressed by all stem cells. This is an indication of the high degree of heterogeneity and plasticity of the gene expression network involved in maintaining diverse populations of multipotent or pluripotent cells in vivo or in vitro. Of these genes, Oct4, Nanog, and Sox2 have been shown to be the central regulatory factors controlling the transcriptional network mediating self-renewal in ESCs (Boyer et al. 2005). Although it is reasonable to assume a role for these genes in adult stem cell (ASC) self-renewal, little evidence has been obtained to demonstrate these gene products are involved in mediating this process in adult stem cell populations. This assessment becomes even more complicated by the discovery of pseudogenes for these factors. At least 6 highly homologous pseudogenes for Oct4 and 10 for Nanog have been identified by nucleotide BLAST (basic local alignment sequence tool) searches (Pain et al. 2005). Human hematopoietic stem cells derived from umbilical cord blood, for example, have been found to express transcripts for pseudogenes of Oct4, but are unable to trigger downstream expression of genes driven by the Oct4 promoter (Redshaw et al.). Thus, assessments based on RT-qPCR results are insufficient to reach
conclusions on gene expression and requires assessments at the protein level. In addition, Oct4 protein has at least two isoforms, Oct4a and Oct4b (Atlasi et al. 2008). The human OCT4 gene can generate at least three transcripts (Oct4a, Oct4b, and Oct4b1) and four protein isoforms (OCT4A, OCT4B-190, OCT4B-265, and OCT4B-164) by alternative splicing and alternative translation initiation. OCT4A is a transcription factor responsible for the pluripotency properties of embryonic stem (ES) cells. While OCT4b cannot sustain ES cell self-renewal, it may respond to cell stresses. Yet, the function of OCT4b1 is still unclear. Lack of distinction of OCT4 isoforms could lead to confusion and controversies on OCT4 in various tissues and cells (Wang et al.). Our assessment of Oct4a includes the use of Oct4a-specific primers expected to exclude all known isoforms and pseudogenes, several Oct4 antibodies including one against the N-terminus unique to Oct4a transcription factor demonstrating nuclear localization and the correct size of the functional transcription factor undistinguishable from that involved in ESC self-renewal and pluripotency. ESC extracts have been used as positive controls in numerous experiments. Overall, Oct4a, Nanog, Sox2, and hTeRT are detected at the mRNA levels and the respective proteins with the correct predicted molecular mass and subcellular localization, are expressed by MIAMI cells, while SSEA4 glycolipid molecules are detected on their membrane using specific antibodies. Furthermore, under low oxygen tension (1-3% pO$_2$) conditions, their levels are significantly increased. While Oct4a, Nanog, and Sox2 have been shown to be required for self-renewal and pluripotency in hESCs, we have not established their role in MIAMI cell self-renewal or pluripotency. In
contrast, hTeRT function in oxygen-stimulated telomere length maintenance in MIAMI cells has been demonstrated (D'Ippolito et al. 2006). Thus, the expression pattern of Oct4a, Nanog, Sox2, hTeRT and SSEA4 in MIAMI cells leads one to predict that these cells might maintain an immature stem cell phenotype and possibly make them capable of self-renewal under low oxygen conditions. In support of this notion, expression of these immature markers declines to undetectable levels upon MIAMI cells engaging in a differentiation program, such as osteoblastic (D'Ippolito et al. 2006), or neural (Tatard et al. 2007). In conclusion, here we present, and discuss the implications of our findings that examine the molecular mechanisms mediating the effects of low oxygen on the maintenance of self-renewal in MIAMI cells, defined by the sustained expression of Oct4, Nanog, Sox2, hTeRT and SSEA4.

