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Building Muscle: The Role of Insulin-Like Growth Factor Binding Protein-6 and Insulin-Like Growth Factors in the Differentiation of Placental Mesenchymal Stem Cells into Skeletal Muscle

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Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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ABSTRACT

Human placenta is a readily available source for isolation of adult mesenchymal stem cell (MSC), for potential use in regenerative therapies. MSC fate is influenced by the microenvironment in vivo, and insulin-like growth factors (IGFs) are critical components of the stem cell niche, as they regulate proliferation and differentiation into different lineages including bone, fat, and skeletal muscle. Insulin-like growth factor binding protein-6 (IGFBP-6), relative to other IGFBPs, has high affinity for IGF2 and is believed to be the main modulator of IGF-2 function. However, the role of IGFBP-6 in muscle development has not been clearly defined. In this study, we investigated the role of IGFBP-6 in different stages of muscle commitment and differentiation using human mesenchymal stem cells derived from the placenta (PMSCs). Our central hypothesis is that IGFBP-6 regulates the maintenance of multipotency in PMSCs and also promotes PMSC differentiation into muscle via intracellular and extracellular interactions in both IGF-dependent and independent mechanisms. We used immunoblotting, immunocytochemistry, ELISA, and ALDH-activity to evaluate IGFBP-6 effects on PMSC muscle differentiation. We showed that PMSCs are capable of differentiating into muscle cells when exposed to muscle-specific differentiation medium characterized by the decrease of pluripotency-associated markers (OCT4 and SOX2) and the gain of expression of muscle markers Pax3/7, MyoD, Myogenin, and Myosin heavy chain in a time-dependent manner and eventually forming multi-nucleated fibers. Extracellular supplementation with IGFBP-6 during culture increased muscle differentiation markers levels in early stages. The opposite effects were observed when IGFBP-6 was silenced and was rescued by increasing IGFBP-6. We also showed that IGFBP-6 had impact on
muscle differentiation in both IGF-dependent and -independent mechanisms. IGF-1 and IGF-2 had different effects on muscle differentiation with IGF-1 promoting multipotency and early commitment to muscle, whereas IGF-2 promoting muscle differentiation. Muscle differentiation required activation of both AKT and MAPK pathways. Interestingly, we demonstrated that IGFBP-6 could compensate for IGF-2 loss and help enhance the muscle differentiation process by triggering predominantly the MAPK pathway independent of activating either IGF-1R or insulin receptor (IR). These findings indicate complex interactions between IGFBP-6 and IGFs in PMSC differentiation into skeletal muscle. The most prominent effects were observed early in the differentiation process, before muscle lineage commitment. This knowledge on how myogenesis can be manipulated using IGFs and IGFBP-6 will aid in the development of improved muscle regeneration therapies using stem cells from human placenta.

**KEYWORDS**

IGF-1, IGF-2, IGFBP-6, IGF-1R, Insulin receptor, OCT4, SOX2, MyoD, Myogenin, Myosin heavy chain, Pax3/7, Placental mesenchymal stem cells, skeletal muscle differentiation.
CO-AUTHORSHIP STATEMENT

 Portions of Chapter one (included in the published review) were written by Dr. Amer Youssef and I, then modified based on review by Dr. Victor Han and Dr. David Hess. Chapter one sections, not included in the review, were written by me and modified based on review by Dr. Victor Han and Dr. David Hess.

 All other chapters were written by me and modified based on review by Dr. Victor Han and Dr. David Hess.
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<table>
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<th>Description</th>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>α</td>
<td>Alpha</td>
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<tr>
<td>β</td>
<td>Beta</td>
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<tr>
<td>m</td>
<td>Milli</td>
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<tr>
<td>μ</td>
<td>Micro</td>
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<td>n</td>
<td>Nano</td>
</tr>
<tr>
<td>λ</td>
<td>Wavelength</td>
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<tr>
<td>AKT</td>
<td>Serine/threonine protein kinase B</td>
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<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase activity</td>
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<td>ALP</td>
<td>Alkaline phosphatase</td>
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<td>Amperes</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblastic growth factor</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>C terminal</td>
<td>Carboxy-terminal</td>
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<tr>
<td>CHO</td>
<td>Chinese Hamster ovary cell line</td>
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<tr>
<td>DEAB</td>
<td>Diethy lam inobenzaldehyde</td>
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<tr>
<td>dH₂O</td>
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<tr>
<td>DMEM-F12</td>
<td>Dulbecco’s modified eagle medium and nutrient</td>
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<tr>
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<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
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<tr>
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<td>ECL</td>
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<td>Ethylene diamine tetraacetic acid</td>
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<td>EGF</td>
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<td>ELISA</td>
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<td>Extracellular signal-regulated kinase</td>
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<td>ES</td>
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<td>LY294002</td>
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<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidyl-inositol-3-kinase</td>
</tr>
<tr>
<td>PMSC</td>
<td>Placenta-derived mesenchymal stem cell</td>
</tr>
<tr>
<td>PPP</td>
<td>Picropodophyllin</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RD</td>
<td>Rhabdomyosarcoma derived</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel Electrophoresis</td>
</tr>
<tr>
<td>SHC</td>
<td>Src-homology 2 containing protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SOX2</td>
<td>SRY-related HMG box 2</td>
</tr>
<tr>
<td>SSEA</td>
<td>Stage specific embryonic antigen</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transduction</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline supplemented with 0.05% Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>U0126</td>
<td>1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
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CHAPTER ONE

INTRODUCTION

This chapter contains portions of a review that was published in Stem Cells International.

1.1 Stem Cells

1.1.1 Overview

Many tissues contain adult multipotent stromal cells, also known as mesenchymal stem cells (MSCs), a cell type with great potential in tissue regeneration therapies. However, the MSC availability in most tissues is limited which demands extensive expansion \textit{in vitro} following isolation. Like many developing progenitor cell types, the function of MSCs is affected by the surrounding microenvironment, and mimicking natural microenvironmental cues that support multipotent or differentiated states \textit{in vivo} is essential for the successful use of MSC in regenerative therapies. Many researchers are, therefore, optimizing cell culture conditions \textit{in vitro} by altering growth factors, extracellular matrices, chemicals, oxygen tension, and pH to enhance stem cell expansion or differentiation. The insulin-like growth factors (IGFs) system has been demonstrated to play an important role in MSC biology to either promote cell division (self-renewal) or to enhance differentiation onset and outcome, depending on the cell culture conditions. In this chapter, we will review the importance of the IGFs system and its signaling partners during development, and describe how these pathways affect differentiation towards multiple lineages in the MSC niche.

Currently, many degenerative diseases which are associated with organ failure are untreatable by pharmaceuticals or organ replacement, have been impacted by the promise of cell-replacement or tissue regenerative therapies (1). Such diseases include endocrine diseases (diabetes), neurodegenerative diseases (Parkinson’s, Alzheimer’s and Huntington’s), and cardiovascular diseases (myocardial infarction and peripheral
vascular ischemia) or chronic conditions in the cornea, skeletal muscle, skin, joints and bones (2). Stem cells have the potential to replace damaged cells, or to send paracrine signals that promote the survival and function of damaged tissues (3). In addition, with the ability to generate induced pluripotent stem cells from the recipient’s own somatic cells (4-6) and the availability of new gene editing technologies (e.g. CRISPR-Cas9 and TALEN) (7,8); the power of stem cell therapy for many genetic and acquired diseases, is closer to reality than ever before.

1.1.2 Adult Mesenchymal Stem Cells (MSCs)

Adult mesenchymal stem cells (MSCs) are multipotent cells that are highly proliferative in culture, adherent to plastic, and differentiate into many cell types including bone, fat, cartilage, and muscle. Unlike embryonic stem cells, MSCs have less ethical controversies and lower tumorigenicity; however, they have restricted differentiation potential (9). Recent research has also demonstrated rare transdifferentiation ability of MSCs from cells of one germ layer to another (10). In addition, MSCs consistently demonstrate an immunomodulatory effect to reduce inflammation and are able to be engrafted successfully in therapy resistant graft-versus-host disease (3). The existence of multipotent stem and progenitor cells in adult tissues was first described in the hematopoietic system by Till and McCulloch in 1961 (11) and was followed by the isolation of MSCs from bone marrow by Friedenstein in 1968 (12). Since then, MSCs have been isolated from many mature organs and tissues including skeletal muscle (13), adipose tissue (14), deciduous teeth (15), umbilical cord blood and placenta (16), peripheral blood (17), and brain (18). Several biological markers characterize MSCs of
different origins to be positive for CD73, CD105, CD29, CD44, CD71, CD90, CD106, CD120a, and CD124, and negative for CD117, CD34, CD45 and CD14 (19-21). MSCs have been demonstrated to differentiate predominantly into mesodermal cells including osteogenic, chondrogenic, adipogenic (22), endothelial (23), and myogenic (24) lineages. Also, MSCs can differentiate towards ectodermal lineages including corneal (25, 26) and neuronal cells in vitro (27).

1.1.3 Placental Mesenchymal Stem Cells (PMSCs)

Placenta is a vital organ for the embryo, acting as the interface between the fetal and maternal environments for the transportation of nutrients and substrates (e.g. oxygen), the removal of metabolic waste, and the production of endocrine hormones essential for the maintenance of pregnancy (28). Unlike the early embryo from which human embryonic stem cells are derived, placenta is a non-controversial discarded tissue that is a readily available source of MSCs for regenerative therapy in human patients (29, 30, 31). At 6-7 days post fertilization, embryonic mesenchymal cells infiltrate the cytotrophoblast layer. These cells in conjunction with trophoblast stem cells, invade the maternal endometrium for implantation and initiation of placental development (32, 33). There are four types of tissues of the placenta from which stem cells can be isolated; amnion, chorion, umbilical cord, and fetal membranes (32, 34, 35), each found in a different niche location with the chorionic villi being the largest source (32, 34).

The MSCs used in this study, were isolated from the main functional unit of the placenta, the chorionic villi (Figure 1.1). Each chorionic villus is composed of three layers of cells: villous trophoblast (syncytiotrophoblasts and cytotrophoblasts), villous stroma
(mesenchymal cells, Hofbauer cells, and fibroblasts), and fetal vascular endothelium (endothelial cells) (36). Chorionic villi-derived cells are the earliest fetal material obtained for prenatal diagnosis of fetal disorders (chorionic villous sampling) (37), and the most versatile stem cell type in the placenta (38). Furthermore, chorionic villi MSCs or placental MSCs (PMSCs) have greater cell proliferation and differentiation capacity in vitro compared to bone marrow MSCs (39, 40).

To define the MSC population in vitro, the International Society for Cellular Therapy established three required criteria, namely that MSC must be a) plastic-adherent when maintained in standard culture conditions, b) express CD105, CD73 (stromal and vascular markers), and CD90 (fibroblast markers) and lack hematopoietic markers such as CD45 and CD34, and c) be capable of differentiation into osteoblasts, adipocytes, and chondroblasts in vitro (39, 41, 42). Therefore, MSCs isolated from the human placenta must be validated before use.

In this study, PMSCs isolated from the chorionic villi of preterm placentae (15-20 weeks) tissue were tested for MSC criteria. Cultured PMSCs were clonally selected, highly proliferative and adherent to plastic, and positive for the mesenchymal markers CD73 and CD105 and did not express the hematopoietic marker c-kit (Appendix 4). Furthermore, PMSCs isolated efficiently differentiated into osteoblasts and adipocytes; confirming multipotent differentiation of PMSCs (Appendix 4). Thus, PMSCs represent a promising cell type for the development of tissue regeneration therapy, due to early ontogeny and multipotent potential.
Figure 1.1. The human placenta is a source of chorionic villi derived placental mesenchymal stem cells (PMSCs). A cross section of the placenta shows different placental structures. The intervillous space is filled with maternal blood from maternal arterioles for nutrient and waste exchange between the fetal and maternal circulation. The umbilical cord carries fetal blood to the fetal capillaries within the chorionic villi, which is the main functional interface between the maternal and fetal environments. There are seven types of cells in the chorionic villi: endothelial cells, pericytes, mesenchymal stem cells, Hofbauer cells, cytotrophoblasts, stromal fibroblasts, and syncytiotrophoblasts. PMSCs are found in the perivascular region of the fetal capillaries within the chorionic villi.

1.1.4 Stem Cell Niche

The stem cell “niche” is a paracellular microenvironment that includes cellular and non-cellular components from local and systemic sources that regulate stem cell multipotency, proliferation, differentiation, survival and localization (43). Stem cells are maintained by the surrounding microenvironment via cues including physical, structural, humoral, paracrine, autocrine, and metabolic interactions (44). Therefore, a combination of microenvironmental signals generated during development, healing or diseases regulate tissue regeneration leading to cell proliferation, differentiation or quiescence (45). This thesis focuses on the role of insulin-like growth factors (IGFs) system in the MSC niche (Figure 1.2).

Insulin-like growth factors are the early effectors that impact cell fate; therefore, controlling the availability of growth factors in the stem cell microenvironment is beneficial for manipulating stem cells in vitro. This study will focus on the effects of insulin like growth factors (IGFs) and their binding proteins (IGFBPs), IGFBP-6 in particular, as components of the stem cell niche.
**Figure 1.2. Stem cell niche.** The stem cell niche is a complex compartment directing mesenchymal stem cell (MSC) differentiation and function via cellular and acellular components. Various signals are exchanged between MSCs, mature stromal cells, other progenitor cell types, and the extracellular matrix containing variable soluble factors, ECM composition, oxygen tension, and pH. Therefore, the MSC niche manipulates the state of MSCs following growth and regeneration demand. IGFs can signal via paracrine/autocrine (produced locally by the tissue) or endocrine (delivered by blood supply) routes and interact with IGF-1 receptor, IGF-2 receptor, or the insulin receptor on MSCs and other cells. IGFBPs (extracellular and/or intracellular) can modify IGF actions and affect their stability and degradation. Other receptors and integrins are also expressed in MSCs and can be affected by extracellular microenvironment. MSC differentiation occurs by the shutdown of pluripotency-associated genes, such as OCT4, SOX2, and NANOG, for the up-regulation of differentiation genes. For example, MSCs can give rise to all mesodermal lineages depending on transcription factor expression to generate adipose, cartilage, bone, and muscle.
1.2  Insulin-like Growth Factor System (IGF)

1.2.1  IGF System: Ligands, Receptors and Binding Proteins

Insulin-like growth factors (IGFs; IGF-1 and -2) are small polypeptides (~7 kDa) that regulate survival, self-renewal, and differentiation of many cell types, including stem cells (46). In the systemic circulation in postnatal life, IGF-1 levels are regulated by growth hormone (GH), which induces IGF expression and release by the liver and accounts for 70-90% of circulating IGFs (47). Knockdown of Igf-1 in the postnatal murine liver resulted in a 75% reduction in circulating IGF-1 levels that was accompanied by a compensatory 4-fold increase in GH, which can lead to insulin resistance (48). Even in the absence of hepatic IGF-1, postnatal growth was not affected in mice. This is likely due to extra-hepatic expression of IGFs in a paracrine/autocrine fashion, such as in the bone, brain, lung, uterus, ovaries, adipose tissue, and muscle (49). Under this condition, serum IGF concentrations were regulated by several factors including gender, age, and nutrition status, leading to a variable concentration range of IGF-1 (264-789 ng/mL) and IGF-2 (702-1042 ng/mL) in healthy individuals (50). In prenatal (embryonic/fetal) life, the regulation of the synthesis of IGF-1 and IGF-2 by many different organs and tissues is not well understood. Most likely, the synthesis is regulated by local (paracrine) factors and cues such as nutrient status, oxygen tension, extracellular matrix, and other growth factors in addition to endocrine factors. Importantly, the IGFs are synthesized as required by the developmental and physiological cues within the extracellular and intracellular environment. It is likely that the fate of mesenchymal stem cells which reside in the paracellular niches in adult tissues is
regulated by IGF signals in the tissue microenvironment.

At the molecular level, IGF-1 shares more than 60% sequence homology with IGF-2 and 50% with pro-insulin (51, 52). IGFs signal mainly via the IGF-1 receptor (IGF-1R), which has the highest binding affinity (Kd of 1 nM) towards IGF-1, with 10-fold lower affinity to IGF-2 (53). IGF-1R is a receptor-tyrosine-kinase (RTK) which shares a structural homology domain with the insulin receptor (IR). In turn, IR is expressed in two isoforms IR-A and IR-B, and can form hybrid receptors (HR-A and HR-B) with the IGF-1R, which bind to both IGFs with variable affinities (54). Unlike IGF-1, IGF-2 binds to its specific receptor, IGF-2R, and, similar to insulin, it can bind to IR-A (55). IGF-1R, IR and HRs are mitogenic RTKs while IGF-2R is not. Therefore, different receptor and ligand combinations can cause variable signaling outcomes, especially in stem cells. Few studies have been reported on the effects of IGF-1 on the growth, differentiation, and migratory capacity of MSCs (56-58); however, the expression of different IGF, insulin, and hybrid receptors and their roles in cell potency and differentiation has not been well studied.

Circulating IGFs are also regulated by binding to six soluble (~30 kDa) IGF-binding proteins (IGFBPs, 1-6), which determine the bioavailability of free IGF ligand in the vicinity of the receptors, thus modifying IGF actions (59). Under normal physiological conditions, IGFs bind IGFBPs with greater affinity than they bind IGF-1R (60-62). IGFBPs interaction with IGFs occurs via non-covalent binding (63) that protects them from degradation, increases their half-life (64, 65), and facilitates delivery to specific tissues. Therefore, IGFBPs play an important role in IGF-regulated cell metabolism, development, and growth (66). In addition, it has become apparent that IGFBPs are
expressed and maintained within the cellular microenvironment and have additional functions independent of regulating IGFs (67). The goal of this thesis is to delineate the role of IGFBPs, specifically IGFBP-6, in MSC fate-decisions.

1.2.2 IGF System: Signaling Cascades

IGF-1R is a trans-membrane tetramer receptor that exists as heterodimers composed of two \( \alpha \) and \( \beta \) hemireceptors linked by disulfide bonds in a \( \beta-\alpha-\alpha-\beta \) structure (68). Upon ligand binding, the downstream signal of IGF-1R is dependent on the differential phosphorylation of its \( \beta \)-subunit and the resultant tyrosine residues become available to initiate mitogenic signals, mainly through the phosphoinositide 3-kinase (PI3K), AKT/PKB and the extracellular signal-regulated kinase (ERK1/2) (66, 69, 70). In this manner, IGF-1R can induce transcriptional activity to promote survival and differentiation of MSCs (71, 72).

Upon activation of the extracellular \( \alpha \) subunits of the IGF-1R, autophosphorylation of tyrosine residues on the \( \beta \)-subunits creates high affinity binding sites for signaling adaptor molecules and substrates. For the ERK1/2 signaling pathway, SHC interacts directly with the IGF-1R\( \beta \) which recruits GRB2 to interact with SOS that subsequently activates c-RAS leading to the sequential phosphorylation of RAF, MEK1/2 and ERK1/2 (51, 73-77). To activate the PI3K/AKT signaling, p85, the regulatory subunit of PI3K, interacts directly with IGF-1R\( \beta \) independent of SHC binding (78). IRS-1 is a main target of the IGF-1R, implicated in the mitogenic effect of IGF-1R, inhibition of apoptosis, and transformation. Conversely, IR-1 down-regulation has been associated with the inhibition of differentiation and the induction of apoptosis (69, 79). The phosphorylation of IRS-1
amplifies the IGF-1R signaling by indirectly recruiting GRB2 to transduce ERK1/2 signaling (73), or p85 to transduce PI3K signaling (80). Therefore, surrounding microenvironmental inputs that define stem cell behavior depend on IGF-1R activation and subsequent signaling cascades.