We initially confirmed and expanded our original observations demonstrating that low oxygen tension (1-3% pO$_2$) not only upregulates mRNA transcripts for Oct4 transcription factor in MIAMI cells (D'Ippolito et al. 2006); but that the specific isoform upregulated was Oct4a, and in addition, Sox2 and Nanog transcripts appeared to be increased, but not to statistically significant levels, under low oxygen tension (3% pO$_2$) (Figure 5A). These data are in support of our previous findings that low oxygen tension (3% pO$_2$) led to an upregulation of mRNAs thought to be responsible for MIAMI stem cell self-renewal; Oct4a/b, Rex1, hTeRT, and Hypoxia Inducible Factor-1$\alpha$ (HIF1$\alpha$), a key mediator in pro-survival pathways during hypoxic conditions. Upregulation of these mRNAs was reported to occur even under osteogenic culture conditions in comparison to cells
grown at 21% oxygen (D'Ippolito et al. 2006). This original observation suggested that MIAMI cells are developmentally immature and that oxygen tension plays a key role in regulating their immature state in vitro. Based on these findings, we expanded our studies to determine that low oxygen tension also stimulates the expression of other known immature markers in MIAMI cells, such as Nanog, Sox2, and specifically Oct4a isoform and whether this stimulation was detectable at the protein level as well.

Protein levels of Oct4a, Nanog, and Sox2 were observed to be increased under low oxygen conditions (Figure 5B) providing further evidence of the role of low oxygen tension (1-3%) in self-renewal of the MIAMI cells. As indicated, all proteins were found to have the predicted molecular mass. The protein most dramatically induced was Sox2, which had the highest but most variable mRNA stimulation (Fig. 5A & B). In addition, Sox2 protein levels decreased to the lowest relative level when the cells were exposed to air. Sox2 levels were also the most affected and decreased to the lowest levels upon LRP5 inhibition (Figure 9D). In contrast, Nanog and Octa decreased upon exposure to air, but some expression was maintained (Figure 5B). This may be indicative of alternate splicing events and expression of splice variants. Interestingly, some cancer cells, such as gastric cancer cells, have been found to express abnormally high levels of Oct4 suggesting it may drive maintenance of an immature and proliferative phenotype (Chen et al. 2009). However, it remains to be established specifically which Oct4 isoform(s) is/are expressed in these tumor cells.
Implication of the Wnt Signaling Pathway Playing a Role in MIAMI Cell Oxygen-Stimulated Self-Renewal

In order to identify the molecular pathways that could be driving the maintenance of the immature phenotype (i.e., self-renewal) as a function of low oxygen tension, we examined pathways known to regulate self-renewal in other stem cell populations as well as key aspects of embryonic development, initially focusing on the Wnt signaling pathway (Hochedlinger et al. 2005). We measured expression levels of LRP5, a key co-receptor of the Wnt signaling pathway and other Wnt mediators and targets such as Wnt3a and CyclinD1 (Figure 5A and 5C). The upregulation of these genes at 3% oxygen in comparison to 21% oxygen provided us with the rationale to pursue examining the canonical Wnt pathway further in the context of low oxygen tension-stimulated self-renewal. In addition, these data set the MIAMI cells apart from MSCs that are routinely cultivated at 21% oxygen, by suggesting that a low oxygen environment stimulates a pathway known to be involved in development and cell growth. We also know that growing MIAMI cells at 3% pO$_2$ is essential to maintaining their neuronal differentiation potential. This is one of the main advantages of growing the cells at low oxygen. Interestingly, Sox2 is required for maintenance (i.e., self-renewal) of neural stem cells, and atmospheric oxygen tension decreased Sox2 expression to the largest extent (Fig. 5B). Growing MIAMI cells at 21% pO$_2$, causes not only a decrease of the immature markers, primarily Sox2, but also a loss of the neuronal potential, thus a decrease in pluripotency (Tatard et al. 2007).
The effects of low oxygen on proliferation with simultaneous inhibition of osteoblastic markers (D'Ippolito et al. 2006) is similar to the Wnt3a-mediated activation of canonical Wnt signaling in MSCs. Boland et al., showed that Wnt3a exposure inhibits MSC osteogenic differentiation and that such an effect was accompanied by an increase in cell number resulting from an increase in proliferation and a decrease in apoptosis, particularly during expansion of undifferentiated MSCs (Boland et al. 2004). We have not only shown that low oxygen tension promotes maintenance of primitive markers in MIAMI cells, but in addition, we have also shown for the first time that Wnt3a stimulates the expression of these ESC pluripotency markers in these cells. Thus, we expanded the observations regarding the role of Wnt3a as an activator of canonical Wnt signaling, as other investigators have shown in human marrow stromal cells (Boland et al. 2004; de Boer et al. 2004), by describing its effect on the expression of immature markers in MIAMI cells. We discovered that MIAMI cells produced Wnt3a to levels ranging between 9 and 10 ng/mL during expansion conditions at both 3% and 21% oxygen tension as detected by ELISA (Figure 6B). These data confirmed our real time quantitative PCR results (Figure 5B) suggesting that Wnt3a was being produced and secreted by MIAMI cells. However, the ELISA quantitative analysis did not detect a difference between Wnt3a production between cells grown at 3% vs. 21% oxygen tension. The similar release of Wnt3a at low and high oxygen suggested that Wnt3a is not the rate-limiting step stimulating the canonical Wnt regulation leading self-renewal in
MIAMI cells, at least at a relatively long time point of 7 days. It is possible, however unlikely, that cells release Wnt3a differently at earlier time points. Although this question still needs to be addressed, an alternative possibility is that the timing and the degree of regulation may be controlled by receptor availability, not the release of Wnt3a. Cells can produce and secrete a protein, however, if its corresponding receptor is not available to utilize it, the induced protein is not used and its physiological role is not relevant. Thus, energy efficiency dictates that the receptor levels could be regulating, at least in part, the outcome resulting from protein production, receptor binding, and activation of the pathway.

With the finding that MIAMI cells release Wnt3a, we sought to determine the dose response of MIAMI cells to increasing levels of the purified ligand. Previous studies had quantified the proliferative response of hMSCs to increasing amount of conditioned medium from Wnt3a-secreting cells (Boland et al. 2004). We treated MIAMI cells with exogenous recombinant mouse (rm) Wnt3a. Mouse Wnt3a shares 96% amino acid identity with the human protein. MIAMI cells responded to the recombinant protein in a dose-dependent fashion at both low and high oxygen tensions. From preliminary studies we selected 5ng/mL as our optimal concentration for subsequent experiments, as this concentration showed the greatest effect at 3% oxygen compared to 21% (Figure 6C). After repeating the growth cell experiments sufficient times for statistical significance we found that cell growth was enhanced by rmWnt3a treatment to similar levels at either 3% or 21% oxygen, an indication that Wnt3a can potentially take precedence
over the differentiation effects of 21% oxygen (Figure 6D). Furthermore, these results further support the notion that Wnt3a is not the rate limiting step stimulated by low oxygen leading to activation of the canonical Wnt signaling.

To confirm activation of canonical Wnt signaling at low oxygen tension even further, we used a TCF/Luciferase reporter plasmid TOPFLASH to test for TCF activity that occurs upon binding with nuclear β-catenin, a critical mediator of Wnt signaling that acts by entering the nucleus. Luciferase activation was measured in cells grown at 3% and 21% oxygen with selected cells receiving treatment with 20mM LiCl, an agonist of the Wnt signaling pathway, for 1 hour just prior to harvesting. Low oxygen (3%) increased Wnt activation in MIAMI cells by 1.35-fold in comparison to 21% oxygen and by 1.74-fold for 3% cells treated with the Wnt agonist LiCl (Figure 7B). Although these increases were modest, it is an indication that low oxygen has a stimulatory effect of the Wnt pathway and with the appropriate stimulation it can increase these effects even further.