1.2.3 The Role of IGFs in Growth and Development

During development, circulating IGF levels correlate proportionally with placental and fetal weight, and reduced levels due to poor maternal-nutrition have been suggested to lead to fetal growth restriction (81). In human pregnancies, IGFs play an early role in promoting proliferation/ differentiation and preventing cell apoptosis of various types of placental cells (72). In mice, global knockout of Igf-1 or Igf-1r causes restricted growth (<60% of wild-type) and premature death of newborn embryos. Most pups with Igf-1r/- are unable to survive due to the lack of functional muscles required for breathing, whereas some mouse lines with Igf-1/- will survive with deficits in major organs (82-84). On the other hand, Igf-2 knockout mice (indistinguishable between homozygous and heterozygous) are viable at 60% birth weight of wild-type (85). Double mutants for Igf-1 and Igf-2 are severely growth-deficient (30% of wild-type) and die shortly after birth due to respiratory failure. Although both IGFs have an additive effect in embryonic development, IGF-1 is more important in postnatal growth, while IGF-2 is important for prenatal feto-placental growth. Hence, IGF-1 and IGF-2 stimulate both proliferation and terminal differentiation of many organs and tissues in developing embryos and in adult life. Since the initial description of Igf-1, Igf-2 and Igf-1r null mice using classic knockout methodology, mice with tissue-specific knockouts of these genes have been
generated using the Cre/loxP conditional knockout system resulting in a range of phenotypes (86, 87). The major differences between the models are in the tissue/cell-specificity and the timing of the null mutation (prenatal or postnatal). Most of the conditional models are generated with gene targeting after birth to allow examination of the role of *Igf-1* and *Igf-1r* in postnatal development without the ability to discriminate null mutation in stem or somatic cells. In classic knockout models, gene targeting occurs in the embryonic stem cells, allowing us to examine the impact of *Igf* genes in stem cells. However, only very few reports have investigated the impact of the knockout of *Igf-1* and *Igf-1r* in mesenchymal stem cells.

### 1.2.4 The Role of IGFs in MSC Multipotency

MSCs isolated from different tissues, such as bone marrow, adipose tissue, placental chorionic villi and fetal membranes, consistently express and secrete IGF-1 and/or IGF-2 *in vitro* (88-93). Ectopic IGF-1 expression in MSCs enhanced proliferation and lowered apoptosis (94). Basic fibroblast growth factor (bFGF) was shown to be required in maintaining stemness and proliferation in hESCs (46, 95) and in MSCs (90, 96, 97). Further investigation showed that this effect was mediated via the IGF system, which is up-regulated by an autonomous expression of IGF-1R, IGF-1, and IGF-2 in umbilical cord MSCs (96). Although both IGF-1 and IGF-2 are involved in mediating stem cell fate changes, IGF-2 appears more prominent than IGF-1 in promoting MSC multipotency. In hESCs, IGF-2 secreted by spontaneously differentiated autologous fibroblast-like cells in response to bFGF was required to maintain hESC pluripotency and self-renewal via IGF-1R signals (46). However, one study showed that hESCs pluripotency/self-renewal
maintenance could be independent of IGF-2 secretion only when MSCs are used as a feeder layer (98). A study in human dental pulp MSCs (hDSCs), confirmed that the IGF-1R is required for MSC multipotency and was regarded as a selection marker for stemness, similar to OCT4 and SOX2 (99). In placental MSCs (PMSCs), IGF-2 is upregulated by low oxygen tension and is required to maintain MSC multipotency (90, 97). Also, in neural stem cells (NSCs), IGF-2 was shown to play an important role in maintaining self-renewal (100) via the A-isof orm of IR (IR-A), independent of IGF-1R or IGF-2R (101). Although IGF-1R and IR can form hybrid receptors, the role of hybrid receptor in maintaining stem cell fate (ESCs or MSCs) and pluripotency is yet to be determined.

1.2.5 Induction of MSC towards Different Lineages

*In vitro*, MSC differentiation can be initiated *via* extrinsic stimulation by growth-factors (102); a process that requires withdrawing maintenance growth factors and adding differentiation-promoting growth factors and chemicals (43, 44). Differentiation factors include: dexamethasone, 3-isobutyl-1-methylxanthine, insulin and indomethacin for adipogenic differentiation (103); TGF-β1, insulin, transferrin, dexamethasone, and ascorbic acid for chondrogenic differentiation (103); insulin, transferrin and selenium for skeletal myogenic differentiation (103, 104); and dexamethasone, ascorbic acid and β-glycerophosphate for osteogenic differentiation (105). Therefore, the same MSC population exposed to different extrinsic stimuli will initiate differentiation towards a specific cell type *via* activation of tissue specific transcription factors, such as SOX5/6/9 for chondrocytes, PPARγ for adipocytes, MyoD family for myoblasts, and
RUNX2/Osterix for osteoblasts (106). Moreover, MSCs have been shown to differentiate into lineages outside of the mesodermal lineage (neural differentiation). These complex differentiation processes have implicated IGFs in fine-tuning transcription factor expression and activity leading to commitment towards alternate lineages from the three-germ layers.

1.2.6 The Role of IGFs in Mesenchymal Stem Cell Fate Specification and Differentiation

1.2.6.1 Osteogenesis

IGF-1 and IGF-2 are secreted by osteocytes for stimulation of bone formation, growth, and metabolism and can prevent apoptosis in a paracrine/autocrine manner (107, 108). Local overexpression of IGF-1 in osteoblasts can accelerate the rate of bone formation and increase the pace of matrix mineralization, which is dependent on IGF-1R (109). Decoupling of IGF-1R signaling from IGF-1 is responsible for reduced proliferation/differentiation in primary osteoblasts from osteoporosis patients, causing bone loss (110). Under non-differentiation conditions, IGF-1 transfected human MSCs are able to up-regulate expression of various osteoblast genes (111). Bone marrow MSCs (BM-MSCs) secrete IGF-1 and generate conditioned media that was sufficient to induce alveolar bone regeneration prior to dental implant placement (112). In dental pulp MSC, IGF-1 was shown to promote mTOR signaling pathway and trigger the expression of RUNX2, OCN, OSX, and COL1 (57). Also, human deciduous teeth MSCs with high osteopotentia express and secrete IGF-2 that is required for differentiation and mineralization (113). Overall, IGF-1 and IGF-2 play a significant role in MSC osteogenic
differentiation and bone health.

1.2.6.2 Myogenesis

L6E9 cells, a myoblast cell line used in myogenesis studies, with IGF-1 induces an initial proliferative response (114). During rapid cell division, myogenic regulatory factors are inhibited, but 30 hours later, the mitogenic effect is suppressed and Myogenin expression and activity are increased. Although, the downstream factors in IGF-mediated differentiation are still under investigation, IGFs can induce myogenic transcription factors expression. Moreover, the overexpression of MyoD (a protein that plays a major role in regulating muscle differentiation) in C3H 10T1/2 mouse embryonic fibroblast cells induced IGF-2 expression, which in turn activated IGF-1R and its downstream target AKT (115). Specifically, IGF-2 was required for the recruitment and induction of myogenic promoters and myogenesis (116). In particular, IGF-2 was required to allow continued recruitment of MyoD-associated proteins at the Myogenin promoter (117). The IGF-2 specific binding protein, IGFBP-6, is expressed during embryonic development in many different tissues including the ossifying bones of the cranium, myoblasts and the motor neurons of the spinal cord (118).

The potential use of MSCs has been previously investigated in treating muscular injury, including myocardial infarction. Rat BM-MSCs release paracrine factors that can be used to treat soleus muscle injury (119). Treatment of MSCs with IGF-1 improved MSC-induced muscle healing by reducing scar formation, increasing angiogenesis, recovery of muscle structure, and improving function (119). Injection of BM-MSC into the cardiac muscle increased the proliferation, migration and inhibited apoptosis of existing cardiac
muscle cells after myocardial infarction; however, IGF-1 did not induce myocardial differentiation of these MSCs (120). In addition, transplantation of IGF-1-primed MSCs attenuated cardiac dysfunction, increased the survival of engrafted MSCs in the ischemic heart, decreased myocardium cell apoptosis, and inhibited the expression of inflammation cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β, and IL-6 (121). The current research into MSCs and IGF in myogenesis is, therefore, more focused on in vivo muscle repair rather than MSC-differentiated myoblast replacement after transplantation.

1.2.6.3 Adipogenesis

In a comparative study of MSCs from different sources including adipose (ASCs), bone marrow (BMSCs), dermal sheath cells (DSCs), and dermal papilla cells (DPCs), IGF-1 had the highest expression and secretion in ASC desired populations (122). Furthermore, IGF-1 was shown to alter MSC fate between osteogenic and adipogenic lineages via its ability to bind and form a complex with the acid-labile subunit (ALS) (123). The loss of this IGF-1/ALS complex shifted differentiation from osteogenesis to adipogenesis (123).

1.2.6.4 Chondrogenesis

In a study of intervertebral disc degeneration, it was shown that IGF-1 and TGF-β3 work in synergy to enhance nucleus pulposus-derived MSCs viability, extracellular matrix biosynthesis and differentiation towards nucleus pulposus cells (124). Although TGF-β signaling is known to be important for chondrocyte-inductive differentiation from MSCs, other studies show that IGF-1 can regulate MSC chondrogenesis independent of TGF-β. In one study, IGF-1 induced chondrogenic differentiation from adipose-derived MSCs as
evident by increased collagen type II, aggrecan, and SOX9 expression (58). Similarly, IGF-1 induced the chondrogenic potential of BM-derived MSCs stimulating proliferation and inducing expression of chondrogenic markers (125). In addition, IRS-1 localization was induced from nuclear to cytoplasmic to shift MSC proliferation to differentiation (126).

1.2.7 Crosstalk between the IGF Axis and Other Signaling Pathways in MSC Proliferation and Differentiation

Crosstalk between signaling pathways are important for stem cells self-renewal and differentiation; however, specific interactions with the IGF system are still being delineated in MSCs. As shown in Figure 1.1, integrins can play an important role in IGF signaling. In particular, IGF-1 directly binds to αvβ3 integrin and induces αvβ3-IGF1-IGF1R ternary complex formation required for phosphorylation, ERK and AKT activation and cell proliferation (127).

During bone formation and osteoblast differentiation, IGF-2 potentiates bone morphogenetic protein-9 (BMP-9) signaling, which belongs to the transforming growth factor-β (TGF-β) superfamily (128). IGF-2 stimulation of PI3K/AKT can potentiate BMP-9-induced activity of alkaline phosphatase (ALP) a marker of early osteogenesis and the expression of later markers such as osteocalcin and osteopontin in MSCs. Interestingly, IGFBP-3 and IGFBP-4 inhibit these effects (128).

During osteoblast differentiation of BM-MSCs, hedgehog (HH) via Gli2 was shown to increase IGF-2 expression acting via the IGF-1R/mTORC2/AKT pathway (129). IGF-2-mediated AKT activation served as a positive feedback loop for enhanced HH
transcriptional output by stabilizing full length Gli2 due to phosphorylation by AKT. In myogenic differentiation, sonic hedgehog (SHH), was also regarded as a positive regulator of IGF-1 signaling with a cooperative additive effect in primary myoblast proliferation and differentiation via the MAPK/ERK and PI3K/AKT pathways (130).

Crosstalk between the IGF system and other pathways has also been explored in cancer stem cells, which may not be dependent on their immediate niche, but can give an insight into normal stem cell signaling (131). In glioma stem cells (GSC), HH via Gli1 up-regulated the transcriptional activation of IRS-1 which increased GSC sensitivity to IGF-1 stimulation (132). In lung adenocarcinoma stem-like cells, IGF-1R-mediated-OCT4 expression to form a complex with β-catenin and SOX2 that was crucial for self-renewal and oncogenic potential (133).

1.2.8 IGF-Expressing MSCs for the Treatment of Terminal Diseases

Paracrine factors, including IGFs, secreted by MSC are shown to play a major role in treating organ-failure diseases. IGF-expressing MSCs enhance proliferation, differentiation and repair of surrounding tissue in kidney, heart, and pancreas (91-93). In kidney ischemic-reperfusion injury, physical interaction between MSC and kidney tissue was required to promote kidney repair (93). Following myocardial infarction, adult human epicardium-derived cells and cardiomyocyte progenitor cells synergistically improve cardiac function, likely instigated by complementary paracrine actions (134). In fact, co-transplantation of unmodified MSC with cardiovascular progenitors elevated the expression of factors promoting cardiac repair. Specifically, IGF-1 promoted expression of pro-survival and angiogenesis genes in human cardiomyocyte progenitors (135).
In STZ-induced hyperglycemic mice, MSC helped in wound healing via IGF-1 secretion (136). IGF-1 in these mice activated PI3K/AKT and GLUT4, which improved glucose uptake and insulin sensitivity, thereby improving diabetic wound healing. In hepatocellular carcinoma (HCC), fetal human MSC conditioned media was used to inhibit cell growth (137). It was discovered that the conditioned media contained high levels of IGFBPs that sequestered IGFs and reduced IGF-1R and AKT activation leading to cell cycle arrest in HCC. These tumor-specific effects were not observed in matched hepatocytes or patient-derived matched normal tissue.

1.2.9 IGFs and in vitro MSC Differentiation

Addition of IGFs into MSC differentiation media leads to an earlier commitment and increased differentiation of MSC into several lineages including osteoblast, adipocyte, and chondrocyte cells (Table 1.1). In this context, a typical concentration of 10-100 ng/mL of IGF-1 is sufficient to activate only the IGF-1R and not the IR. On the other hand, not much attention is given to receptor binding affinity of IGF-1R versus IR when insulin is used in maintenance or differentiation conditions. Commercial media for stem cell research include non-physiological concentrations of insulin (0.5, 5, 10 µg/mL); 100-1,000 x higher than the highest insulin concentration in serum (138). High concentrations of insulin (≥1 µg/mL) not only activate IR but also activate IGF-1R (53, 138). Therefore, using high insulin concentrations in defined media, which can be a substitute for IGFs, cannot distinguish whether the effect is mediated via the IGF-1R or IR signaling pathways and studies describing the effect of IGFs in growth and/or differentiation of stem cells in “defined medium” should recognize this potential confounding effect.
Table 1.1 MSC differentiation protocols with IGFs towards different lineages.
Example of MSC differentiation protocols included IGFs in their differentiation media formulations.

<table>
<thead>
<tr>
<th>MSC Population</th>
<th>Differentiation</th>
<th>Protocol</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human dental pulp stem cells</td>
<td>Osteoblast-like</td>
<td>Osteogenic media supplemented with 0.1 µmol/L dexamethasone, 10 nmol/L b-</td>
<td>(57)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>glycerophosphate and 50 µg/mL ascorbic acid and 100 ng/mL IGF-1</td>
<td></td>
</tr>
<tr>
<td>Stromal vascular fraction of adipose tissue - human</td>
<td>Adipocyte-like</td>
<td>StemPro® Adipogenesis Differentiation Kit supplemented with 10 ng/mL of IGF-1.</td>
<td>(139)</td>
</tr>
<tr>
<td>adipose stem/progenitor cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human adipose-derived mesenchymal cells</td>
<td>Chondrocyte-like</td>
<td>DMEM high glucose supplemented with 1% FBS, 0.1 mM ascorbic acid-2- phosphate, 10⁻⁷</td>
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</tr>
<tr>
<td></td>
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<td>dexamethasone, 6.25 µg/ml transferrin, 6.25 ng/ml selenous acid, 10 ng/ml recombinant human TGF-β1, 100 ng/ml recombinant human IGF-1.</td>
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1.3 Insulin-Like Growth Factor Binding Protein-6 (IGFBP-6)

IGFBP-6 is a 30 kDa, secreted, O-linked glycoprotein, which unlike other IGFBPs has a significantly higher affinity (100 fold), for IGF-2 than IGF-1 (67, 140). IGFBP-6 has three structural domains: the C terminal domain, the L domain (for linker or labile) and the N terminal domain. The C-terminal domain of IGFBP-6 shows a high degree of mobility. Interestingly, the site on IGF-2 where IGFBP-6 binds overlaps with that of the IGF-1R binding site, giving a structural basis for IGFBP-6’s ability to inhibit IGF-2 binding to the IGF-1R (141). Although highly conserved, the N-terminal domain of
IGFBP 1-5 contains a GCGCC motif which is not conserved in IGFBP-6. In IGFBP-6 the two adjacent cysteine residues are missing, resulting in different disulphide linkages than the other binding proteins (142). This difference appears to be a contributing factor to the unique preference of IGFBP-6 for IGF-2 (142).

In addition to being a secreted protein, IGFBP-6 is also subject to proteolysis. Studies in Madin-Darby canine kidney (MDCK) cells, an epithelial cell line derived from canine kidney, suggest that IGFBP-6 proteolysis and the conformational change that accompanies IGFBP-6 binding to IGF-2 may prolong degradation rates (143). The balance between proteolysis and glycosylation of IGFBP-6 helps maintain stability in IGF-2 binding and thus IGF-2 function modulation.

1.3.1 IGFs, IGFBP-6 and Myogenesis

Myogenesis is the process by which undifferentiated cells differentiate into muscle cells. Muscle cells develop from the mesoderm layer of the developing embryo. Myogenesis can be divided into three distinct stages. After completing each of these stages, the differentiating cells express specific proteins. The expression of these proteins indicates which stage the developing cell has passed. After specification once the cells become muscle precursors, they express Pax3, Pax7, Gli2, and Six1 and undergo commitment to become myoblast cells. Committed myoblasts express myogenic regulatory factors (MRFs) as well as muscle commitment markers such as Pax3 and Pax7. After commitment, cells undergo terminal differentiation and become myocytes that express early muscle differentiation markers (Myf-5, MyoD, and Myogenin) followed by late
muscle lineage differentiation marker myosin heavy chain (MHC) and the formation of multi-nucleated fibres (144-148).

The myogenic regulatory factors (MRFs) each serve distinct but overlapping roles in muscle development. Mice develop skeletal muscle normally when either Myf-5 or MyoD are lacking. The loss of myf-5 results in an up-regulation of MyoD (149, 150). However, when both are missing, myoblasts do not form, suggesting that Myf-5 and MyoD have overlapping roles and can compensate for the loss of the other (151). The loss of Myf-5 and MyoD together is perinatal lethal due to respiratory failure from a total lack of muscles in the trunk. Myogenin is also necessary for survival. When myogenin is lacking in mice, the mutations are also perinatal lethal, and the mice die shortly after birth due to severe muscle deficiency and respiratory failure (152).

The IGF family has been shown to have a role in muscle development. One clear demonstration is loss-of-function studies that have been performed. In IGF-1 or IGF-1R knock-out mice the embryos die prematurely because the pups are unable to survive because functional muscle is completely lacking and thus they are unable to breathe (82, 86). When MyoD is expressed in C3H 10T1/2 mouse embryonic fibroblast cells, there is an induction of IGF-2 expression and activation of the IGF-1R (116). After muscle injury, IGF-1 enhances regeneration while inhibiting IGF-1 reduces the number of regenerating myofibers in vivo (153). IGF-2 is expressed abundantly in the developing skeletal muscle and is the major growth factor for muscle growth, differentiation, and regeneration (154, 155). These studies suggest an important role for IGF peptides and the IGF-1R in the regulation of myogenesis. However, the downstream factors involved in IGF stimulated differentiation still remain elusive.
IGFBP-6 is expressed during embryonic development in myoblasts (156). Previous studies in the Han Laboratory showed that rhabdomyosarcoma cells (RD cells) treated with mutant (non-nuclear) IGFBP-6 undergo terminal differentiation at a rate of 0.05% compared to 0% of the un-transfected RD cells or wild-type IGFBP-6 expressing cells, as measured by the immunoreactivity of myosin heavy chain (157). This percentage is significant since control (un-transfected or empty vector transfected) RD cells and wild-type IGFBP-6 expressing RD cells never underwent myogenesis. No previous studies have been performed on the role of IGFs and IGFBP-6 on PMSCs differentiation into skeletal muscle.