**Confirmation of Wnt Signaling Pathway Playing a Role in Low-Oxygen Stimulated MIAMI Cell Self-Renewal**

The stabilization and nuclear translocation of β-catenin is the primary evidence to demonstrate active Wnt signaling in cells (Eberhart et al. 2001). Thus, we resolved to assess oxygen-stimulated nuclear localization of β-catenin in MIAMI cells. We analyzed levels of serine and threonine phosphorylation of β-catenin in relation to total levels of β-catenin in 293T cells (human embryonic kidney cells) and MIAMI cells (Figure 8A). Activation of canonical Wnt signaling decreases β-catenin phosphorylation at serine and threonine residues allowing it to migrate to the nucleus, demonstrating the presence of this pathway in MIAMI cells and its
activation. As expected, 293T cells receiving the LiCl agonist treatment had less phosphorylated β-catenin at its serine/threonine residues in comparison to 293T cells without LiCl treatment (Figure 8A, lanes 1 and 2). MIAMI cells grown at 3% and 21% oxygen tension have approximately the same levels of serine/threonine-phosphorylated β-catenin. However, with respect to the total amount of β-catenin present, the 3% oxygen samples have less serine/threonine-phosphorylated β-catenin present (Figure 8A, lanes 3 and 4). Thus, at low oxygen MIAMI cells have a higher ratio of unphosphorylated to serine/threonine-phosphorylated β-catenin, an important observation since the accumulation of cytoplasmic β-catenin is necessary for its subsequent translocation into the nucleus for Wnt activation.

We demonstrated the low oxygen mediated activation of canonical Wnt signaling by showing that exposure of MIAMI cells to low oxygen tension (3%), increased the nuclear accumulation of β-catenin about 2.1-fold compared to cells exposed to 21% oxygen (Fig. 8B, lanes 5 vs. 6). Just as LiCl can stimulate the Wnt pathway, exogenous treatment with Wnt ligands should do so as well if all components of the pathway are intact and functional. Based on this rationale, we treated the MIAMI cells with recombinant mouse (rm) Wnt3a (the human protein was not yet available at the time of study). We found that MIAMI cells grown at 3% and 21% oxygen tension for 7 days in the presence of 5ng/mL rmWnt3a exhibited a 1.4-fold increase in total β-catenin in the cytoplasm (Figure 8B, lanes 1 vs. 3). Within the nucleus, cells grown at 21% oxygen tension showed a 2.3-fold increase in total β-catenin with rmWnt3a treatment (Figure 8B, lanes 5 vs. 7).
The levels of low oxygen- vs. Wnt3a-stimulated nuclear accumulation of β-catenin were to similar extents, suggesting that low oxygen activates canonical Wnt signaling to levels comparable to those resulting from ligand activation. Our findings suggest that Wnt3a treatment may mimic the effects of low oxygen in MIAMI cells by causing the 21% cells to behave, to a certain extent, as if they were grown at 3% oxygen. Although our results are not conclusive, we are confident that the application of exogenous Wnt ligand has influence, much in the same way as low oxygen does, on the growth of these cells, thus implicating the ability of Wnt3a and/or oxygen to serve as effectors in altering a molecular mechanism involved in cell growth. However, we should also consider that low oxygen may activate other pathways (i.e., Notch, REST) in addition to canonical Wnt, which may be involved and necessary for other aspects of MIAMI cell self-renewal and growth.

During active canonical Wnt signaling, β-catenin migrates to the nucleus and binds a member of the TCF family of transcription factors activating downstream Wnt gene targets. We investigated potential β-catenin binding partners by studying the effect of low oxygen on nuclear β-catenin binding activity using immunoprecipitation and Western Blot analysis. We pulled down TCF4 protein binding partners under non-denaturing conditions using TCF4-specific antibodies, separated the complexes in a gel, and probed for β-catenin. In samples from MIAMI cells grown at 3% and 21% oxygen tension, we found that the 3% - low oxygen samples display at least 2.0-fold more association between β-catenin and TCF4 (Figure 8C) compared to the 21% samples. This increased
β-catenin/TCF4 binding interaction provides further evidence for canonical Wnt signaling stimulation at low oxygen tension in MIAMI cells. The low oxygen stimulated β-catenin/TCF4 binding appeared to be of greater magnitude than the β-catenin nuclear localization (2.0-fold vs. 2.1-fold), suggesting a higher affinity of β-catenin for TCF4 at low oxygen tensions, or that different β-catenin partners may be preferentially bound at lower or higher oxygen tensions. We could not find suitable antibodies to examine the potential interaction of β-catenin and other TCF proteins, though these studies warrant further analyses.