The need to find a stem cell population with the proper niche factors that will eliminate the difficulties related to other stem cells used before to try and treat muscle diseases like Duchene muscular dystrophy in tissue regeneration therapy is important. Therefore, I propose to further delineate the extracellular and intracellular molecular mechanisms that trigger the process of skeletal muscle differentiation from placental mesenchymal stem cells by IGFBP-6 and the IGF system.

1.4 Thesis Rationale

The expression of IGFBP-6 is associated with non-proliferative states such as cell differentiation (158). Also, IGFBP-6 over-expression in cells has either a growth-promoting or a growth-inhibitory effect, depending on the cell type (159, 160). To date, no studies have been performed on IGFBP-6 and PMSCs. Moreover, the conflicting data about IGFBP-6 in other cell lines raises questions about how this binding protein will function in PMSCs and how it affects the fate of these cells.
During development, IGF-2 and IGFBP-6 are expressed abundantly in developing muscle cells and are required for myogenesis (155). Furthermore, IGF-1 and IGF-2 are expressed by skeletal muscle cells during muscle repair in response to muscle injury (154, 155).

Testing if myogenesis can be manipulated by the IGF system, specifically IGFBP-6 expression, and delineating the mechanisms involved during muscle differentiation will help us understand the complex processes of embryonic development and improve muscle regeneration therapies. As no previous studies have been performed on IGFBP-6 and PMSCs, my novel research project is focused on understanding IGFs and IGFBP-6 role in PMSC differentiation into skeletal muscle (Figure 1.3).

Increasing IGFBP-6 in the PMSC extracellular microenvironment is expected to sequester IGF-2 and prevent MSC differentiation. Conversely, silencing of IGFBP-6 expression is expected to increase PMSC differentiation, as a result of a greater availability of IGF-2 (117, 161). In addition, IGFBP-6 is known to be present in the cytoplasm and in the nuclei (162, 163) suggesting that IGFBP-6 may have IGF-2 independent functions.

Knowledge of IGFBP-6 effects on muscle differentiation will help explain the mechanisms controlling stem cell fate in the presence of IGFs and improve strategies for skeletal muscle regeneration therapies using placental mesenchymal stem cells in diseases, such as muscular dystrophy, to improve muscle regeneration.
1.5 Hypothesis and Objectives

Our central hypothesis is that IGFs and IGFBP-6 regulate the differentiation of PMSCs into skeletal muscle via intracellular and extracellular mechanisms.

This hypothesis will be addressed in the following 4 aims:

1. To evaluate the extracellular and intracellular effects of IGFBP-6 on the differentiation of PMSCs into skeletal muscle.

2. To determine the interaction between IGFs and IGFBP-6 on PMSCs skeletal muscle differentiation.

3. To investigate the IGF-dependent and IGF-independent actions of IGFBP-6 on PMSCs muscle differentiation.

4. To identify the signal transduction mechanisms of IGFBP-6 on muscle differentiation by PMSCs.

In these studies, we showed that the human placenta is a valid source of multipotent MSCs and that the IGF signaling axis, specifically involving IGFBP-6, is important in muscle differentiation. Understanding IGFs and IGFBP-6 effects on muscle differentiation will help improve strategies for skeletal muscle regeneration therapies using stem cells to treat muscle degeneration.
1.6 Summary and Conclusions

IGFs are one of the earliest growth factors to be expressed in the pre-implantation embryo and putatively act as autocrine/paracrine factors in many developing cell types, including stem cells. They form an important component of the stem cell niche. Their expression is ubiquitous in many cell types; however, they are most abundant in cells and tissues of mesodermal origin. Thus, MSCs are both the source and target of IGFs during development, and likely play important roles in determining cell fate. Recent evidence also suggests the potential discriminating roles of IGFs in MSCs. As MSCs are being investigated in the context of cellular replacement and regenerative therapies, delineating the roles of endogenous as well as exogenous IGFs in MSC growth and differentiation will be critical in developing these cellular therapies to treat degenerative diseases.
Figure 1.3. Myogenic lineage progression. Muscle development is regulated by a number of transcription factors. Six1/4 and Pax3/7 are master regulators of early myocytes lineage commitment to the myogenic lineage, whereas Myf5, MyoD, and MyoG represent early differentiation markers. Expression of the terminal differentiation genes, required for the fusion of myocytes and the formation of myotubes, is mediated by myosin heavy chain (MHC).

1.7 REFERENCES


Esposito DL, et al. Tyr(612) and Tyr(632) in human insulin receptor substrate-1 are important for full activation of insulin-stimulated phosphatidylinositol 3-kinase activity and translocation of GLUT4 in adipose cells. Endocrinology, 2001; 142(7): p. 2833-2840.


CHAPTER TWO

Insulin-like Growth Factor Binding Protein-6 Alters Myogenic Differentiation of Human Placental Mesenchymal Stem Cells

A version of this chapter has been submitted for publication in Stem Cell International. Article ID 2348485.
2.1 INTRODUCTION

Unlike embryonic stem cells which are derived from the early embryo, placental mesenchymal stem cells (PMSCs) are derived from human placentae which are usually discarded, and therefore are readily available source of adult stem cells for use in tissue regenerative therapies in human patients (1,2,3). PMSCs are available in large numbers in vitro (3). Importantly, the pathways activated by these cells during differentiation into specific cell types in vitro and in vivo may provide important information on the developmental and regenerative processes of tissues and organs during embryogenesis and later development in adults.

Skeletal muscle development is a highly coordinated, step-wise process utilizing a series of transcription factors, and structural and enzymatic proteins that mark the different stages of skeletal muscle development. During myogenesis, committed progenitors differentiate into muscle lineage by up-regulating myogenic regulatory factors (MRFs) as well as muscle commitment transcription factors (Pax3 and Pax7), followed by the expression of early muscle cell structural components (MyoD and Myogenin) (4). After commitment, cells fuse together to form multinucleated fibers and express muscle specific proteins, such as myosin heavy chain (MHC) (4). It is believed that in recovery and regeneration after muscle injury in the adult, this process is recapitulated.

The insulin-like growth factor (IGF) family of peptides regulates cell growth, differentiation and the maintenance of cell survival through several signal transduction pathways (5). This family includes two IGF peptides (IGF-1 and IGF-2), three cell surface receptors (type-1, type-2 IGF receptors, and insulin receptor) and six IGF binding
proteins (IGFBPs) (5). IGF-1 and IGF-2 are circulating peptides that function as potent mitogens for many cell types, mediated by binding to IGF-1R, a membrane receptor tyrosine kinase (6). IGFBPs are carriers for IGFs in the circulation and in the extracellular fluid compartment (7), protecting them from degradation (6, 8), delivering them to specific tissues, and modulating the biological actions of IGFs.

IGFBP-6 is a 30 kDa secreted protein, and unlike other IGFBPs, has a significantly higher affinity (~70-100 fold) for IGF-2 than IGF-1 (9, 10). IGFBP-6 has been demonstrated to modulate IGF-2 activity via inhibition of IGF-2 binding to the IGF-1R or directly independent of IGF-2 (11, 12). IGF binding proteins, including IGFBP-6, are also localized within the intracellular environment, suggesting that IGFBPs may have biological actions independent of the IGFs (13).

The IGF family has been shown previously to play a major role in muscle development. IGF-1R knockout mice die soon after birth due to breathing difficulties due to lack of functional respiratory muscles (14, 15). IGF-2 is expressed abundantly in the developing skeletal muscle and is a major factor for muscle growth, differentiation, and regeneration (16). When IGF-2 is knocked down, myogenesis does not occur (16). During development, IGFBP-6 is expressed abundantly in developing muscle cells and is also required for myogenesis (16). Previous studies in our laboratory have described IGF-independent functions of IGFBP-6 on regulating Ku proteins and thus effecting cell fate in a skeletal muscle cell line (17). Other studies have shown that IGFBP-6 inhibits angiogenesis but promotes migration in an IGF-independent manner (11).
To our knowledge, no previous studies have reported on the biological roles of IGFBP-6 in the differentiation of MSCs into the muscle lineage. In this study, we characterized the effects of IGFBP-6 on the differentiation of PMSCs into skeletal muscle.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of PMSCs

PMSC isolation and experiments were conducted with the approval from the Health Sciences Research Ethics Board of Western University (REB# 12154). Informed consent was obtained from healthy women undergoing therapeutic termination of pregnancy, and the PMSCs used in this study were isolated from one 15 week preterm placental tissue. After surgery, chorionic villi were dissected, washed, and minced with surgical scissors and forceps, and then subjected to enzymatic digestion with collagenase IV (369 IU/mg), hyaluronidase (999 IU/mg) (Sigma-Aldrich, Oakville, ON), and DNase I (2,000 IU/mg) (Hoffmann-LaRoche, Mississauga, ON) for 10 minutes at room temperature, followed by 0.05% trypsin (Gibco/Invitrogen, Mississauga, ON) for 5 minutes at room temperature. The sample was then washed for 10 minutes with 10% FBS in DMEM/F12 medium and the resulting single cell suspension was separated by density centrifugation over a Percoll (Sigma-Aldrich, Oakville, ON) discontinuous gradient using a modified protocol by Worton et al. (18).

2.2.2 Cell Culture

Cells from Percoll gradient fractions #3 and #4 were plated on to T75 flasks, cultured, and maintained using DMEM/F12 media supplemented with 15% FBS serum.
(Gibco/Invitrogen, Mississauga, ON) and FGF-2 (50 ng/mL) (Sigma-Aldrich, Oakville, ON) containing 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 0.25 µg/mL Amphotericin-B. The non-adherent cells were discarded at the time of media change, which was performed every 72 hours. The adherent cells were cultured until they reach 90% confluence. Cells were then passaged 1:2 approximately once per week using 0.05% Trypsin for 10 min at 37°C for 3 passages. Passaged cells were stored at -80°C in 1 mL of freezing media (30% FBS and 10% DMSO in DMEM/F12 media). When needed, vials were thawed and cells were resuspended in normal culture media (25 ng/mL FGF-2 and 15% FBS in DMEM/F12).

2.2.3 Muscle Differentiation

Cells were plated in muscle growth media (fetal bovine serum 0.05 mL/mL, fetuin 50 µg/mL, epidermal growth factor 10 ng/mL, basic fibroblast growth factor 1 ng/mL, insulin 10 µg/mL, and dexamethasone 0.4 µg/mL) for 48 hours before changing to the skeletal muscle differentiation media, which is a proprietary serum-free medium containing 10 µg/mL insulin (PromoCell, Heidelberg, Germany). Cells were grown in six-well plates for 14 days in a standard tissue culture incubator at 37°C in 5% CO₂.

2.2.4 IGFBP-6

Recombinant human IGFBP-6 (PROSPEC, East Brunswick, NJ) was resuspended in sterile MilliQ-H₂O and supplemented to the media at a concentration of 375 ng/mL. IGFBP-6 concentration was determined by a dose response experiment using PMSCs in muscle differentiation media (Figure S2.1A). IGFBP-6 was added every 3 days at the
time of media change because that was the time it took for IGFBP-6 secreted levels to be lower than the control (Figure S2.1B).

2.2.5 Down-Regulation of IGFBP-6 Expression by siRNA

To silence endogenous IGFBP-6 expression in PMSCs, siRNA against IGFBP-6 (Santa Cruz Biotechnology, Dallas, TX) was used. 8 μL of Lipofectamine (Invitrogen, Mississauga, ON) with either 8 μL of scrambled or IGFBP-6 siRNA was added to 100 μL of DMEM/F12 media (Invitrogen, Mississauga, ON) for 40 minutes at room temperature; the final concentration of siRNA was 80 nM. The siRNA solution was then added to 60% confluent cells and incubated for 5 hours at 37°C. Muscle growth media (1.5 mL) was added to the cells for 48 hours and subsequently replaced with 2 mL of muscle differentiation media. New siRNA was added every 3 days during the change of media and the experiment was performed for 7 days.

2.2.6 Immunocytochemistry

PMSCs were grown on glass cover slips (80-90% confluence), stained with primary antibodies (1:100), and incubated at 4°C overnight. To detect markers of cell potency, OCT4 (N-19: sc-8628) (Santa Cruz Biotechnology, Dallas, TX) was used. To detect markers of muscle differentiation, Myosin heavy chain (H-300: sc-20641) (Santa Cruz Biotechnology, Dallas, TX) was used. To detect IGFBP-6, antibody H-70: sc-13094 (Santa Cruz Biotechnology, Dallas, TX) was used. Primary antibodies were washed using 0.1% Tween-20 in PBS (3 times for 5 minutes). Cells were then incubated in the dark with the corresponding secondary AlexaFluor conjugated antibody (1:200). The secondary antibodies used were AlexaFluor 488 or AlexaFluor 568 conjugated antibodies.
The secondary antibody was washed 0.1% Tween-20 in PBS, and the nuclear stain (Hoechst dye) was added for 7 minutes and then rinsed. The cover slips were mounted and images were taken using a confocal microscope (Zeiss, Germany). Each antibody was performed in triplicate.

2.2.7 Cell Lysate Preparation

Following PMSC treatments, cells were washed with DPBS (Gibco/Invitrogen, Mississauga, ON) and 200 μL of (1x) cell lysis buffer (Cell Signaling Technologies, Burlington, ON) mixed with protease and phosphatase inhibitors (1:100) were added to the cells. Cells were detached using a cell scraper and the lysate was sonicated for 15 seconds. Finally, samples were centrifuged for 15 minutes at 14,000 rpm at 4°C, and the supernatant was collected.

2.2.8 Immunoblotting

Cell lysates containing 20 µg of protein were added to 6x SDS gel loading buffer (1% β-mercaptoethanol, 1% SDS, 30% glycerol, 0.0012% bromophenol blue, Tris HCl 0.28 M, pH 6.8). Samples were boiled for 5 minutes at 95°C, then placed on ice for 3 minutes, and centrifuged at 3,000 rpm for 20 seconds before loading. Samples were resolved by molecular weight using 10% SDS polyacrylamide gels transferred onto poly-vinyl-dene-fluoride (PVDF) membranes (Bio-Rad, Hercules, CA) using a Trans-Blot Turbo (Bio-Rad, Hercules, CA) with an optimized protocol depending on protein size. Membranes were blocked with 5% non-fat-dry milk, gently shaking for 1 hour at room temperature in Tris-HCl buffer saline pH 8.0 with 0.1% Tween-20 (TBS-T). Blots were then washed with TBS-T (3 times for 10 minutes) followed by incubation at 4°C overnight with
specific primary antibodies in 5% BSA or 5% non-fat-dry milk in TBS-T following the manufacturer’s protocol. To detect markers of cell potency markers, antibodies for OCT4 (N-19: sc-8628) (Santa Cruz Biotechnology, Dallas, TX), and SOX2 (2683-1) (Epitomics, Burlington, ON) were used. To detect markers of muscle differentiation, Pax3/7 (E-10: sc-365613), MyoD (M-318: sc-760), Myogenin (F5D: sc-12732), and Myosin heavy chain (H-300: sc-20641) (Santa Cruz Biotechnology, Dallas, TX) were used. To detect IGFBP-6, antibody H-70: sc-13094 (Santa Cruz Biotechnology, Dallas, TX) was used. For loading control, pan-Actin Ab-5 (Thermo Fisher Scientific, Fremont, CA) was used.

Then membranes were washed and incubated for 1 hour at room temperature with the corresponding secondary HRP-conjugated antibody. The secondary antibodies used were HRP-conjugated goat anti-rabbit (#170-6515) or anti-mouse (#170-6516) (Bio-Rad, Hercules, CA) or donkey anti-goat antibody (Santa Cruz Biotechnology, Dallas, TX).

Resolved protein bands were detected using chemiluminescence and images were taken using the VersaDoc Imager (Bio-Rad, Hercules, CA). Western blots were performed in triplicate.

### 2.2.9 Quantification of IGFBP-6 and IGF-2 Secretion by Enzyme-Linked Immunosorbent Assay (ELISA)

Human IGFBP-6 (RayBiotech®, Burlington, ON) and IGF-2 (ALPCO, Salem, NH) ELISA kits were used to measure the amount of IGFBP-6 and IGF-2 secreted into the conditioned media for 14 days. Standards and samples were loaded into the wells (in triplicate) and the immobilized antibody bound IGFBP-6 or IGF-2 present in the sample.
The wells were washed and biotinylated anti-human antibody was added. After washing, HRP-conjugated streptavidin was added; then a TMB substrate solution was used to develop a blue color in proportion to the amount of IGFBP-6 or IGF-2 bound. Then, a Stop Solution was added, which changed the color from blue to yellow, and the intensity was measured at 450 nm using Multiskan Ascent plate reader and analysis software.

2.2.10 Aldehyde Dehydrogenase (ALDH) Activity

ALDH-activity, a conserved progenitor cell function, was assessed by flow cytometry for 14 days. Using the Aldefluor™ assay (Stem Cell Technologies, Vancouver, BC), as per the manufacturer’s instructions. Briefly, 5 μL of activated Aldefluor reagent was added to 1 mL of cell suspension and incubated for 45 minutes at 37°C. Cells were washed and resuspended in 500 μL of ice-cold Aldefluor assay buffer and ALDH-activity was measured using flow cytometry. As a negative control, Aldefluor™ DEAB reagent was used. Samples were run in triplicate.

2.2.11 Statistical Analysis

All experiments were performed in triplicate from one 15 week placental tissue (technical replicate). GraphPad Prism Software 5.0 was used to generate all graphs and analyses. A two-way ANOVA followed by a Bonferroni’s multiple comparison test or a one-way ANOVA followed by a Student’s t-test was used to calculate significant differences when p<0.05. Graphic representation values are presented as mean ± SEM (shown as variance bars).
2.3 RESULTS

2.3.1 PMSCs can Differentiate into Skeletal Muscle

To determine if PMSCs can differentiate into skeletal muscle, PMSCs were grown under muscle differentiation conditions for up to 14 days. Compared to PMSCs grown in non-differentiating conditions (10% FBS), differentiated PMSCs showed muscle morphology as early as day 1 post-differentiation (compaction and elongated appearance) (Figure 2.1A and 2.1B), and cells continued to differentiate forming multi-nucleated fibers at day 14 (Figure 2.1C, 2.1D and S2.2A). Associated with these morphological changes, pluripotency-associated marker (OCT4) immunoreactivity appeared low (Figure 2.1E, 2.1F, and S2.2B), and muscle differentiation marker (MHC) immunoreactivity was high (Figure 2.1G and 2.1H) when compared to control cells (10% FBS). In addition, PMSCs under muscle differentiation conditions showed lower cell counts per field compared to undifferentiated controls (Figure S2.2C).

Under muscle differentiation conditions, PMSCs decreased pluripotency-associated protein levels of OCT4 and SOX2. OCT4 levels were significantly reduced at day 1 compared to control with further decrease at 14 days post-differentiation (Figure 2.2A). In addition, SOX2 levels were lowered and nearly diminished by day 14 in cells under muscle differentiation conditions compared to control (Figure 2.2B). Muscle commitment marker Pax3/7 level was increased at day 7, followed by a decrease at day 14 in PMSCs under muscle differentiation conditions compared to control (Figure 2.2C); suggesting that PMSCs under muscle differentiation conditions are committed to the muscle lineage and are proceeding to muscle differentiation. This was confirmed by the
protein levels of muscle markers (MyoD, MyoG, and MHC), that increased significantly over time under muscle differentiation conditions (Figure 2.2D, 2.2E, and 2.2F). Collectively, these findings indicated that PMSCs differentiate into skeletal muscle under differentiating culture conditions, and this cell differentiation model could be consistently used to study muscle development in vitro.