We found that the cell cycle protein, and direct Wnt target gene, CyclinD1, was stimulated at 3% oxygen and this stimulation was even more evident when we compare CyclinD1 levels in the nucleus (Fig. 8D lanes 5 vs. 6, ≥80-fold); although the nuclear levels overall were lower than the cytoplasmic levels (Fig. 8 lanes 5 & 6 vs. 1 & 2). In contrast, Wnt3a treatment had a minor stimulatory effect only evident in the cytoplasmic levels at 21% oxygen, but it was both nuclear and cytoplasmic at 3% oxygen (Figure 8D). These results suggest that low oxygen is a more potent stimulator of CyclinD1 expression than Wnt3a in MIAMI cells, again indicating that other pathways may be activated by low oxygen leading to CyclinD1 stimulation in addition to canonical Wnt signaling.

We also examined OCT4a/b protein; another potential direct target of the canonical Wnt pathway and an immature marker of MIAMI cells at low oxygen tension. As expected, low oxygen increased nuclear levels of Oct4 preferentially (Figure 8E, lanes 5 vs. 6, 1.3-fold), but to our surprise Wnt treatment increased 8.6-fold nuclear OCT4 levels at 21% oxygen (Fig. 8E, lanes 3 vs. 7); whereas a
6.2-fold increase was measured in the 3% oxygen samples (Fig. 8E, lanes 4 vs. 8). The lower fold difference at 3% oxygen may be due to the contribution of low oxygen to Oct4 nuclear localization and further suggests that low oxygen stimulates nuclear localization of OCT4. Overall, low oxygen stimulated nuclear localization of Oct4 in the presence or absence of Wnt3a. However, Wnt3a treatment dramatically decreased cytoplasmic levels of Oct4. This may be a previously unappreciated effect of this ligand on MSCs. Although the antibody used for these Western Blots recognized a protein of the predicted molecular mass of Oct4a (360 amino acids, 45-48kDa), we decided to confirm the effect of low oxygen on nuclear localization of Oct4a using antibodies specific to the unique Oct4a amino terminus combined with immunofluorescence.

OCT4a has already been shown to be present in the nuclei of pluripotent stem cells and absent from non-pluripotent somatic cells (Atlasi et al. 2008). We used immunofluorescence (Figure 8F) in an attempt to confirm our Western Blot results (Figure 8E). We detected a stronger nuclear signal in the 3% oxygen MIAMI cells in comparison to the 21% cells (Figure 8F), suggesting that low oxygen increases the nuclear active form of OCT4 protein, OCT4a, a key pluripotent transcription factor in ESCs and downstream target of canonical Wnt signaling in some systems. One plausible explanation for these results is that low oxygen causes a shift in the proportion and accumulation of the Oct4 splice isoforms not readily distinguishable in the Western Blot experiments. However, immunofluorescence indicates an increase in the nuclear accumulation of the Oct4a isoform in response to low oxygen, not evident in the Western Blot
experiments. This result was essential in establishing a link between low oxygen, Wnt signaling and immature markers in MIAMI cells.

Canonical Wnt activation requires the presence of the co-receptors, Lrp5 or Lrp6 (Badders et al. 2009). Increased expression of LRP5 has been shown to be sufficient for the activation of canonical Wnt signaling pathway (Zhang et al. 2009). We initially observed a 2- to 3-fold stimulation of LRP5 mRNA at low oxygen tension (Fig 5 A & C). We detected both LRP5 and LRP6 proteins by Western Blot in MIAMI cells.

Interestingly, LRP5 was minimally stimulated (1.1-fold) after culturing the cells for 7-days at 3% oxygen (Figure 9A). This small fold increase may be due to the proteins reaching steady-state levels after 7-days in culture blunting the effect of low oxygen stimulation. This apparent discrepancy may be resolved by quantifying the changes in LRP5 levels at different periods of time, from a few hours to a few days, after exposing the cells expanded at 21% oxygen to a 3% oxygen atmosphere. In light of our ELISA data that show equal secretion of Wnt3a at low and high oxygen tension (Figure 6A) and the Lrp5 mRNA (2-3-fold) and protein (1.1-fold) data which show oxygen stimulation, we decided to examine the possibility that Lrp5 could be the rate-limiting step in the low oxygen-induced Wnt activity we have observed in our cells. We knocked-down expression of Lrp5 with sequence specific-siRNA. One hundred nM of siLrp5 produced the most consistent results by dropping Lrp5 and Oct4a mRNA transcript levels (Figure 9B). Initial experiments performed showed a substantial reduction in mRNA levels of self-renewal and Wnt genes; Lrp5, Oct4a, Sox2,
Nanog, CyclinD1, and hTeRT in comparison to the negative siRNA control (Figure 9C). Subsequent experiments reflect the variability in mRNA transcript levels for these genes in MIAMI cells; however, Oct4a (p=0.05), Sox2 (p=0.01), and Lrp5 (p=0.03) were found to be consistently reduced when Lrp5 was knocked down (Figure 9D). Interestingly, these three genes are expressed upstream of the other genes studied here (Nanog, CyclinD1, and hTeRT). Oct 4 and Sox2 must be expressed to act together to bind the Nanog promoter for its activation in an autoregulatory loop (Boyer et al. 2005). CyclinD1 is a known downstream target of Wnt; canonical Wnt signaling must terminate before it can be stimulated (Tetsu et al. 1999). Recently, TeRT has been shown to be associated with β-catenin in the nucleus in mouse epithelial stem cells. Park and colleagues found an unanticipated role for telomerase reverse transcriptase as a cofactor and transcriptional modulator of the Wnt/β-catenin signaling pathway. Using chromatin immunoprecipitation of endogenous TeRT protein from mouse gastrointestinal tract epithelial cells, they demonstrated that TeRT physically occupied the promoters of Wnt-target genes (Park et al. 2009). In sum, the expression of Nanog, TeRT, and CyclinD1 most likely occurs after the expression of Oct4, Sox2 and Lrp5. Further studies that take into consideration a more delayed response from these genes will have to be conducted to determine an accurate timing of their expression.

Western Blot analysis was used to determine the effect of siLrp5 on the expression of Lrp5 and OCT4a/b proteins. There was a complete depletion in LRP5 protein (Figure 9E, top blot, lane 4) in comparison to the siRNA negative
control (Figure 9E, top blot, lane 3). Unexpectedly, we found that OCT4 protein expression was minimally affected after transient Lrp5 knockdown (Figure 9F). One way to explain this unexpected result is that OCT4 protein turnover occurs slowly and requires a more sustained Lrp5 inhibition than the time allotted in these experiments. Cells were initially transfected with siLrp5, left for 3 days to grow, then collected and transfected once again. Protein lysates were collected after this second transfection with siLrp5. However, we decided to investigate the possibility that siLrp5 may modify the ratio of nuclear/cytoplasmic OCT4 isoforms.

Sub-cellular localization of OCT4 isoforms by immunofluorescence determined that OCT4b protein accumulated outside of the nucleus in siLrp5-treated cells (Figure 9F, right panels). Nuclear localization of OCT4 was more evident in the negative siControl (Figure 9F, left panels) indicating the dependence of the transcriptionally active form, OCT4a, on canonical Wnt signaling in a low oxygen environment. This observation presents the possibility that loss of Lrp5 may alter the splicing mechanism of OCT4 allowing for more of the OCT4b splice variant and less of the OCT4a to be produced.