We used the Aldefluor\textsuperscript{TM} assay to determine the frequency of primitive progenitor cells with high ALDH-activity. In this context, high ALDH-activity is a conserved characteristic of proliferative progenitor cells of multiple lineages (19-21). As differentiation occurs towards a more mature cellular phenotype, ALDH-activity is reduced. Compared to PMSCs grown under non-differentiation conditions, there was a decrease in the frequency of cells with high ALDH-activity (ALDH\textsuperscript{+} cells) under muscle differentiation conditions at days 1 to 14 (Figures 2.3A to 2.3D). Moreover, ALDH-activity was also decreased over time when cultured under control conditions (10% FBS) (Figure 2.3E). These findings suggested that PMSCs comprised of a heterogeneous population that slowly differentiated during maintenance in standard culture conditions, and PMSCs stimulated to differentiate into skeletal muscle immediately decreased ALDH-activity at earlier time points.
Figure 2.1. PMSCs cultured under muscle differentiation conditions showed muscle morphology with lower OCT4 and higher MHC at 14 days post-differentiation. (A, B) Compared to cells cultured under standard conditions in 10%FBS, PMSCs grown in muscle differentiation media showed skeletal muscle morphology as early as day 1 post-differentiation (20X). (C, D) At 14 days post-differentiation, PMSCs grown in muscle differentiation media showed multi-nucleated skeletal muscle fiber formation (20X). 40X magnification is shown in the bottom right corner. White arrows indicate multi-nucleated muscle cells. (E-H) Cells grown in muscle differentiation media, showed less OCT4 (green-Alexa 488, λ-488 nm) and more MHC immunoreactivity (red-Alexa 568, λ-568 nm), when compared to PMSCs in 10% FBS at 14 days post-differentiation (10X). Nuclei, were stained with Hoechst dye (blue, λ=340 nm). Experiment was performed in triplicate from one placental tissue.
Figure 2.2. PMSCs cultured under muscle differentiation conditions increased levels of muscle markers (Pax3/7, MyoD, MyoG, and MHC) and decreased OCT4 and SOX2 levels. Compared to PMSCs cultured under control conditions, (A) OCT4 was decreased at 1 and 14 days under muscle differentiation conditions. (B) SOX2 levels were decreased at each time point under muscle differentiation conditions. (C) Pax3/7 was increased at day 7 and decreased by day 14 under muscle differentiation conditions. (D) MyoD, (E) MyoG, and (F) MHC were increased at each time point under muscle differentiation conditions. Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 comparing control to muscle differentiation conditions, or #P<0.05, ##P<0.01, ###P<0.001 comparing the same treatment over time.
(A)  
DEAB  
Day 1  
Control (10% FBS)  
Muscle Differentiation  
ALDH  

(B)  
Day 3  
Control (10% FBS)  
Muscle Differentiation
(C)  

**Day 7**  
Control (10% FBS)  

**Muscle Differentiation**  

(D)  

**Day 14**  
Control (10% FBS)  

**Muscle Differentiation**  

(E)  

**ALDH**  

- Control  
- Muscle Differentiation  

% of Cells (%)  

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*Significant differences indicated by asterisks: ***p < 0.001, ###p < 0.0001*
Figure 2.3. PMSCs cultured under skeletal muscle differentiation conditions showed a decreased frequency of cells with high ALDH-activity. Representative flow cytometry dot plots showing the frequency of PMSC with high ALDH-activity with Aldefluor and an inhibitor of ALDH (DEAB) or with ALDH alone when cultured under control (10% FBS) or muscle differentiation conditions at (A) day 1, (B) day 3, (C) day 7, (D) and day 14. (E) Compared to PMSCs cultured under control conditions, PMSCs cultured under differentiated conditions showed significantly decreased frequency of cells with high ALDH-activity. Even under control culture conditions, PMSCs showed diminished ALDH-activity over time. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine ***P<0.001 comparing control to muscle differentiation conditions, or ###P<0.001 comparing the same treatment over time.
2.3.2 PMSCs Cultured under Muscle Differentiation Conditions Showed Higher IGFBP-6 Expression, with Higher IGF-2 Secretion

PMSCs cultured under skeletal muscle differentiation conditions were investigated to determine if they expressed IGFBP-6. Using immunocytochemistry, PMSCs cultured under differentiation conditions showed high intracellular IGFBP-6 immunoreactivity compared to PMSCs cultured under control conditions (Figure 2.4A, 2.4B, and S2.3). Using immunoblotting at multiple time points, IGFBP-6 protein levels were increased in PMSCs under differentiation conditions. Following day 2 of differentiation, IGFBP-6 levels gradually decreased in PMSCs cultured under differentiation conditions but remained higher than time-matched controls (Figure 2.4C). Using ELISA detection in PMSC conditioned media, both IGFBP-6 and IGF-2 secretion was increased at every time point with muscle differentiation conditions compared to PMSCs cultured under control conditions (10% FBS), confirming that developing muscle cells secrete IGFBP-6 and IGF-2 into the extracellular space (Figure 2.4D and 2.4E). Therefore, the synthesis of IGFBP-6 and IGF-2 increased as the cells became more differentiated into skeletal muscle.
Figure 2.4. PMSCs cultured under skeletal muscle differentiation conditions showed increased IGFBP-6 expression and secretion. (A, B) PMSCs cultured under muscle differentiation conditions showed higher IGFBP-6 staining (red-Alexa, λ=568 nm) when compared to PMSCs under control conditions (10% FBS) at 14 days post-differentiation. Nuclei, were stained with Hoechst dye (blue, λ=340 nm). (C) IGFBP-6 protein levels in PMSCs cultured under differentiation conditions were increased at each time point compared to control conditions. Under muscle differentiation conditions, IGFBP-6 levels peaked at 2 days post-differentiation and gradually decreased from day 3 to 14. Protein levels were quantified by densitometry and normalized to β-Actin. (D) IGFBP-6 and (E) IGF-2 secretion into the media was increased under muscle differentiation conditions compared to control conditions. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 comparing control to muscle differentiation conditions, or #P<0.05, ##P<0.01, ###P<0.001 comparing the same treatment over time.
2.3.3 IGFBP-6 Affects Multipotency of the Developing Muscle Cells from PMSCs before Muscle Commitment

To test the effects of extracellular IGFBP-6 on PMSC differentiation to muscle, recombinant human IGFBP-6 was supplemented to the media. Addition of extracellular IGFBP-6 into the culture media increased intracellular IGFBP-6 detection by western blots, suggesting that recombinant human IGFBP-6 induced a positive feedback effect or was taken up by the differentiating cells (Figure 2.5A). Furthermore, stimulation in IGFBP-6 increased OCT4 and SOX2 levels (Figure 2.5B, and 2.5C). Interestingly, IGFBP-6 supplementation also increased Pax3/7 levels suggesting enhanced PMSC commitment towards the skeletal muscle lineage (Figure 2.5D). Finally, IGFBP-6 treatment increased the levels of muscle-specific markers, MyoD, MyoG, and MHC at the earlier time points with a decline over time in the prolonged presence of increased extracellular IGFBP-6 compared to un-supplemented muscle differentiation conditions (Figure 2.5E, 2.5F, and 2.5G). Collectively, these data suggested that IGFBP-6 promoted PMSC commitment to the muscle lineage as an immediate effect but maintained pluripotency-associated markers and delayed muscle differentiation at later time points, as seen with the decreased protein level of muscle differentiation markers.

Due to the fact that both potency and differentiation markers increased by IGFBP-6 treatment in a time-dependent manner, we tested the cells for ALDH-activity to determine the frequency of PMSCs that maintained high ALDH progenitor phenotype. Under muscle differentiation conditions, extracellular IGFBP-6 supplementation increased ALDH-activity at day 1 (Figure 2.6A), day 3 (Figure 2.6B), and day 7 (Figure 2.6C), but not at day 14 (Figure 2.6D). After analysis of 3 independent samples from one
placenta, increased retention of high ALDH progenitor cell frequency was consistently observed after IGFBP-6 treatment (Figure 2.6E), suggesting that IGFBP-6 addition prolonged primitive progenitor phenotype in PMSCs cultured under muscle differentiation conditions. Further immunocytochemistry analyses at day 14, revealed that compared to un-supplemented conditions, PMSCs treated with IGFBP-6 showed more muscle compaction (Figure 2.7A, 2.7B, and S2.4A) with fewer cells per field (Figure S2.4B). Furthermore, OCT4 and MHC immunoreactivity appeared equivalent with or without IGFBP-6 supplementation (Figures 2.7C to 2.7F). ALDH-activity results further suggested that IGFBP-6 treatment maintained PMSCs potency before PMSC commitment to the muscle lineage. Collectively, these findings need to be further investigated to confirm the identity of cells over -time.
Figure 2.5. PMSCs treated with IGFBP-6 increased pluripotency-associated and muscle differentiation markers. IGFBP-6, OCT4, SOX2, Pax3/7, MyoD, MyoG, and MHC protein levels were quantified within PMSCs grown in muscle differentiation media with or without IGFBP-6 (375 ng/mL) supplementation. (A) IGFBP-6 treatment increased IGFBP-6 levels as compared to PMSCs grown in muscle differentiation media only. IGFBP-6 treatment also increased pluripotency-associated markers (B) OCT4 and (C) SOX2 levels. (D) IGFBP-6 treatment increased muscle lineage commitment marker Pax3/7 at each time point. Muscle differentiation markers (E) MyoD, (F) MyoG, and (G) MHC levels increased with IGFBP-6 treatment at early time points (1-3 days) but showed reduced levels at later time points (7-14 days). Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 comparing treatments, or †P<0.05, ‡P<0.01, §§P<0.001 comparing the same treatment over time.
(A)  

Muscle Differentiation

Muscle Differentiation (+) IGFBP-6

Day 1

Muscle Differentiation  

Day 3

Muscle Differentiation (+) IGFBP-6
(C)

Day 7
Muscle Differentiation

Muscle Differentiation
(+1) IGFBP-6

(D)

Day 14
Muscle Differentiation

Muscle Differentiation
(+1) IGFBP-6

(E)

ALDH

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<th>7</th>
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<td>10</td>
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<td>1</td>
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<td>20</td>
<td>***</td>
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</tbody>
</table>

** Annotation

*** Annotation
Figure 2.6. IGFBP-6 treatment increased the frequency of PMSCs with high ALDH-activity. Representative flow cytometry dot plots with Aldefluor and an inhibitor (DEAB) or with ALDH alone in PMSCs cultured under muscle differentiation conditions with or without IGFBP-6 addition at (A) day1, (B) day 3, (C) day 7, and (D) day 14. (E) At days 1, 3, and 7, PMSCs treated with IGFBP-6 showed increased frequency of cells with high ALDH-activity. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, ***P<0.001.
Figure 2.7. PMSCs treated with IGFBP-6 showed similar immunofluorescence for OCT4 and MHC at 14 days under muscle differentiation conditions. Compared to PMSCs cultured under muscle differentiation alone, (A, B) PMSCs treated with IGFBP-6 showed high cell compaction at day 14 (10X). (C-F) PMSCs were immunoreactive for OCT4 (green-Alexa, λ-488 nm) and MHC (red-Alexa, λ-568 nm), with no change in immunoreactivity with IGFBP-6 treatment. Nuclei were stained with Hoechst dye (blue, λ=340 nm). Images are representative of 3 technical replicate.
2.3.4 IGFBP-6 Knockdown Delayed PMSC Differentiation into Muscle Cells

To evaluate the effects of IGFBP-6 silencing on pluripotency-associated and muscle differentiation markers in PMSCs, IGFBP-6 knockdown by siRNA was used during muscle differentiation over 7 days. As predicted, PMSC expression of IGFBP-6 was decreased for 1-2 days after IGFBP-6 knockdown compared to scrambled siRNA control. However, IGFBP-6 levels were equivalent to scrambled controls by day 3. Re-administration of IGFBP-6 siRNA at day 3 prolonged IGFBP-6 reduction, but IGFBP-6 returned to control levels by day 6 (Figure 2.8A), indicating that siRNA treatment resulted in transient IGFBP-6 silencing. Alongside IGFBP-6 knockdown, we observed a reduction in pluripotency-associated markers for OCT4 (Figure 2.8B) and SOX2 (Figure 2.8C), concomitant with reduced IGFBP-6 levels, suggesting that IGFBP-6 may be important for maintain potency which needs to be further investigated. In contrast, levels of the muscle commitment marker Pax3/7 were increased during IGFBP-6 knockdown at day 1 and 2 but returned to control levels by day 3 (Figure 2.8D). Similarly, levels of the muscle lineage differentiation markers MyoD, MyoG, and MHC were all decreased at early time points after IGFBP-6 knockdown (Figures 2.8E to 2.8G). Increased protein levels of muscle commitment marker and reduced levels of muscle differentiation markers suggest that endogenous IGFBP-6 knockdown initiated PMSCs commitment to the muscle lineage but delayed muscle differentiation. However, IGFBP-6 knockdown did not change cell morphology when compared to the control at day 7 post-treatment (Figure 2.9A and 2.9B) which needs to be further investigated.

On the other hand, IGFBP-6 knockdown decreased IGFBP-6 production and secretion as IGFBP-6 levels were reduced in PMSC conditioned media at all-time points as measured
by ELISA (Figure 2.9C). In addition, the frequency of cells with high ALDH-activity, was decreased with IGFBP-6 knockdown compared to control (scrambled siRNA) at days 1 and 3 (Figure 2.10).
Figure 2.8. IGFBP-6 knockdown in PMSCs under muscle differentiation conditions reduced muscle markers protein levels. PMSCs were treated with siRNAs against IGFBP-6, or scrambled siRNA control every 3 days in muscle differentiation media. (A) IGFBP-6 levels were significantly decreased at 1 and 2 days after siRNA treatment but recovered to control levels at day 3. When IGFBP-6 siRNA was reintroduced at day 3, there was a significant decrease for up to 5 days which returned to control levels at day 6. IGFBP-6 siRNA treatment decreased (B) OCT4 and (C) SOX2 levels at the early time points then returned to control levels at day 5. In contrast, muscle cell commitment marker (D) Pax3/7 was increased at days 1 and 2 when IGFBP-6 was knocked-down. Muscle differentiation markers: (E) MyoD, (F) MyoG, and (G) MHC levels were decreased at early time points but recovered to control levels by day 5. Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 comparing siRNA treatments, or #P<0.05, ##P<0.01, ###P<0.001 comparing the same treatment over time.
Figure 2.9. IGFBP-6 siRNA treatment maintained PMSCs cell morphology and inhibited IGFBP-6 secretion. PMSC skeletal muscle morphology was maintained for 7 days under muscle lineage differentiation conditions with (A) scrambled siRNA or (B) IGFBP-6 siRNA treatment. (C) IGFBP-6 secretion was decreased at each time point with IGFBP-6 siRNA treatment that was applied every 3 days. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine ***P<0.001.
(A) DEAB

Day 1
Si (Control)

ALDH

ALDH

(IGFBP-6)

(B) ALDH

Day 3
Si (Control)

ALDH

ALDH

(IGFBP-6)
Figure 2.10. IGFBP-6 siRNA in PMSCs cultured under muscle differentiation conditions decreased the frequency of cells with high ALDH-activity. Representative flow cytometry dot plots with Aldefluor and an inhibitor of ALDH (DEAB) or with ALDH alone of PMSCs treated with IGFBP-6 siRNA at (A) day 1, (B) day 3, and (C) day 7 under muscle differentiation conditions. (D) PMSCs treated with IGFBP-6 siRNA showed significantly reduced frequency of cells with high ALDH-activity. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine ***P<0.001.
2.4 DISCUSSION

Stem cell research has progressed in recent years and the promise of using stem cells in tissue regeneration and cellular therapies is closer to becoming a reality in the clinical field (22, 23). However, before they can be used reliably and safely in regenerative medicine, it is essential to understand how factors within the stem cell microenvironment influence lineage commitment and differentiation as stem cell fate is altered by the culture conditions in vitro (24). In addition, most current cellular therapies utilize multipotent stem cells already poised to generate a desired lineage of committed progenitor cells by culturing them under specific differentiation conditions prior to therapy. Congenital muscular dystrophies represent a potential genetic disorder that may be amenable to cellular therapies due to accessibility and possible incorporation of new functional skeletal muscle cells into diseased tissues after transplantation (25, 26). Results from this study are the first to provide insight into how IGFBP-6 can be used to modulate muscle lineage commitment and differentiation from readily-available PMSCs in vitro.

The human placenta is usually discarded, but it represents a rich source of adult mesenchymal stem cells for the development of regeneration therapies (2, 3, 27). Due to early ontogeny, PMSCs demonstrate greater expansion capacity in vitro as compared to mesenchymal stem cells isolated from adult bone marrow (1). They also demonstrate low tumorigenicity (28) and high immunotolerance capacity to reduce the possibility of triggering an immune response (29). Thus, placental stem cells could provide a readily available source of MSCs for future clinical applications.
The IGF family plays a central role in muscle development, differentiation, growth, and regeneration (14-16, 30, 31). IGFBPs are the carriers for IGFs in the circulation (7), protecting them from degradation (6, 32), and delivering them to specific tissues and thus modulating the biological actions of IGFs. Also, IGFBPs increase the half-life of the IGF peptides in the circulation and control their access to the IGF-1R, thus playing an important role in IGF regulated cell metabolism, development, and growth. In recent years, it has become apparent that the IGFBPs have functions independent of IGFs (8).

Several IGF-binding proteins have been shown to be important in myogenesis and are expressed in developing muscle cells. Ren et al. reported that in C2C12 myoblast cells and in primary skeletal muscle cells, IGFBP-5 acts in an IGF-dependent manner to promote myogenesis by binding to IGF-2 and promoting its interaction with the IGF-1R (33). Knockdown of IGFBP-5 impaired myogenic differentiation by reducing myogenin, myosin heavy chain, and IGF-2 expression (33). In L6E9 skeletal myoblasts, IGFBP-4 and IGFBP-6 were accumulated during myogenesis, with IGFBP-4, not IGFBP-6, inhibiting IGF-1 induced muscle differentiation (34). These findings suggested the important role of IGFBPs in the differentiation of both primary cell lines of skeletal muscle lineage. Our study is the first to demonstrate the role of IGFBP-6, which is specific for the embryonic IGF, IGF-2, in muscle development using PMSCs.

The aim of this study was to characterize the effects of IGFBP-6 on early differentiation stages before PMSCs commit to the muscle lineage. When PMSCs were cultured under muscle differentiation conditions, they showed the capacity to differentiate into multi-nucleated fibers. The biological effects of IGFBP-6 on this differentiation process, as determined by markers of multipotency (OCT4 and SOX2), muscle commitment
(Pax3/7), and differentiation (MyoD, MyoG, and MHC) were significantly changed at early time points. Thus, suggesting that IGFBP-6 induced muscle differentiation and could potentially be used to guide skeletal muscle regeneration using stem cell therapy.

IGFBP-6 was highly expressed in developing muscle cells (35, 36), however, its role in muscle development is unclear. Previous studies from our laboratory using human fetal tissues have demonstrated that IGFBP-6 mRNA is expressed abundantly in the skeletal muscle, heart and skin, and prevalent in the regions of active cellular division and differentiation, suggesting that the protein is synthesized in these tissues and has autocrine/paracrine actions in developing cells (37). In another study from our laboratory, we reported that IGFBP-6 mRNA was expressed in low abundance in the chorionic villi of placenta during the second and third trimesters (38), suggesting that this IGFBP-6 is expressed in PMSCs and that the expression is increased only when PMSCs are induced to differentiate into a specific lineage such as skeletal muscle.

The findings in this current study suggest that PMSCs in the developing myotome express IGFBP-6 during differentiation, indicating that IGFBP-6 is an integral protein during muscle development. In fact, as muscle differentiation progressed in vitro, intracellular IGFBP-6 decreased gradually as seen with IGFBP-6 protein levels due to the capacity to secrete IGFBP-6 into the culture medium. These results indicate that IGFBP-6 may have both intracellular and extracellular effects on muscle development. Thus, IGFBP-6 activities may switch from intracellular IGF-independent actions to more paracrine IGF-dependent or IGF-independent actions as muscle differentiation occurs. Interestingly, the addition of IGFBP-6 to the culture medium increased cellular IGFBP-6 (intracellular or cell-associated) with a concurrent increase in pluripotency-associated
markers OCT4 and SOX2. The increase in intracellular IGFBP-6 suggests that IGFBP-6 was likely internalized or associated with the cell surface which needs to be further investigated.