In order to examine the possibility that other pathways could be directing β-catenin to the nucleus leading to activation of Wnt targets, we investigated two alternative pathways that could potentially act in this way; HGF/c-Met and Pim. The phosphorylation state of β-catenin involved in HGF-mediated signaling occurs at tyrosine residues, namely, tyrosine-654; not at serine/threonine residues known to be phosphorylated in canonical Wnt signaling (Lilien et al.)
To rule out the possibility that the β-catenin we have detected in the nucleus of MIAMI cells at low oxygen tension is not the tyrosine-phosphorylated form, we looked for its presence in our cells. Using immunofluorescence and antibodies specific to tyrosine-654-phosphorylated β-catenin we were able to clearly visualize that although tyrosine-654-phosphorylated β-catenin is present in MIAMI cells at low oxygen tension, it is not localized to the nucleus (Figure 10B). The tyrosine-654-phosphorylated β-catenin that is present within the cytoplasm of MIAMI cells is most likely being utilized for migration as described in other systems by Lilien and colleagues (Lilien et al. 2005; Park et al. 2009).

As previously indicated, Pim serine/threonine kinases are transcriptionally regulated enzymes which phosphorylate and inactivate GSK3β, leading to accumulation of cytoplasmic β-catenin and subsequent β-catenin nuclear translocation. This pathway by-passes the ligand-dependent stimulation of the Wnt pathway while eliciting the same downstream results. We found that low oxygen tension increased Pim2 levels (Figure 10C). These data suggest that Pim2 could indeed play a role in nuclear localization of β-catenin leading to self-renewal in MIAMI cells at 3% oxygen tension. Further studies designed to investigate the downstream effects of over-expression and inhibition of Pim2 would need to be conducted before any conclusions can be drawn. However, activation of Pim2 does not exclude the evidence that supports Wnt activation in MIAMI cells at low oxygen tension and its effect on self-renewal targets.

Like Wnt, the Notch and REST pathways have also been shown to be involved in the self-renewal of developmentally immature pluripotent stem cells,
although they act through their own distinct mediators. Notch has been linked to hypoxica inducible factor, HIF1α. To determine whether or not these pathways could be playing a role in the self-renewal of MIAMI cells at low oxygen tension, we measured the expression of key mediators along these pathways. Initial experiments hinted at upregulation of Notch genes in MIAMI cells. Subsequently, we looked at the protein expression of the notch intracellular domain (NICD). As previously indicated, NICD is cleaved and sent to the nucleus upon activation of the Notch pathway and can associate with HIF1α once in the nucleus. We found evidence that NICD does indeed localize to the nucleus in MIAMI cells at low oxygen tension (Figure 11A), an increase that is coordinated with an increase in HIF1α in the nucleus in the same cells. Albeit the detection of HIF1α protein is difficult as its degradation begins within minutes upon exposure to atmospheric oxygen (Berra et al. 2001). Densitometric analysis indicated a 3.2-fold increase of NICD and a 3.8-fold increase of HIF1α in response to low oxygen. Thus, Notch signaling is indeed activated at low oxygen tension in MIAMI cells. However, further studies are required to determine the potential role Notch in mediating low oxygen-induced self-renewal.