When extracellular IGFBP-6 was supplemented into PMSC cultured under muscle differentiation conditions, the muscle commitment marker, Pax3/7 was increased at all-time points of the study, while muscle differentiation markers increased only at the earliest time points. As differentiation progressed, IGFBP-6 treatment delayed myogenic differentiation as demonstrated by decreased muscle differentiation markers MyoD, MyoG and MHC. These findings together with the higher OCT4 and SOX2 levels indicate that IGFBP-6 promotes the commitment of PMSCs towards the muscle lineage, while the prolonged presence delays the muscle differentiation process. Therefore, IGFBP-6 may be playing a dual role in both PMSC multipotency and differentiation towards skeletal muscle, with greater impact before muscle commitment has occurred.

Moreover, increased IGFBP-6 in the MSC microenvironment is expected to reduce the bioavailability of IGF-2 due to its high affinity for the peptide, confirmed by IGF-2 ELISA (Figure S2.5A). Thus, it is likely that increased IGF-2 secretion by differentiating muscle cells will have a biologic impact on muscle development, which was further investigated in chapter three.

Knockdown of IGFBP-6 using siRNA decreased both intracellular and secreted IGFBP-6. This knockdown resulted in decreased OCT4 and SOX2 levels, suggesting that IGFBP-6 enhances the multipotent progenitor cell phenotype in PMSCs. In contrast, the early increase in Pax3/7 in response to IGFBP-6 silencing indicates an earlier
commitment towards the myogenic lineage. Increased Pax3/7 may be correlated to the presence of increased extracellular IGF-2 (Figure S2.5B). In contrast, the muscle differentiation markers MyoD, MyoG, and MHC were all reduced after IGFBP-6 knockdown, suggesting IGFBP-6 is required for the muscle differentiation processes. Therefore, IGFBP-6 loss leads to an early commitment towards the myogenic lineage but delayed differentiation. However, there results needs to be further investigated using a more stable knockdown of IGFBP-6 because IGFBP-6 siRNA was transient and the IGFBP-6 levels returned to control. Moreover, the IGFBP-6 knockdown was performed for 7 days and a longer time point (14 days) may provide additional information to help delineate the results.

Overall, we have demonstrated that IGFBP-6 has both endogenous and exogenous actions that can promote or inhibit PMSC multipotency or differentiation. Extracellular IGFBP-6 facilitated muscle lineage commitment while a prolonged exposure inhibited late stage differentiation. Therefore, endogenous IGFBP-6 may be required for maintaining PMSC multipotency, delaying commitment, and enhancing late stage differentiation. This could occur through different possible pathways in a time-dependent manner that needs to be further investigated to confirm the mechanisms of IGFBP-6 effects on PMSCs muscle differentiation. IGFBP-6 addition before PMSCs commit to the muscle lineage may cause IGFBP-6 to bind to another muscle protein and form a complex to activate myogenesis. IGFBP-6 may bind to an inhibitor of myogenesis and either prevent it from inhibiting muscle differentiation or enhance the inhibitors functions. IGFBP-6 may be affecting myogenesis through IGF-1R and its downstream signaling pathways (PI3K or MAPK) or independent of IGF binding.
There is still a lot of work to be done to determine the mechanism for IGFBP-6 effects on PMSCs myogenesis and to confirm the outcomes from this study as experiments were performed from one preterm placental tissue (15 weeks) and increasing the sample number to have biological replicates will help confirm the results and better clarify the data.

In conclusion, PMSCs differentiate into skeletal muscle cells under appropriate niche conditions, and this process is enhanced by the increase in extracellular IGFBP-6 and delayed by silencing IGFBP-6 expression (Figure 2.11). The balance between endogenous and exogenous levels of IGFBP-6 controls muscle differentiation, and since IGFBP-6 has intracellular and extracellular effects, the response may occur dependent or independent of IGFs (particularly IGF-2).
Figure 2.11. Model of IGFBP-6 functions on PMSCs differentiation into skeletal muscle. PMSCs under normal growth conditions (10% FBS) express high levels of pluripotency-associated markers OCT4 and SOX2. As these cells commit towards the skeletal muscle lineage, increased IGFBP-6 correlated with increased Pax3/7 that decreased as differentiation markers (MyoG, MyoD, and MHC) were increased. Both committed and differentiated muscle cells continued to express and secrete IGFBP-6. As IGFBP-6 increased, there was an increase in multipotency markers, as well as, an earlier commitment and differentiation towards the muscle lineage. Thus, IGFBP-6 was required for maintaining multipotency and enhancing muscle commitment and differentiation.
2.5 REFERENCES


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Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells 2006; 24:462-71.


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Appendix 1
Figure S2.1. IGFBP-6 levels in response to IGFBP-6 supplementation in PMSCs under skeletal muscle differentiation conditions. (A) PMSCs cultured under muscle differentiation conditions showed increased IGFBP-6 protein levels, using western blots, in response to different doses of recombinant human IGFBP-6 protein supplementation with 375 ng/mL and 450 ng/mL having the highest band intensity. (B) IGFBP-6 secretion into the media was increased with the supplementation of recombinant human IGFBP-6 protein (375 ng/mL) that reduced by time and was lower compared to control at day 3. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta (15 weeks). Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine ***P<0.001.
Figure S2.2. PMSCs cultured under muscle differentiation conditions showed the formation of multi-nucleated fibers and lower cell count compared to control. (A) At 14 days post-differentiation, PMSCs grown in muscle differentiation media showed multi-nucleated skeletal muscle fiber formation (40X). Black arrows indicate the multi-nucleated muscle. (B, C) Cells grown in muscle differentiation media, showed less OCT4 (green-Alexa 488, λ=488 nm) when compared to PMSCs in 10% FBS at 14 days post-differentiation (20X). Nuclei, were stained with Hoechst dye (blue, λ=340 nm). (D) PMSCs under muscle differentiation conditions showed lower cell count per field compared to control. Data is presented as the mean ± SEM of 15 different fields from 3 independent experiments from one preterm placenta. One-way ANOVA followed by a Student’s t-test, **P<0.01.
Figure S2.3. PMSCs cultured under skeletal muscle differentiation conditions showed increased IGFBP-6 expression. (A, B) PMSCs cultured under muscle differentiation conditions showed higher IGFBP-6 staining (red-Alexa, λ-568 nm) when compared to PMSCs under control conditions (10% FBS) at 14 days post-differentiation (20X). Nuclei, were stained with Hoechst dye (blue, λ=340 nm). Images are representative of 3 technical replicates.
Figure S2.4. PMSCs under muscle differentiation conditions treated with IGFBP-6 showed more muscle compaction with lower cell count compared to cells under muscle differentiation alone. Data is presented as the mean ± SEM of 15 different fields from 3 independent experiments from one preterm placenta (15 weeks). One-way ANOVA followed by a Student’s t-test, **P<0.001.
Figure S2.5: IGF-2 secretion in PMSCs treated with IGFBP-6 or IGFBP-6 siRNA under muscle differentiation conditions. (A) IGF-2 levels secreted into the media were significantly decreased at each time point after IGFBP-6 addition compared to the control. (B) After treatment with siRNA against IGFBP-6 compared to controls (scrambled siRNA), IGF-2 levels increased at the first 48 hours with siRNA treatment applied every 3 days. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta (15 weeks). Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.001.
CHAPTER THREE

Insulin-Like Growth Factor-2 and Insulin-Like Growth Factor Binding Protein-6 Promote the Differentiation of Placental Mesenchymal Stem Cells into Skeletal Muscle
3.1 INTRODUCTION

Insulin-like growth factor (IGF) system regulates cell growth, differentiation, migration, and cell survival through the integration of several receptor-dependent signal transduction pathways (1). The IGF family consists of two IGF ligands, IGF-1 and IGF-2, three cell surface receptors, type-1, type-2 IGF receptors, and the insulin receptor, and six IGF binding proteins (IGFBPs) (1). IGF-1 and IGF-2 are circulating peptides, which bind IGF-1R, a ligand-activated receptor tyrosine kinase (1, 2). IGFBPs act as carriers for IGFs in the circulation (3), protecting them from degradation (2, 4), and regulating the biological actions of IGFs by delivering them to specific tissues. The IGF family plays an important role in fetal and placental development by stimulating proliferation, differentiation and survival of various types of placental cells (5). IGFs are vital in cell growth, development, and cell-fate changes through several mitogen activation cascades (6-9). The IGF family is also important in muscle development as IGFs maintain muscle cell viability, promote hypertrophy, and stimulate differentiation in cultured myoblasts (9). The importance of IGFs and the IGF-1R in skeletal muscle development was determined using loss-of-function animal models. IGF-1R knockout mice die soon after birth as functional respiratory muscle was deficient and pups were unable to breathe (10, 11). Furthermore, IGF-1 and IGF-2 were expressed by skeletal muscle cells during muscle repair in response to injury and exercise (12, 13). IGFs are the only factors known to promote both muscle cell proliferation and differentiation (13). In response to muscle injury, adult skeletal muscle regenerates by expressing myogenic regulatory factors (MRFs) (13). During myogenesis, committed muscle cells differentiate into the muscle lineage by expressing muscle commitment markers, Pax3 and Pax7, which in turn up-
regulate MyoD and myogenin (14). After commitment, myoblasts fuse together and form multinucleated fibers that express myosin heavy chain (MHC) (14).

After muscle injury, IGF-1 enhanced regeneration while loss of IGF-1 activity using neutralizing antibodies caused a reduction in the number of regenerating myofibers in vivo (15). IGF-2 which up-regulates its own gene expression during myogenesis in a positive feedback loop (12), is expressed abundantly in the developing skeletal muscle and is the major growth factor for muscle growth, differentiation, and regeneration (12, 13). When IGF-2 is inhibited, myogenesis does not occur (16). In fact, IGF-2 is required to allow continued recruitment of MyoD-associated proteins at the Myogenin promoter (17). Moreover, in cultured myoblasts, IGFs stimulate terminal differentiation through an autocrine pathway dependent on IGF-2 secretion (18).

IGFBPs also play a role in fetal and placental development as they are expressed during different stages of development in time-specific and cell-specific manner. Although, loss of function studies targeting single IGFBP genes have not yielded significant phenotypic changes in the fetus or placenta, it is thought that these subtle changes in development are possible biologic compensation by other IGF-binding proteins. IGFBP-6, a member of the IGF binding protein family, is expressed abundantly in developing muscle cells and is required for myogenesis (13). It is a unique peptide among the IGFBPs due to its higher binding affinity to IGF-2 versus IGF-1 (~70 to 100 fold) (19-22). The most commonly reported function of IGFBP-6 is the modulation of IGF-2 activity. IGFBP-6 binds IGF-2 in the circulation and prevents IGF-2 from binding to the cell surface receptors, and modulates IGF-2 bioavailability in vitro and in vivo (23, 24). IGFBP-6 can also bind and localize IGF-2 at the cell surface, enhancing IGF-2 actions by delivering IGF-2 to the
IGF-1R (24). However, the mechanisms controlling the multiple actions of IGFBP-6 remain unclear. Previous studies in Chapter 3, showed that both extracellular and intracellular IGFBP-6 were expressed in PMSCs during myogenesis. In this study, we determined whether IGFBP-6 impact on PMSCs differentiation into skeletal muscle is dependent or independent of IGF-1 and IGF-2 binding (particularly IGF-2).

3.2 MATERIALS AND METHODS

3.2.1 PMSC Isolation

PMSC isolation and experiments were conducted with the approval from the Health Sciences Research Ethics Board of Western University (REB# 12154). Informed consent was obtained from healthy women undergoing therapeutic termination of pregnancy, and the PMSCs used in this study were isolated from one 15 week preterm placental tissue. After surgery, chorionic villi were dissected, washed, and minced with surgical scissors and forceps, and then subjected to enzymatic digestion with collagenase IV (369 IU/mg), hyaluronidase (999 IU/mg) (Sigma-Aldrich, Oakville, ON), and DNase I (2,000 IU/mg) (Hoffmann-LaRoche, Mississauga, ON) for 10 minutes at room temperature, followed by 0.05% trypsin (Gibco/Invitrogen, Mississauga, ON) for 5 minutes at room temperature. The sample was then washed for 10 minutes with 10% FBS in DMEM/F12 medium and the resulting single cell suspension was separated by density centrifugation over a Percoll (Sigma-Aldrich, Oakville, ON) discontinuous gradient using a modified protocol by Worton et al. (25).
3.2.2 Cell Culture

Cells from Percoll gradient fractions #3 and #4 were plated on to T75 flasks, cultured, and maintained using DMEM/F12 media supplemented with 15% FBS serum (Gibco/Invitrogen, Mississauga, ON) and FGF-2 (50 ng/mL) (Sigma-Aldrich, Oakville, ON) containing 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 0.25 µg/mL Amphotericin-B. The non-adherent cells were discarded at the time of media change, which was performed every 72 hours. The adherent cells were cultured until they reach 90% confluence. Cells were then passaged 1:2 approximately once per week using 0.05% Trypsin for 10 min at 37°C for 3 passages. Passaged cells were stored at -80°C in 1 mL of freezing media (30% FBS and 10% DMSO in DMEM/F12 media). When needed, vials were thawed and cells were resuspended in normal culture media (25 ng/mL FGF-2 and 15% FBS in DMEM/F12).

3.2.3 Muscle Differentiation and IGF treatment

Cells were plated in muscle growth media (fetal bovine serum 0.05 mL/mL, fetuin 50 µg/mL, epidermal growth factor 10 ng/mL, basic fibroblast growth factor 1 ng/mL, insulin 10 µg/mL, and dexamethasone 0.4 µg/mL) for 48 hours before changing to the skeletal muscle differentiation media, which is a proprietary serum-free medium containing 10 µg/mL insulin (PromoCell, Heidelberg, Germany) for 14 days with or without 100 ng/mL of either IGF-1 or IGF-2 (Cell Signaling Technology, Danvers, MA). IGFs were added every 3 days at the time of media change. Cells were grown in six well plates in a standard tissue culture incubator at 37°C in 5% CO₂.
3.2.4 Down-Regulation of IGF-2 Expression by siRNA

To silence endogenous IGF-2 expression in PMSCs, siRNA against IGF-2 (Santa Cruz Biotechnology, Dallas, TX) was used. 8 μL of Lipofectamine (Invitrogen, Mississauga, ON) with either 8 μL of scrambled or IGF-2 siRNA was added to 100 μL of DMEM/F12 media (Invitrogen, Mississauga, ON) for 40 minutes at room temperature. The siRNA solution was added to 60% confluent cells and incubated for 5 hours at 37°C. Muscle growth media (1.5 mL) was added for 48 hours and subsequently replaced with 2 mL of muscle differentiation media (PromoCell, Heidelberg, Germany). New siRNA was added every 3 days during the change of media for 14 days.

3.2.5 IGFBP-6

Recombinant human IGFBP-6 (PROSPEC, East Brunswick, NJ) was added to the media (375 ng/mL) every 3 days at the time of media change. The IGFBP-6 concentration and time of supplementation was determined as shown in Chapter 2 (Figure S2.1).

3.2.6 Cell Lysate Preparation

Following PMSC treatments, cells were washed with DPBS (Gibco/Invitrogen, Mississauga, ON) and 200 μL of (1x) cell lysis buffer (Cell Signaling Technologies, Burlington, ON) mixed with protease and phosphatase inhibitors (1:100) were added to the cells. Cells were detached using a cell scraper and the lysate was sonicated for 15 seconds. Finally, samples were centrifuged for 15 min at 14,000 rpm at 4°C, and the supernatant was collected.
3.2.7 Immunoblotting

Cell lysates containing 20 µg of protein were added to 6x SDS gel loading buffer (1% β-mercaptoethanol, 1% SDS, 30% glycerol, 0.0012% bromophenol blue, Tris HCl 0.28 M, pH 6.8). Samples were boiled for 5 minutes at 95°C, then placed on ice for 3 minutes, and centrifuged at 3,000 rpm for 20 seconds before loading. Samples were resolved by molecular weight using 10% SDS polyacrylamide gels transferred onto poly-vinyl-dene-fluoride (PVDF) membranes (Bio-Rad, Hercules, CA) using a Trans-Blot Turbo (Bio-Rad, Hercules, CA) with an optimized protocol depending on protein size. Membranes were blocked with 5% non-fat-dry milk, gently shaking for 1 hour at room temperature in Tris-HCl buffer saline pH 8.0 with 0.1% Tween-20 (TBS-T). Blots were then washed with TBS-T (3 times for 10 minutes) followed by incubation at 4°C overnight with specific primary antibodies in 5% BSA or 5% non-fat-dry milk in TBS-T following the manufacturer’s protocol. To detect stem cell-associated potency markers, antibodies for OCT4 (N-19: sc-8628) (Santa Cruz Biotechnology, Dallas, TX), SOX2 (2683-1) (Epitomics, Burlington, ON), and Nanog (3369-1) (Epitomics, Burlington, ON, CAN) were used. To detect markers of muscle differentiation, Pax3/7 (E-10: sc-365613), MyoD (M-318: sc-760), Myogenin (F5D: sc-12732), and Myosin heavy chain (H-300: sc-20641) (Santa Cruz Biotechnology, Dallas, TX) were used. To detect IGFBP-6, antibody H-70: sc-13094 (Santa Cruz Biotechnology, Dallas, TX) was used. To detect IGF-2, antibody H-103: sc-5622 (Santa Cruz Biotechnology, Dallas, TX) was used. For loading control, pan-Actin Ab-5 (Thermo Fisher Scientific, Fremont, CA) was used.

Then membranes were washed and incubated for 1 hour at room temperature with the corresponding secondary HRP-conjugated antibody. The secondary antibodies used were
HRP-conjugated goat anti-rabbit (#170-6515) or anti-mouse (#170-6516) (Bio-Rad, Hercules, CA) or donkey anti-goat antibody (Santa Cruz Biotechnology, Dallas, TX).

Resolved protein bands were detected using chemiluminescence and images were taken using the VersaDoc Imager (Bio-Rad, Hercules, CA). Western blots were performed in triplicate.

### 3.2.8 Immunocytochemistry

PMSCs were grown on glass cover slips (80-90% confluence) for 14 days, stained with primary antibodies (1:100), and incubated at 4°C overnight. To detect stem cell-associated potency markers, OCT4 antibody (N-19: sc-8628) (Santa Cruz Biotechnology, Dallas, TX) was used. To detect markers of muscle differentiation, Pax3/7 (E-10: sc-365613), MyoD (M-318: sc-760), and Myosin heavy chain (H-300: sc-20641) (Santa Cruz Biotechnology, Dallas, TX) were used. To detect IGFBP-6, antibody H-70: sc-13094 (Santa Cruz Biotechnology, Dallas, TX) was used. Primary antibodies were washed using 0.1% Tween-20 in PBS (3 times for 5 minutes); cells were then incubated in the dark with the corresponding secondary AlexaFlour conjugated antibody (1:200). The secondary antibodies used were green-Alexa 488 or red-Alexa 568 (Invitrogen, Mississauga, ON). The secondary antibody was washed with 0.1% Tween-20 in PBS, and the nuclear stain (Hoechst dye) was added for 7 minutes and then rinsed. The cover slips were mounted and images were taken using a confocal microscope (Zeiss, Germany). Each antibody was in triplicates.
3.2.9 Quantification of IGFBP-6 and IGF-2 Secretion by Enzyme-Linked Immunosorbent Assay (ELISA)

Human IGFBP-6 (RayBiotech®, Burlington, ON) and IGF-2 (ALPCO, Salem, NH) ELISA kits were used to measure the amount of IGFBP-6 and IGF-2 secreted into PMSC conditioned media for 14 days. Standards and samples were loaded into the wells (in triplicate) and the immobilized antibody bound IGFBP-6 or IGF-2 present in the sample. The wells were washed and biotinylated anti-human antibody was added. After washing, HRP-conjugated streptavidin was added; then a TMB substrate solution was used to develop a blue color in proportion to the amount of IGFBP-6 or IGF-2 bound. The Stop Solution changes color from blue to yellow, and the intensity was measured at 450 nm using Multiskan Ascent plate reader and analysis software.

3.2.10 Aldehyde Dehydrogenase (ALDH) Activity

ALDH-activity, a conserved progenitor cell function, was assessed by flow cytometry for 14 days. Using the Aldefluor™ assay (Stem Cell Technologies, Vancouver, BC), as per the manufacturer’s instructions. Briefly, 5 μL of activated Aldefluor reagent was added to 1 mL of cell suspension and incubated for 45 minutes at 37°C. Cells were washed and resuspended in 500 μL of ice-cold Aldefluor assay buffer and ALDH-activity was measured using flow cytometry. As a negative control, Aldefluor™ DEAB reagent was used. Samples were run in triplicate.

3.2.11 Statistical Analysis

All experiments were performed in triplicate from one 15 week placental tissue (technical replicates). GraphPad Prism Software 5.0 was used to generate all graphs and analyses. A
two-way ANOVA followed by a Bonferroni’s multiple comparison test or a one-way ANOVA followed by a Student’s t-test was used to calculate significant differences when p<0.05. Graphic representation values are presented as mean ± SEM (shown as variance bars).

3.3 RESULTS

3.3.1 IGF-1 Affects PMSC Multipotency and Differentiation into Skeletal Muscle

To test the effects of exogenous IGF-1 on PMSC multipotency and differentiation to muscle cells, IGF-1 was supplemented to muscle differentiation media (100 ng/mL) every third day for up to 14 days. Immunocytochemistry analyses at day 14 revealed that PMSCs treated with IGF-1 showed qualitatively increased Pax3/7, decreased MyoD, and no change in OCT4 and MHC immunoreactivity (Figure 3.1). Moreover, IGF-1 treatment increased the total number of cells per field compared to un-supplemented controls (Figure S3.1) suggesting the IGF-1 stimulated proliferation which needs to be further investigated.

Western blots were used to determine the protein levels of potency and muscle differentiation markers. Pluripotency-associated marker OCT4 levels were not changed by IGF-1 supplementation (Figure 3.2A), while both SOX2 (Figure 3.2B) and Nanog (Figure 3.2C) levels were reduced at early time points compared to un-supplemented muscle differentiation conditions. In contrast, IGF-1 supplementation increased Pax3/7 levels at 7 and 14 days suggesting PMSCs’ commitment to the muscle lineage (Figure 3.2D). Finally, IGF-1 supplementation decreased the levels of muscle-specific
differentiation marker, MyoD, at day 14 (Figure 3.2E), but did not change MyoG and MHC levels (Figures 3.2F and 3.2G), compared to PMSCs cultured under un-supplemented muscle differentiation conditions.

ALDH-activity of PMSCs was determined for the frequency of PMSCs with conserved phenotype of early progenitor cells (26-28). Under muscle differentiation conditions, IGF-1 supplementation increased the frequency of cells with high ALDH-activity at all-time points compared to PMSCs under muscle differentiation alone (Figures 3.3A to 3.3D) suggesting that extracellular IGF-1 prolonged progenitor cell phenotype in PMSCs under muscle differentiation conditions.

3.3.2 IGF-2 Affects PMSCs Multipotency and Differentiation into Skeletal Muscle

The impact of IGF-2 in PMSC multipotency and differentiation into skeletal muscle was investigated by adding IGF-2 (100 ng/mL) to the muscle differentiation media. Using immunocytochemistry, PMSCs cultured under muscle differentiation conditions supplemented with IGF-2 showed no change in OCT4, Pax3/7, and MyoD immunoreactivity (IR) (Figure 3.1). In contrast, muscle differentiation marker MHC immunoreactivity (IR) was increased (Figure 3.1) at 14 days after IGF-2 treatment compared to untreated controls, with fewer cells per field (Figure S3.1) suggesting that IGF-2 enhanced the terminal muscle differentiation process.

Using western blots, we demonstrated that IGF-2 supplementation also increased the protein levels of pluripotency-associated marker OCT4 at days 3, 7, and 14 compared to PMSCs cultured in muscle differentiation media alone (Figure 3.2A), whereas other
pluripotency-associated markers SOX2 (Figure 3.2B) and Nanog (Figure 3.2C) were decreased. In contrast, IGF-2 supplementation increased the protein levels of the muscle lineage commitment marker Pax3/7 and the muscle differentiation markers MyoD, MyoG, and MHC levels compared to untreated controls (Figures 3.2D to 3.2G).

Moreover, IGF-2 supplementation of PMSCs under muscle differentiation conditions decreased the frequency of cells with high ALDH-activity compared to PMSCs under untreated muscle differentiation condition at all time points (Figure 3.3A to 3.3D), suggesting that IGF-2 promoted the differentiation of PMSCs into skeletal muscle.
Figure 3.1. PMSCs treated with IGF-2 showed qualitatively increased MHC immunoreactivity (IR) at 14 days. Compared to PMSCs cultured under muscle differentiation alone, PMSCs treated with IGF-1 or IGF-2 showed similar OCT4 immunoreactivity (red-Alexa, λ-568 nm). However, IGF-1 supplementation increased Pax3/7 IR (green-Alexa, λ-488 nm), decreased MyoD IR, with no change in MHC IR (red-Alexa, λ-568 nm). In contrast, IGF-2 treatment did not alter Pax3/7 IR (green-Alexa, λ-488 nm) or MyoD IR, but increased MHC IR (red-Alexa, λ-568 nm). Nuclei, stained with Hoechst dye (blue, λ=340 nm). Immunocytochemistry was performed in triplicate with each antibody.
Figure 3.2. IGF-1 and IGF-2 supplementation differently affected levels of pluripotency-associated and muscle differentiation markers in PMSC. (A) IGF-1 treatment had no effect on OCT4 levels, while IGF-2 treatment increased OCT4 levels at 3, 7, and 14 days compared to muscle differentiation alone. (B) SOX2 and (C) Nanog levels were decreased after IGF-1 treatment at early time points only, whereas IGF-2 treatment decreased SOX2 levels until day 7 and at all-time points for Nanog. (D) Pax3/7 levels were increased at 7 and 14 days and did not decrease after IGF-1 treatment, while IGF-2 treatment increased Pax3/7 levels at all-time points but decreasing with time. (E) MyoD, (F) MyoG, and (G) MHC protein levels did not change with IGF-1 supplementation, except for a decrease in MyoD levels at day 14. In contrast, IGF-2 treatment increased muscle differentiation marker levels at all-time points. Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 comparing control to muscle differentiation conditions, or ###P<0.001 comparing the same treatment over time.
Figure 3.3. PMSCs cultured under skeletal muscle differentiation conditions treated with IGF-1 showed an increased frequency of cells with high ALDH-activity, while cells treated with IGF-2 showed a decreased frequency. Representative flow cytometry dot plots showing the frequency of PMSC with high ALDH-activity when cultured under muscle differentiation conditions with or without either IGF-1 or IGF-2 at (A) day 1, (B) day 3, (C) day 7, and (D) day 14. DEAB treated controls were used to establish the ALDH gate (data not shown). (E) Compared to PMSCs under muscle differentiation conditions, cells treated with IGF-1 showed increased frequency of cells with high ALDH-activity, while cells treated with IGF-2 showed decreased frequency of cells with high ALDH-activity. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, ***P<0.001.
3.3.3 Extracellular IGFs Altered IGFBP-6 Levels

Using immunocytochemistry performed at day 14, IGF-1 treatment decreased IGFBP-6 immunoreactivity; whereas IGF-2 increased IGFBP-6 immunoreactivity in PMSCs treated under muscle differentiation conditions (Figure 3.4A). Using western blots, we showed increased IGFBP-6 levels with IGF-1 treatment at early time points, which coincided with delayed muscle commitment as indicated previously by higher Pax3/7 levels (Figure 3.1D). In contrast, IGF-2 did not increase IGFBP-6 levels until day 14 compared to PMSCs under muscle differentiation only (Figure 3.4B). When compared to PMSCs treated with IGF-1, IGF-2 treatment increased IGFBP-6 levels at 7 and 14 days (Figure 3.4B) suggesting that IGF-2 treatment stimulated IGFBP-6 synthesis after PMSCs commitment to the muscle lineage, whereas IGF-1 effect on IGFBP-6 synthesis occurred before PMSCs commitment to muscle.

To investigate the effects of IGF-1 and IGF-2 on IGFBP-6 secretion into conditioned media under muscle differentiation conditions, IGFBP-6 concentration in the PMSC media was measured using ELISA. With IGF-1 treatment, IGFBP-6 concentration was increased at early time points (day 1 and 3) and decreased at later time points (days 7 and 14) compared to control PMSCs under muscle differentiation conditions (Figure 3.4C). In contrast, IGF-2 treatment increased IGFBP-6 secretion throughout differentiation until day 14 (Figure 3.4C). Therefore, both IGFs increased IGFBP-6 synthesis by PMSCs under muscle differentiation conditions with the effect of IGF-1 short lived and IGF-2 for a long duration.
Figure 3.4. IGFBP-6 expression is altered by IGF-1 and IGF-2 treatment in PMSCs cultured under skeletal muscle differentiation conditions. (A) 14 days post-differentiation, PMSCs grown in muscle differentiation media were immunoreactive for IGFBP-6 (red-Alexa, λ-568 nm). IGF-1 decreased IGFBP-6 immunoreactivity and IGF-2 increased IGFBP-6 IR (nuclei stained with Hoechst dye, blue, λ=340 nm). (B) IGFBP-6 levels were increased by IGF-1 treatment at days 1 and 3 compared to PMSCs under muscle differentiation alone, however they were reduced at days 7 and 14. IGF-2 treatment did not change IGFBP-6 levels until day 14 when they were increased compared to controls. IGFBP-6 levels were quantified by densitometry and normalized to β-Actin. (C) IGFBP-6 secretion into conditioned media was increased with both IGF-1 (day 1 and 3) and IGF-2 (day 1 to 7) compared to controls. However, secreted IGFBP-6 levels were significantly decreased at 7 and 14 days with IGF-1 treatment; and remained increased with IGF-2 treatment. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to control (PMSCs under muscle differentiation), or #P<0.05, ###P<0.001 comparing between IGF-1 or IGF-2 treatments.
3.3.4 Extracellular IGFBP-6 Maintains Muscle Differentiation by PMSCs in the Absence of IGF-2

To evaluate the role of endogenous IGF-2 on PMSCs differentiation into skeletal muscle, IGF-2 mRNA was silenced using siRNA every 3 days for up to 14 days. PMSCs with IGF-2 knockdown showed lower muscle compaction compared to control (scrambled siRNA) at day 14 (Figure 3.5A, 3.5B and S3.2). The addition of IGFBP-6 together with IGF-2 siRNA permitted PMSCs compact muscle morphology at 14 days (Figure 3.5B and 3.5C). As expected, IGF-2 levels were decreased by IGF-2 knockdown compared to scrambled siRNA control. However, IGF-2 protein levels were equivalent to control levels at day 14 although IGF-2 siRNA was administered every 3 days (Figure 3.5D) indicating that siRNA-treatment was transient. Furthermore, IGF-2 levels remained significantly low at all of the time points by the addition of IGFBP-6 with IGF-2 knockdown and did not return to control levels (Figure 3.5D), suggesting an interaction between IGF-2 and IGFBP-6.

After IGF-2 knockdown, IGFBP-6 levels were increased until day 3 compared to scrambled siRNA control (Figure 3.5E), suggesting greater availability of IGFBP-6 demonstrated by an increase in secreted IGFBP-6 (Figure S3.3). As expected, IGFBP-6 protein levels were increased at each time point during IGF-2 siRNA treatment alongside extracellular IGFBP-6 supplementation compared to controls (Figure 3.5E).

Concurrent with IGF-2 knockdown, we observed a decrease in pluripotency-associated marker OCT4 levels until day 3 with an increase at day 14 compared to scrambled siRNA control (Figure 3.6A). In contrast, SOX2 levels did not change (Figure 3.6B). Furthermore, the addition of IGFBP-6 with IGF-2 knockdown reduced both OCT4
(Figure 3.6A) and SOX2 (Figure 3.6B) levels. The protein levels of muscle lineage differentiation markers MyoD (Figure 3.6C) and MHC (Figure 3.6D) were decreased significantly after IGF-2 knockdown indicating a critical role for IGF-2 in PMSC differentiation into skeletal muscle. In contrast, IGFBP-6 supplementation alongside IGF-2 knockdown significantly increased MyoD and MHC levels at 7 and 14 days compared to siRNA scrambled control or IGF-2 knockdown alone (Figure 3.6C and 3.6D).

Knockdown of IGF-2 expression in PMSCs significantly decreased the frequency of cells with high ALDH-activity compared to control (scrambled siRNA) at day 1 (Figure 3.7). In contrast, the addition of IGFBP-6 together with IGF-2 knockdown further decreased the frequency of cells with high ALDH-activity at all-time points compared to siRNA scrambled control or IGF-2 siRNA treatment (Figure 3.7) Collectively, these data suggest that IGFBP-6 reduced progenitor cell phenotype under muscle differentiation conditions and maintained the differentiation of PMSCs towards skeletal muscle in the absence of IGF-2.
Figure 3.5. IGF-2 knockdown with siRNA inhibited PMSCs differentiation into skeletal muscle and adding extracellular IGFBP-6 with the treatment helped rescue muscle compaction. (A) Compared to PMSCs under muscle lineage differentiation conditions with siRNA scrambled control, (B) PMSCs under muscle differentiation conditions treated with IGF-2 siRNA showed less skeletal muscle compaction at 14 days (10X). (C) Extracellular IGFBP-6 supplementation with IGF-2 knockdown enhanced the PMSCs muscle compaction at 14 days compared to IGF-2 siRNA. The white arrows indicate muscle compaction. (D) IGF-2 levels by PMSCs treated with IGF-2 siRNA under differentiation conditions were reduced and returned to control levels by day 14, while adding IGFBP-6 with IGF-2 knockdown maintained lower IGF-2 levels. (E) IGFBP-6 protein levels increased until day 3 with IGF-2 knockdown compared to siRNA scrambled control. IGFBP-6 addition with IGF-2 knockdown increased IGFBP-6 protein levels at each time point. Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to scrambled siRNA control, or #P<0.05, ###P<0.001 compared to IGF-2 siRNA.
Figure 3.6. IGF-2 knockdown decreased muscle differentiation markers, while adding IGFBP-6 partially increased the markers. (A) OCT4 levels were reduced with IGF-2 knockdown until day 3 and increased at day 14. Adding IGFBP-6 alongside IGF-2 siRNA reduced OCT4 protein levels at all-time points compared to siRNA scrambled control. In contrast, (B) SOX2 levels did not change with IGF-2 knockdown. But when IGFBP-6 was added with IGF-2 silencing, the levels were reduced from 1-3 days compared to control and IGF-2 knockdown. Protein levels of muscle differentiation markers: (C) MyoD and (D) MHC decreased with IGF-2 siRNA and IGFBP-6 supplementation increased the levels. Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to scrambled siRNA control, or #P<0.05, ###P<0.001 compared to IGF-2 siRNA.
Figure 3.7. IGF-2 knockdown and extracellular IGFBP-6 addition in PMSCs under muscle differentiation conditions decreased the frequency of cells with high ALDH-activity. Representative flow cytometry dot plots showing the frequency of PMSCs with high ALDH-activity when cultured under muscle differentiation conditions with or without either IGF-2 siRNA or IGF-2 siRNA and extracellular IGFBP-6 at (A) day 1, (B) day 3, (C) day 7, and (D) day 14. DEAB treated controls were used to establish the ALDH gate (data not shown). (E) Compared to PMSCs under muscle differentiation conditions (siRNA scrambled control), cells treated with IGF-2 siRNA showed decreased frequency of cells with high ALDH-activity at day 1, while adding extracellular IGFBP-6 with IGF-2 siRNA showed decreased frequency of cells with high ALDH-activity at each time point. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine **=P<0.01, ***P<0.001 compared to scrambled siRNA control, or ###P<0.001 compared to IGF-2 siRNA.
3.4 DISCUSSION

It is believed that if stem cells are to be used successfully in cell-based therapies for specific diseases, the cells must be induced to differentiate towards a progenitor cell of a desired lineage (e.g. skeletal muscle for muscular dystrophy) \(^{(29)}\) prior to therapy. In addition, adequate cell numbers will be needed for effective therapy. Human placenta, which is usually discarded, is a source of adult mesenchymal stem cells with functional capacity similar to bone marrow \(^{(30-33)}\). PMSCs also demonstrate low tumorigenicity with higher immunotolerance after transplantation, making them an ideal cell type for tissue regeneration therapies \(^{(34-35)}\).

The IGF system is important for muscle development, growth, regeneration, and differentiation \(^{(10, 11, 13, 36, 37)}\). IGF-2 plays an important role during C2C12 differentiation and is considered the main myogenic factor in myoblast cells \(^{(38)}\). In C3H 10T1/2 fibroblasts converted by MyoD transfection to myoblasts, there was an increase in the mRNA expression and protein levels of IGF-2 during the differentiation stage \(^{(16)}\). Taken together, these findings show the important role of the IGF family in muscle cell differentiation.

IGF-binding proteins act as carriers for IGF-1 and IGF-2 in the circulation, facilitating ligand delivery to specific tissues and controlling access to the IGF receptors \(^{(2, 3, 39)}\). Also, IGFBPs are expressed by many cell types, including skeletal muscle, and have been demonstrated to have functions dependent and independent of IGF binding \(^{(4)}\). For example, IGFBPs are expressed within developing muscle cells and are important in myogenesis \(^{(39, 40)}\). In osteoblasts, IGFBP-6 has been shown to modulate cell growth by
reducing the bioavailability of IGF-2 in the bone microenvironment (41). When L6E9 cells (a myoblast cell line used to study late myogenesis) are stimulated with IGF-1, these cells initiated an initial proliferative response. During this time of rapid cell division the myogenic regulatory factors were inhibited. Approximately 30 hours later, there was a stimulation of myogenin expression (42). In mouse C2C12 cells and C2 satellite cell line, there was a greater IGF-2 mRNA than IGF-1; 2000-folds to 20 folds respectively (12, 19, 43). This study is the first to show the effects of IGF-1, IGF-2, in combination with IGFBP-6 on human PMSCs differentiation into skeletal muscle \textit{in vitro}.

The aim of this study was to characterize the effects of IGFs on the differentiation of PMSCs into skeletal muscle and to delineate their interactions with IGFBP-6. From our data, we showed that IGF-1 can promote an early increase in IGFBP-6 expression before PMSCs commit to the muscle lineage, a requirement that delayed muscle lineage commitment. This was confirmed by the increased levels of muscle commitment marker, Pax3/7, and decreased muscle differentiation marker (MyoD) after IGF-1 treatment. On the other hand, IGF-2 treatment increased both IGFBP-6 and OCT4 levels. These results are in agreement with previous studies in Chapter two, showing IGFBP-6 positive effects on OCT4 levels. Also, the muscle differentiation markers (MyoD, MyoG, and MHC) were increased at later time points with IGF-2 treatment, confirming that IGF-2 enhanced PMSCs muscle differentiation, unlike IGF-1 (Figure 3.8). Increased OCT4 levels occurred alongside a decrease in SOX2 and Nanog levels which is expected in a mesodermal differentiation (44); OCT4 is needed for differentiation as it supports down-regulating pluripotency, and when deficient, cells are not able to differentiate (45).
In L6A1 myoblasts, IGFBP-6 inhibited muscle differentiation stimulated by IGF-2 but not IGF-1 (46). In contrast, IGFBP-6 was required for PMSCs differentiation into skeletal muscle and modulated both multipotency and muscle markers levels, as well as IGF-2 secretion (Chapter Two). Therefore, we focused on the role of IGFBP-6 on IGF-2 in PMSCs under muscle differentiation conditions.