We also investigated any involvement of the REST pathway by measuring gene expression of NRSF/REST and its co-activator, coREST and REST protein. We found that both REST and coREST genes are upregulated at low oxygen tension in MIAMI cells as well as the REST protein. The nuclear localization shown in Figure 11C is indicative of its activity; adding further to the complexity of interactions involved in Wnt signaling.
We have proposed two possible mechanisms describing the interplay by which these pathways may be regulating self-renewal at low oxygen tension in MIAMI cells (Figure 12A and B). Taken together, the data show that low oxygen tension in MIAMI cells causes an upregulation of LRP5 gene expression. With decreased serine/threonine phosphorylation, β-catenin accumulates in the cytoplasm and translocates to the nucleus. There, β-catenin associates with the co-activator TCF4, triggering the activation of Wnt downstream targets (Oct4, Sox2, Nanog, CyclinD1). More specifically, the ratio of splice variants OCT4a to OCT4b appears to be altered. The observation of similar levels of total OCT4 protein by Western Blot on siLRP5-transfected MIAMI cells indicates that although OCT4 protein is maintained, its localization is affected as seen by immunofluorescence. Another gene, hTeRT is also increased by low oxygen tension. It is possible that the observed increase may be indicative of its greater promoter co-occupancy of some of the Wnt downstream genes (Park et al. 2009), co-stimulating their expression. However, binding assays will have to be performed to examine this possibility to determine how hTeRT might fit in with our model in Figure 12A.

We have shown that low oxygen tension has an effect on the Notch and REST pathways. How they impact β-catenin and its nuclear translocation and how they fit into our model in Figure 12A is not yet clear. Each of these pathways are of particular interest to the study of stem cell self-renewal at low oxygen tension. It is known that Notch has a direct relationship with the hypoxic response by binding HIF1α in the nucleus and REST is a direct target of the canonical Wnt pathway and a known transcriptional repressor of neuronal genes. However, the
preliminary experiments shown here must be followed by more extensive studies to understand the link to self-renewal in MIAMI cells. Such studies should include knocking-down the expression of Notch and REST to assess downstream Wnt and self-renewal targets, specifically, Oct4a, Sox2, Nanog and hTeRT. Our model in Figure 12A shows how the initial stimulation of self-renewal could begin with low oxygen tension. Investigation of the mediators specifically involved in the hypoxic response could elucidate the mechanism by which the depicted pathways interact. In fact, it is possible that it is one of the hypoxia-inducible factors that is the link that allows for cross-talk to occur between these pathways. HIF1α shares considerable sequence homology with another hypoxia-inducible factor, HIF2α, which has been shown to regulate OCT4 in mice. A knock-in strategy was used by Covello and colleagues to demonstrate that targeted replacement of oxygen-regulated transcription factor HIF1α with HIF2α resulted in expanded expression of OCT4 (Covello, 2006) giving credence to the notion that hypoxia is actively involved in OCT4-related mechanisms.

An alternative model shown in Figure 12B by-passes canonical Wnt altogether and acts through Pim2 (a tyrosine kinase that inhibits GSK3β). The possible scenario involving low oxygen-induced Pim2 provides an alternative means by which β-catenin could accumulate then migrate into the nucleus in a canonical Wnt – like manner. This model does not depend on the LRP5 receptor nor the APC complex to inhibit GSK3β as canonical Wnt signaling does. Rather, Pim2 comes in mid-stream to phosphorylate and inhibit GSK3β, thus allowing for β-
catenin to escape degradation, build up in the cytoplasm and migrate to the nucleus with the same downstream effects of canonical Wnt signaling.

In sum, our studies have shown, as displayed in Figures 12A and 13, that LRP5 is a key player in regulating canonical Wnt signaling and its self-renewal effects under low oxygen conditions in MIAMI cells. We have shown evidence that this pathway most likely is not working alone, rather, a complex interaction involving Notch and REST is more reasonable. Finally, we have shown that at the crux of this problem is oxygen. How it influences stem cell behavior whether in its presence or absence could have major implications on our understanding of stem cell self-renewal.
Figure 12. Proposed Models Depicting Interplay of Self-Renewal Pathways in MIAMI cells at Low Oxygen Tension. A) Model A; Wnt, Notch, and REST pathways act in parallel or sequence to stimulate the nuclear localization of β-catenin. B) Model B; Low oxygen acts on Pim2 to stimulate the nuclear localization of β-catenin.
Figure 13. Summary of Data. Flow chart of observations made in MIAMI cells at low oxygen tension. Mediators indicated are participants in the Wnt, Notch, and REST pathways.
REFERENCES


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