When IGF-2 was knocked down using siRNA, myogenesis was inhibited, and adding IGFBP-6 helped recover the muscle differentiation process; supported by muscle morphology and differentiation markers. Moreover, IGFBP-6 expression has been previously associated with non-proliferative states and inhibition of IGF-2 dependent tumor cell growth in rhabdomyosarcoma, neuroblastoma, and colon cancer (47). More specifically, neuroblastoma cells undergo a decrease in both cell proliferation and tumorigenic potency as a result of exogenous IGFBP-6 expression; as IGFBP-6 sequesters IGF-2 preventing a mitogenic response in tumor cells (48, 49). These results are in agreement with our findings, as IGF-2 protein levels were reduced with IGFBP-6 extracellular addition and IGFBP-6 secretion into the media was higher after IGF-2 knockdown.

Previous studies in C2C12 cells showed that as muscle differentiation progressed, IGF-2 stimulated its own expression and inhibited IGF-1 expression in a time- and dose-dependent manner (12). In our study, there was a decrease in the amount of secreted IGF-2 to the media after IGF-1 treatment in PMSCs under muscle differentiation conditions; indicating an intricate balance between IGF-1 and IGF-2 expression exists in the niche during myogenesis from PMSCs (Figure S3.4).
Placenta development is dependent on the IGF system, including IGF-1 and IGF-2 (50). The importance of IGFs in the human placenta is well defined in mediating growth and differentiation of the different cells of the chorionic villi (5, 51). In the human placenta, IGF-2 mRNA is expressed in the villous mesenchymal core, where PMSCs reside (50). IGF-2 also plays a role in the placenta at the early gestation, whereas IGF-1 is associated in later gestation (52). In PMSCs, results from this study, showed that IGF-1 and IGF-2 had different effects on PMSCs differentiation into skeletal muscle in a time-dependent manner. The role of IGF1 and IGF-2 in PMSCs differentiation into skeletal muscle is not clearly defined and this study is the first to show the different effects between IGF-1 and IGF-2. However, there is still a lot of work to be done to clearly define IGFs role and determine the mechanism of the effects on PMSCs myogenesis and to confirm the outcomes from this study, as experiments were performed from one preterm placental tissue (15 weeks) and increasing the sample number to have biological replicates will help confirm the results and better clarify the data.

In conclusion, PMSC differentiation into skeletal muscle is regulated by IGFs. IGF-1 delays PMSC commitment towards the muscle lineage while IGF-2 enhanced myogenesis. IGFBP-6 was also required in this differentiation process and extracellular addition of IGFBP-6 alongside IGF-2 inhibition rescued PMSCs muscle differentiation. Since IGFBP-6 has both intracellular as well as extracellular effects, we show that effects on muscle differentiation are both IGF-dependent and IGF-independent (particularly IGF-2). Overall, treating PMSCs with IGFs and IGFBP-6 can regulate and improve PMSC differentiation into skeletal muscle for tissue regeneration. Further investigation of the balance between IGFs and IGFBP-6 is required to better understand PMSC-
mediated myogenesis and testing the effects of these factors and their interactions with the IGF-1R and insulin receptor are warranted to further delineate the signaling mechanisms governing muscle lineage development and regeneration.

**Figure 3.8. Model of IGFs and IGFBP-6 functions during PMSCs myogenesis.** During myogenesis, PMSCs lose pluripotency-associated markers (OCT4, SOX2, and Nanog) and gain muscle commitment marker (Pax3/7) that decreased as muscle differentiation markers increase (MyoG, MyoD, MHC). Committed and differentiated muscle cells expressed IGF-1, IGF-2, and IGFBP-6. Extracellular IGF-1 increased IGFBP-6 protein levels before PMSCs muscle commitment, resulting in a delayed PMSCs muscle commitment and differentiation. However, IGF-2 extracellular increase resulted in an increase in IGFBP-6 after commitment to the muscle lineage, resulting in full muscle lineage differentiation. Increased IGF-2 and IGFBP-6 levels also had a positive effect on OCT4 levels, but SOX2 and Nanog levels were decreased.
3.5 REFERENCES


Appendix 2
Figure S3.1. PMSCs under muscle differentiation conditions treated with IGF-1 showed higher cell count, while IGF-2 treated PMSCs were lower compared to PMSCs in muscle differentiation alone. Data is presented as the mean ± SEM of 15 different fields from 3 independent experiments from one preterm placenta (15 weeks). One-way ANOVA followed by a Student’s t-test was performed to determine ***P<0.001 comparing to muscle differentiation.
Figure S3.2. IGF-2 siRNA treatment inhibited PMSCs differentiation into skeletal muscle and adding extracellular IGFBP-6 with the treatment helped rescue muscle compaction. (A) Compared to PMSCs under muscle lineage differentiation conditions with siRNA scrambled control, (B) PMSCs under muscle differentiation conditions treated with IGF-2 siRNA showed less skeletal muscle compaction at 14 days (20X). (C) Extracellular IGFBP-6 supplementation with IGF-2 knockdown enhanced PMSCs muscle compaction at 14 days compared to IGF-2 siRNA (shown with the white arrow). Images are representative of 3 technical replicate.
Figure S3.3. IGFBP-6 levels secreted to the media increased with IGF-2 knockdown compared to muscle differentiation using ELISA. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test, ***=P<0.001.
Figure S3.4. PMSCs treated with IGF-1 showed decreased IGF-2 levels secreted into the media compared to muscle differentiation. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test, *P<0.05, ***=P<0.001.
CHAPTER FOUR

Insulin-Like Growth Factor Binding Protein-6 Promotes the Differentiation of Placental Mesenchymal Stem Cells into Skeletal Muscle Independent of the Insulin-Like Growth Factor Receptor-1 and Insulin Receptor
4.1 INTRODUCTION

Skeletal muscle comprises one-half of the human body (1). The development of skeletal muscle is a complex multi-step process, starting with the generation of myogenic precursors from mesodermal stem cells and ending with terminal differentiation and the commitment of myoblasts into myofibers (2). During myogenesis, muscle stem cells commit to muscle lineage by up-regulating muscle commitment markers (Pax3/7). As Pax3/7 subsequently decreases, early muscle differentiation markers (MyoD and Myogenin) begin to be expressed (3). The committed muscle cells then start to fuse and form multinucleated fibers, which express the late muscle differentiation marker, myosin heavy chain (MHC) (3). During muscle repair, a similar process is thought to occur whereby satellite cells become activated, migrate towards injured muscle, and begin the differentiation process to replace injured myofibers (4).

IGFs are important components of the skeletal muscle microenvironment and are required for muscle growth during development and regeneration after injury (1, 5, 6). IGFs regulate MyoD and Myogenin gene expression, but the mechanism is not completely understood (1). When mice are injected with IGF-1, there is an enhancement in muscle mass (hypertrophy) (7, 8). Moreover, IGF-1R null mice show profound muscle hypoplasia and die prematurely soon after birth due to breathing difficulties resulting from atrophy of diaphragm and respiratory muscles (9).

Following binding of IGFs to IGF-1R or IR, IRS-1 and IRS-2 are phosphorylated, then PI3K-AKT-mTOR and MAPK pathways are activated (10). Therefore, crosstalk between the different receptor tyrosine-kinase (RTK) pathways can lead to different cellular
responses and signaling outcomes. Also, the presence of target effectors and the timing of their activation are important in determining cell fate decisions towards proliferation or differentiation (11).

During muscle differentiation, MAPK signals play an important role (12). Marshall et al. reported that a prolonged activation of ERK1/2, leads to differentiation, whereas, a transient activation of ERK1/2 leads to proliferation, as it is not sufficient to elevate the levels of nuclear ERK1/2 (13). Therefore, the availability of growth factors in the microenvironment and the receptors they activate determine stem cell fate through the signaling intermediates activated. Furthermore, it is known that IGFs mediate and induce myogenesis by directly activating the myogenin gene promoter. However, when the PI3K inhibitor, LY294002, which acts upstream of AKT signaling is introduced, IGF is no longer able to induce myogenesis or enhance the expression of myogenin (14). Therefore, the direct effects of the IGF stimulation on the myogenin promoter are also mediated via the actions of PI3K via AKT signaling. Additionally, IGF-1R signaling through PI3K was shown to up-regulate myogenin expression leading to an enhanced myogenesis (14); and also regulated basal levels of IGF-1 and IGF-2 genes during myogenesis (14, 15). C2BP5 myoblast differentiation was still achieved when transfected by recombinant adenoviruses expressing MyoD in the absence of IGFs (16). When MyoD-transfected C2BP5 cells were treated with LY294002, the transcriptional activity of MyoD, Myogenin, and MHC was not inhibited but the myofibers were smaller and thinner with fewer nuclei (16). Collectively, these studies suggested that IGF-activated PI3K-AKT and MAPK pathways are both important for myoblast differentiation (17).

IGFs interact with insulin for metabolism, survival, proliferation, and differentiation of
many cell types either through IGF-1R or the insulin receptor (IR) or IGF-1R-IR hybrid receptor (18-20). Both the IGF-1R and IR, are tyrosine protein kinases that activate multiple signaling transduction pathways (20, 21). PI3K-AKT pathway but not the MAPK is activated by insulin (21). It is known that each ligand binds to its respective receptor with higher affinity and to the other receptor or hybrid receptors with lower affinity. While IGFs play a major role in cellular proliferation, differentiation and survival, and insulin has a major role in metabolism, their functions are interchangeable depending on the concentration of the peptide in the extracellular space.

In RD rhabdomyosarcoma and LIM 1215 colon cancer cells, mutant IGFBP-6 that does not bind to IGF-2 induces cellular migration, suggesting an IGF-independent function of IGFBP-6 (22). Inhibition of ERK1/2 but not AKT impeded cellular migration (22). We have previously reported that IGFBP-6, which has high affinity to IGF-2 (23, 24), stimulates a multipotent profile and an early commitment to the muscle lineage in PMSCs (Chapter Three). Furthermore, the impact of extracellular IGFBP-6 and silencing of endogenous IGFBP-6 suggest that the biologic actions of IGFBP-6 occur in both IGF-dependent and IGF-independent mechanisms (19, 25-27). The mechanisms of IGF-dependent and IGF-independent actions are not yet delineated. In this study, we demonstrated that the biologic actions of IGFBP-6 on PMSCs differentiation into skeletal muscle occur independently of either IGFs or insulin signaling through IGF-1R or IR.
4.2 MATERIALS AND METHODS

4.2.1 PMSC Isolation

PMSC isolation and experiments were conducted with the approval from the Health Sciences Research Ethics Board of Western University (REB# 12154). Informed consent was obtained from healthy women undergoing therapeutic termination of pregnancy, and the PMSCs used in this study were isolated from one 15 week preterm placental tissue. After surgery, chorionic villi were dissected, washed, and minced with surgical scissors and forceps, and then subjected to enzymatic digestion with collagenase IV (369 IU/mg), hyaluronidase (999 IU/mg) (Sigma-Aldrich, Oakville, ON), and DNase I (2,000 IU/mg) (Hoffmann-LaRoche, Mississauga, ON) for 10 minutes at room temperature, followed by 0.05% trypsin (Gibco/Invitrogen, Mississauga, ON) for 5 minutes at room temperature. The sample was then washed for 10 minutes with 10% FBS in DMEM/F12 medium and the resulting single cell suspension was separated by density centrifugation over a Percoll (Sigma-Aldrich, Oakville, ON) discontinuous gradient using a modified protocol by Worton et al. (28).

4.2.2 Cell Culture

Cells from Percoll gradient fractions #3 and #4 were plated on to T75 flasks, cultured, and maintained using DMEM/F12 media supplemented with 15% FBS serum (Gibco/Invitrogen, Mississauga, ON) and FGF-2 (50 ng/mL) (Sigma-Aldrich, Oakville, ON) containing 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 0.25 µg/mL Amphotericin-B. The non-adherent cells were discarded at the time of media change, which was performed every 72 hours. The adherent cells were cultured until they reach
90% confluence. Cells were then passaged 1:2 approximately once per week using 0.05% Trypsin for 10 min at 37°C for 3 passages. Passaged cells were stored at -80°C in 1 mL of freezing media (30% FBS and 10% DMSO in DMEM/F12 media). When needed, vials were thawed and cells were resuspended in normal culture media (25 ng/mL FGF-2 and 15% FBS in DMEM/F12).

4.2.3 Muscle Differentiation and Treatments

Cells were plated in muscle growth media (fetal bovine serum 0.05 mL/mL, fetuin 50 µg/mL, epidermal growth factor 10 ng/mL, basic fibroblast growth factor 1 ng/mL, insulin 10 µg/mL, and dexamethasone 0.4 µg/mL) for 48 hours before changing to the skeletal muscle differentiation media, which is a proprietary serum-free medium containing 10 µg/mL insulin (PromoCell, Heidelberg, Germany) for 14 days. Cells were grown in six-well plates in a standard tissue culture incubator at 37°C in 5% CO₂.

PMSCs were treated every 3 days with: 200 nM of the IGF-IR inhibitor, PPP (Santa Cruz Biotechnology, Dallas, TX), 25 µM of the AKT inhibitor LY294002 (Santa Cruz Biotechnology, Dallas, TX), 10 µM of the ERK1/2 inhibitor U0126 (Santa Cruz Biotechnology, Dallas, TX), or 10 µM of the IR inhibitor HNMPA (Santa Cruz Biotechnology, Dallas, TX) under muscle differentiation conditions. Treatment concentrations for LY294002, U0126, and HNMPA were determined by a dose response experiment using PMSCs in muscle differentiation media (Figure S4.1).

For IGFBP-6 supplementation with the inhibitors, recombinant human IGFBP-6 (PROSPEC, East Brunswick, NJ) was added to the media (375 ng/mL) every 3 days at
the time of media change. The IGFBP-6 concentration and time of supplementation was determined as shown in Chapter 2 (Figure S2.1).

4.2.4 Cell Lysate Preparation

Following PMSC treatments, cells were washed with DPBS (Gibco/Invitrogen, Mississauga, ON) and 200 μL of (1x) cell lysis buffer (Cell Signaling Technologies, Burlington, ON) mixed with protease and phosphatase inhibitors (1:100) were added to the cells. Cells were detached using a cell scraper and the lysate was sonicated for 15 seconds. Finally, samples were centrifuged for 15 min at 14,000 rpm at 4°C, and the supernatant was collected.

4.2.5 Immunoblotting

Cell lysates containing 20 μg of protein were added to 6x SDS gel loading buffer (1% β-mercaptoethanol, 1% SDS, 30% glycerol, 0.0012% bromophenol blue, Tris HCl 0.28 M, pH 6.8). Samples were boiled for 5 minutes at 95°C, then placed on ice for 3 minutes, and centrifuged at 3,000 rpm for 20 seconds before loading. Samples were resolved by molecular weight using 10% SDS polyacrylamide gels transferred onto poly-vinyl-diene-fluoride (PVDF) membranes (Bio-Rad, Hercules, CA) using a Trans-Blot Turbo (Bio-Rad, Hercules, CA) with an optimized protocol depending on protein size. Membranes were blocked with 5% non-fat-dry milk, gently shaking for 1 hour at room temperature in Tris-HCl buffer saline pH 8.0 with 0.1% Tween-20 (TBS-T). Blots were then washed with TBS-T (3 times for 10 minutes) followed by incubation at 4°C overnight with specific primary antibodies in 5% BSA or 5% non-fat-dry milk in TBS-T following the manufacturer’s protocol. To detect markers of cell potency, antibodies for OCT4
antibody (N-19: sc-8628) (Santa Cruz Biotechnology, Dallas, TX), and SOX2 (2683-1) (Epitomics, Burlington, ON) were used. To detect markers of muscle differentiation, Pax3/7 (E-10: sc-365613), MyoD (M-318: sc-760), Myogenin (F5D: sc-12732), and Myosin heavy chain (H-300: sc-20641) (Santa Cruz Biotechnology, Dallas, TX) were used. To detect IGFBP-6, antibody H-70: sc-13094 (Santa Cruz Biotechnology, Dallas, TX) was used. For loading control, pan-Actin Ab-5 (Thermo Fisher Scientific, Fremont, CA) was used. To detect the activated signaling molecules, we used: phospho-p44/42 MAPK (#4377), p44/42 MAPK (#9102), phospho-AKT (Ser473, #4051), and AKT (#9272) (Cell Signaling Technologies, Burlington, ON).

Then membranes were washed and incubated for 1 hour at room temperature with the corresponding secondary HRP-conjugated antibody. The secondary antibodies used were HRP-conjugated goat anti-rabbit (#170-6515) or anti-mouse (#170-6516) (Bio-Rad, Hercules, CA) or donkey anti-goat antibody (Santa Cruz Biotechnology, Dallas, TX).

Resolved protein bands were detected using chemiluminescence and images were taken using the VersaDoc Imager (Bio-Rad, Hercules, CA). Western blots were performed in triplicate.

### 4.2.6 Immunocytochemistry

PMSCs were grown on glass cover slips (80-90% confluent) for 14 days, stained with primary antibodies (1:100), and incubated at 4°C overnight. To detect markers of cell potency, OCT4 antibody (N-19: sc-8628) (Santa Cruz Biotechnology, Dallas, TX) was used. To detect markers of muscle differentiation, Myosin heavy chain (H-300: sc-20641) (Santa Cruz Biotechnology, Dallas, TX) was used. To detect IGFBP-6, antibody
H-70: sc-13094 (Santa Cruz Biotechnology, Dallas, TX) was used. Primary antibodies were washed using 0.1% Tween-20 in PBS (3 times for 5 minutes); cells were then incubated in the dark with the corresponding secondary AlexaFluor conjugated antibody (1:200). The secondary antibodies used were AlexaFluor 488 or AlexaFluor 568 conjugated antibodies (Invitrogen, Mississauga, ON). The secondary antibody was washed using 0.1% Tween-20 in PBS (3 times for 5 minutes), and the nuclear stain (Hoechst dye) was added for 7 minutes and then rinsed. The cover slips were mounted and images were taken using a confocal microscope (Zeiss, Germany). Each antibody was immunostained in triplicates.

4.2.7 Quantification of IGFBP-6 and IGF-2 by Enzyme-Linked Immunosorbent Assay (ELISA)

Human IGFBP-6 (RayBiotech®, Burlington, ON) and IGF-2 (ALPCO, Salem, NH) ELISA kits were used to measure the amount of IGFBP-6 and IGF-2 secreted into conditioned media at days 1, 3, 7, and 14. Standards and samples were loaded into the wells (in triplicate) and the immobilized antibody bound IGFBP-6 or IGF-2 present in the sample. The wells were washed and biotinylated anti-human antibody was added. After washing, HRP-conjugated streptavidin was added; then a TMB substrate solution was used to develop a blue color in proportion to the amount of IGFBP-6 or IGF-2 bound. The Stop Solution changes color from blue to yellow, and the intensity was measured at 450 nm using Multiskan Ascent plate reader and analysis software.
4.2.8 Aldehyde Dehydrogenase Activity (ALDH)

ALDH-activity, a conserved progenitor cell function, was assessed by flow cytometry at days 1, 3, 7, and 14. Using the Aldefluor\textsuperscript{TM} assay (Stem Cell Technologies, Vancouver, BC), as per the manufacturer’s instructions. Briefly, 5 μL of activated Aldefluor reagent was added to 1 mL of cell suspension and incubated for 45 minutes at 37°C. Cells were washed and resuspended in 500 μL of ice-cold Aldefluor assay buffer and ALDH-activity was measured using flow cytometry. As a negative control, Aldefluor\textsuperscript{TM} DEAB reagent was used. Samples were run in triplicate (technical replicate).

4.2.9 Statistical Analysis

All experiments were performed in triplicate from one 15 week placental tissue (technical replicates). GraphPad Prism Software 5.0 was used to generate all graphs and analyses. A two-way ANOVA followed by a Bonferroni’s multiple comparison test or a one-way ANOVA followed by a Student’s t-test was used to calculate significant differences when p<0.05. Graphic representation values are presented as mean ± SEM (shown as variance bars).

4.3 RESULTS

4.3.1 IGF-1R and IGFBP-6 are required for PMSCs Differentiation into Skeletal Muscle

To evaluate the effects of IGF-1R inhibition on pluripotency-associated and muscle differentiation markers in PMSCs under muscle differentiation conditions, PPP (IGF-1R
auto phosphorylation inhibitor) was used during PMSCs muscle differentiation for 14 days with/without IGFBP-6 supplementation every 3 days. Using immunocytochemistry, PPP treatment alone showed no changes in OCT4, IGFBP-6, and MHC immunoreactivity at 14 days compared to muscle differentiation alone (Figure 4.1A). However, adding IGFBP-6 together with PPP treatment showed higher IGFBP-6 and MHC immunoreactivity at day 14 compared to PPP treatment alone (Figure 4.1A). Moreover, PMSCs treated with IGFBP-6 together with PPP under muscle differentiation conditions showed decreased cell numbers per field compared to muscle differentiation alone or PPP alone, suggesting that IGFBP-6 may enhance muscle differentiation independent of IGFs (Figure 4.1B).

As determined by immunoblotting, the presence of PPP decreased IGFBP-6 protein levels at 14 days (Figure 4.2A). Pluripotency-associated markers (OCT4 and SOX2) levels were decreased by PPP treatment compared to muscle differentiation alone (Figure 4.2B and 4.2C). The muscle commitment marker Pax3/7 levels were decreased by PPP treatment at 7 and 14 days (Figure 4.2D). Similarly, muscle lineage differentiation markers MyoD and MyoG protein levels were decreased at 7 and 14 days (Figure 4.2E and 4.2F). In contrast, MHC levels were reduced at all-time points after PPP treatment compared to muscle differentiation (Figure 4.2G). Overall, PPP treatment significantly delayed muscle lineage commitment and differentiation in vitro.

To determine whether IGFBP-6 could rescue PMSCs differentiation into skeletal muscle during IGF-1R inhibition, extracellular IGFBP-6 was added to the culture alongside PPP supplementation. As predicted, IGFBP-6 levels increased after co-administration of IGFBP-6 with PPP at day 14 compared to the inhibitor alone (Figure 4.2A). Also, OCT4
protein levels were increased at 14 days with the combined treatments, while SOX2 levels were not changed compared to the inhibitor alone (Figure 4.2B and 4.2C). Furthermore, IGFBP-6 supplementation with PPP, increased the levels of muscle lineage differentiation markers Pax3/7, MyoD, MyoG, and MHC from 3-14 days compared to PPP alone (Figures 4.2D to 4.2G). These findings suggest that IGFBP-6 may be an important regulator of skeletal muscle differentiation and its action, in part, occurred without activating IGF-1R signaling and independent of IGF.

Downstream of the IGF-1R signaling, the presence of PPP during muscle differentiation caused a significant reduction in p-AKT levels at 7 days and in p-ERK1/2 levels at 7 and 14 days when compared to muscle differentiation alone (Figure 4.3A and 4.3B). In contrast, IGFBP-6 increased both p-AKT and p-ERK1/2 protein levels at all-time points in the presence of PPP under muscle differentiation conditions compared to PPP alone indicating that IGFBP-6 may trigger MAPK signal transduction cascade independent of IGFs (Figure 4.3A and 4.3B).

In the presence of PPP, IGFBP-6 secretion into the conditioned media was increased compared to muscle differentiation (Figure 4.4A) whereas IGF-2 secretion was reduced at days 3 and 7 (Figure 4.4B).
Figure 4.1. PMSCs treated with extracellular IGFBP-6 and PPP showed qualitatively more IGFBP-6 and MHC immunofluorescence with no change in OCT4 expression at 14 days compared to PPP alone. (A) Compared to PMSCs cultured with PPP alone, PMSCs treated with IGFBP-6 and PPP showed no change in OCT4 immunoreactivity (green-Alexa, λ-488 nm), but increased IGFBP-6 and MHC immunoreactivity (red-Alexa, λ-568 nm) compared to PPP alone. Nuclei, stained with Hoechst dye (blue, λ=340 nm). Each antibody was performed in triplicate. (B) PMSCs treated with PPP and IGFBP-6 showed reduced cell counts at 14 days compared to PMSCs in muscle differentiation media alone and PMSCs with the inhibitor only. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta and cell count was from 15 different fields. One-way ANOVA with a Student’s t-test was performed to determine ***P<0.001 compared to muscle differentiation, or ###P<0.001 compared to PPP alone.
Figure 4.2. PMSCs treated with the IGF-1R inhibitor, PPP, decreased pluripotency-associated and muscle differentiation markers. (A) PPP treatment decreased IGFBP-6 protein levels at day 14 as compared to PMSCs grown in muscle differentiation media only. IGFBP-6 supplementation with PPP increased IGFBP-6 levels at 1 and 14 days compared to PPP alone. PPP treatment also decreased the protein levels of the pluripotency-associated markers (B) OCT4 and (C) SOX2. When IGFBP-6 was added with PPP, OCT4 levels increased at 14 days. (D) PPP treatment decreased the protein levels of muscle lineage commitment marker Pax3/7 at 7 and 14 days. IGFBP-6 supplementation with PPP increased Pax3/7 from 3-14 days compared to PPP alone. (E, F) Muscle differentiation markers, MyoD and MyoG, levels were decreased at 7 and 14 days, and adding IGFBP-6 with PPP reversed these effects. (G) Conversely, MHC protein levels were reduced with PPP treatment at all-time points compared to muscle differentiation. IGFBP-6 supplementation with PPP increased MHC levels from 3-14 days compared to PPP alone. Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to muscle differentiation conditions, or #P<0.05, ##P<0.01, ###P<0.001 compared to PPP.
Figure 4.3. PMSCs treated with IGF-1R inhibitor (PPP) showed reduced p-AKT and p-ERK1/2 levels that was reversed by IGFBP-6 addition. (A, B) PMSCs treated with PPP showed lower protein levels of p-AKT p-ERK1/2 at the later time points when compared to PMSCs under muscle differentiation conditions alone. When IGFBP-6 was added to PPP, p-AKT and p-ERK1/2 levels increased at all-time points compared to PPP alone. Protein levels were quantified by densitometry and normalized to total AKT or total ERK1/2. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to muscle differentiation conditions, or #P<0.05, ###P<0.001 compared to PPP.
Figure 4.4. PMSCs treated with PPP showed increased IGFBP-6 secretion but decreased IGF-2 secretion. (A) IGFBP-6 secretion was increased after PPP treatment at all-time points compared to muscle differentiation conditions alone. (B) IGF-2 secretion was reduced at 3 and 7 days compared to muscle differentiation conditions alone. IGFBP-6 with PPP did not have an additional effect. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to muscle differentiation conditions alone.
4.3.2 IGFBP-6 is required for PMSCs Muscle Differentiation after Inhibition of the PI3K Pathway

To better understand downstream signaling of the IGF-1R, LY294002 was used to inhibit PI3K signaling pathway. LY294002 alone reduced differentiated muscle morphology at 7 days (Figure 4.5 and S4.2). However, the addition of IGFBP-6 with LY294002 delayed these changes until day 14 post-differentiation (Figure 4.5 and S4.2). Using immunoblotting, IGFBP-6 expression was decreased at day 1 and 14 in the presence of LY294002, and remained decreased despite IGFBP-6 supplementation (Figure 4.6A). Furthermore, LY294002 treatment reduced the protein levels of the pluripotency-associated markers OCT4 (Figure 4.6B) at day 1 and SOX2 (Figure 4.6C) at all-time points as compared to muscle differentiation alone. After IGFBP-6 supplementation with LY294002 treatment, OCT4 levels were maintained higher at all-time points; while SOX2 expression was higher at day 14 compared to LY294002 treatment alone (Figure 4.6B and 4.6C). Muscle lineage markers MyoD, MyoG, and MHC levels decreased with LY294002 treatment as compared to muscle differentiation alone (Figures 4.6D to 4.6F). IGFBP-6 addition with LY294002 treatment increased MyoD protein levels at days 1 and 3 (Figure 4.6D); while MHC levels were increased compared to LY294002 treatment alone (Figure 4.6F). These results indicated that muscle commitment occurred earlier in the presence of IGFBP-6 with LY294002 treatment. To confirm this hypothesis, Pax3/7, the muscle commitment marker expression was tested. Pax3/7 protein levels were increased after IGFBP-6 addition with LY294002 (Figure 4.6G), suggesting an earlier commitment to the muscle lineage when IGFBP-6 was present.

We used the Aldefluor™ assay to determine the frequency of progenitor cells with high
ALDH-activity, a more primitive progenitor phenotype. Compared to PMSCs under muscle differentiation alone, there was a decrease in the frequency of cells with high ALDH-activity (ALDH\(^+\) cells) in PMSCs treated with LY294002 until day 7 (Figure 4.7). Moreover, IGFBP-6 with LY294002 treatment reduced the frequency of cells with high ALDH-activity at day 1 but was maintained at a higher number compared to LY294002 alone at day 3. Therefore, IGFBP-6 prolonged progenitor phenotype in PMSCs when PI3K pathway is inhibited under muscle differentiation conditions.
Figure 4.5. PMSCs treated with LY294002 reduced differentiated muscle compaction at 7 and 14 days, while IGFBP-6 with LY294002 delayed muscle compaction changes to 14 days. PMSCs treated with LY294002, a PI3K inhibitor upstream of AKT, under muscle differentiation conditions showed less skeletal muscle morphology at 7 and 14 days; but the addition of IGFBP-6 with LY294002 delayed these changes until day 14 (10X). Images are representative of 3 independent experiments from one preterm placenta.
Figure 4.6. LY294002 treatment reduced IGFBP-6, pluripotency-associated and muscle differentiation markers. (A) IGFBP-6 protein levels decreased with LY294002 at day 1 and 14 as compared to muscle differentiation alone and adding extracellular IGFBP-6 with the treatment did not cause additional changes to IGFBP-6 levels. (B, C) PMSCs treated with LY294002 decreased OCT4 (at day 1) and SOX2 (at each time point) compared to muscle differentiation, whereas IGFBP-6 addition with LY294002 increased OCT4 expression at 3, 7, and 14 days and at day 14 for SOX2 compared to LY294002 alone. Muscle lineage differentiation markers protein levels were reduced with LY294002 for (D) MyoD from 3-14 days, (E) MyoG at 7 and 14 days, and (F) MHC at all-time points. IGFBP-6 supplementation with LY294002 increased MyoD levels at 1 and 3 days and MHC levels at 3, 7, and 14 days compared to LY294002 treatment. (G) Pax3/7 protein levels decreased with LY294002 at day 7. IGFBP-6 addition with the inhibitor increased Pax3/7 levels until day 7. Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to muscle differentiation conditions, or #P<0.05, ##P<0.01, ###P<0.001 compared to LY294002, or @@@P<0.001 compared to muscle differentiation with IGFBP-6.
Figure 4.7. PMSCs treated with LY294002 and LY294002 with IGFBP-6 under skeletal muscle differentiation conditions decreased the frequency of cells with high ALDH-activity. Representative flow cytometry dot plots showing the frequency of PMSCs with high ALDH-activity when cultured under muscle differentiation conditions with or without LY294002 or LY294002 and IGFBP-6 at (A) day 1, (B) day 3, (C) day 7, and (D) day 14. DEAB treated controls were used to establish the ALDH gate (data not shown). (E) Compared to PMSCs under muscle differentiation conditions, cells treated with LY294002 showed a decreased frequency of cells with high ALDH-activity at 1, 3, and 7 days. IGFBP-6 with LY294002 treatment decreased frequency of cells with high ALDH-activity only at day1, and a significantly increased ALDH+ cells at day 3 compared to LY294002 treatment alone. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, ***P<0.001 compared to muscle differentiation, or ##P<0.01, ###P<0.001 compared to LY294002 alone.
4.3.3 MAPK signaling is required for PMSC Differentiation into Skeletal Muscle

To test the downstream signaling of the IGF-1R via the MAPK pathway, U0126 was used to inhibit MAPK signaling, which phosphorylates ERK1/2. PMSCs treated with U0126 under muscle differentiation conditions showed reduced muscle cell compaction from 3 to 14 days with a change in muscle morphology compared to PMSCs under muscle differentiation alone. IGFBP-6 supplementation with U0126 treatment showed similar morphology to U0126 alone (Figure 4.8). Using immunoblotting, IGFBP-6 levels were reduced with U0126 treatment and adding IGFBP-6 did not increase IGFBP-6 levels, indicating that MAPK is an important pathway for IGFBP-6 production (Figure 4.9A). OCT4 levels were reduced at day 1 by U0126 alone or U0126 with IGFBP-6 addition; however, U0126 with IGFBP-6 treatment maintained higher levels of OCT4 at 7 and 14 days until a significant decrease at day 14 compared to U0126 alone (Figure 4.9B). In contrast, pluripotency-associated marker SOX2 protein levels were decreased by U0126 until 7 days and were increased by IGFBP-6 at 7 and 14 days compared to U0126 alone (Figure 4.9C). Early and late muscle lineage differentiation markers MyoD, MyoG, and MHC protein levels were significantly reduced after day 3 with U0126 and adding IGFBP-6 with U0126 did not reverse these effects (Figures 4.9D to 4.9F).

PMSCs treated with U0126 under muscle differentiation conditions decreased the frequency of cells with high ALDH-activity compared to PMSCs under untreated muscle differentiation condition at 1, 3, and 7 days (Figure 4.10). In contrast adding IGFBP-6 with U0126 treatment increased the frequency of cells with high ALDH-activity compared to PMSCs treated with U0126 alone.
Figure 4.8. PMSCs treated with U0126 under muscle differentiation conditions showed reduced muscle compaction at 3 days. PMSCs under muscle differentiation conditions with U0126, a MEK inhibitor upstream of ERK1/2, showed less skeletal muscle differentiation at 3, 7, and 14 days with a change in cell morphology at 14 days compared to muscle differentiation (10X). Images are representative of 3 independent experiments from one preterm placenta.
Figure 4.9. PMSCs treated with U0126 under muscle differentiation conditions reduced protein levels of IGFBP-6, pluripotency-associated and muscle differentiation markers. (A) U0126 decreased IGFBP-6 protein levels and adding IGFBP-6 with U0126 did not cause additional changes to IGFBP-6 levels. (B) Pluripotency-associated marker OCT4 protein levels reduced with U0126 treatment at day 1 with increased levels at 7 days. IGFBP-6 addition with U0126 increased OCT4 levels at 3 and 7 days compared to U0126 treatment alone. (C) SOX2 protein levels were lower at 1, 3, and 7 days compared to muscle differentiation. IGFBP-6 addition with U0126 decreased SOX2 levels at day 1 with an increase at 7 and 14 days compared to U0126. (D-F) Muscle differentiation markers MyoD, MyoG, and MHC protein levels were decreased at the later time points with U0126 and adding IGFBP-6 with U0126 did not change these effects. Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to muscle differentiation, or *P<0.05, **P<0.01, ***P<0.001 compared to U0126.
Figure 4.10. PMSCs treated with U0126 and U0126 with IGFBP-6 under skeletal muscle differentiation conditions decreased the frequency of cells with high ALDH-activity. Representative flow cytometry dot plots showing the frequency of PMSCs with high ALDH-activity when cultured under muscle differentiation conditions with or without either U0126 or U0126 and extracellular IGFBP-6 at (A) day 1, (B) day 3, (C) day 7, and (D) day 14. DEAB treated controls were used to establish the ALDH gate (data not shown). (E) U0126 treatment showed decreased frequency of cells with high ALDH-activity at 1, 3, and 7 days compared to muscle differentiation alone. IGFBP-6 supplementation with U0126 increased frequency of cells with high ALDH-activity at 1, 3, and 7 days compared to U0126. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, ***P<0.001 compared to muscle differentiation, or ##P<0.01, ###P<0.001 compared to U0126.
To test the role of insulin receptor (IR) signaling in the differentiation of PMSCs into skeletal muscle, HNMPA, was used to block IR kinase activity. Neither HNMPA nor HNMPA with IGFBP-6 impacted differentiated cell morphology when compared to muscle differentiation conditions alone. However, HNMPA treatment delayed muscle differentiation (less compaction) at day 14, compared to control treatment (Figure 4.11 and S4.3).

Intracellular IGFBP-6 levels were unchanged by HNMPA except for a reduction at day 7, however adding IGFBP-6 with HNMPA increased IGFBP-6 protein levels at 3, 7, and 14 days (Figure 4.12A). HNMPA did not change protein levels of pluripotency-associated markers (OCT4 and SOX2), but that addition of extracellular IGFBP-6 with HNMPA increased both markers at days 7 and 14 compared to PMSCs under muscle differentiation conditions and PMSCs treated with HNMPA (Figure 4.12B and 4.12C).

Additionally, muscle lineage differentiation markers (MyoD, MyoG, and MHC) levels were decreased at the later time points with HNMPA compared to PMSCs under muscle differentiation conditions, and extracellular IGFBP-6 increased MyoG and MHC levels at 7 and 14 days compared to HNMPA alone (Figures 4.12D to 4.12F).
Figure 4.11. PMSCs under muscle differentiation conditions treated with HNMPA show delayed muscle compaction at 14 days. PMSCs under muscle differentiation conditions, with HNMPA or HNMPA with extracellular IGFBP-6, showed minimal change in skeletal muscle morphology and density at day 14 when compared to muscle differentiation (10X). Images are representative of 3 independent experiments from one preterm placenta.
Figure 4.12. PMSCs treated with HNMPA under muscle differentiation conditions reduced muscle differentiation markers with no change in pluripotency-associated markers. (A) HNMPA treatment decreased IGFBP-6 protein levels at 7 days as compared to muscle differentiation. IGFBP-6 supplementation with HNMPA increased IGFBP-6 levels at 3, 7, and 14 days. (B, C) HNMPA treatment did not change pluripotency-associated markers OCT4 and SOX2 protein levels. IGFBP-6 with HNMPA treatment increased the levels at 7 and 14 days. (D-F) HNMPA treatment decreased muscle lineage differentiation markers MyoD, MyoG, and MHC protein levels at the later time points (3-14 days). IGFBP-6 addition with HNMPA treatment increased MyoG and MHC levels at 7 and 14 days compared to HNMPA treatment alone. Protein levels were quantified by densitometry and normalized to β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to muscle differentiation conditions, or #P<0.05, ##P<0.01, ###P<0.001 compared to HNMPA.
4.4 DISCUSSION

The promise of using stem cells in treating diseases is becoming closer to be used in the clinic (29, 30). Still, understanding the niche factors and their influence on stem cell proliferation and differentiation in vitro is essential before stem cells can be used safely in regenerative medicine applications (31). Muscle differentiation is a multistep process, starting with commitment to the muscle lineage and ending by the formation of multinucleated myotubes (2). The IGF family is an essential early niche factor for stem cell survival, growth, proliferation, and differentiation (23). It is also important in the skeletal muscle niche, with a major role in muscle development (5, 6, 9). IGFBP-6 is expressed in the developing cells (1). We have demonstrated that the balance between intracellular and extracellular IGFBP-6 levels is required for modulating muscle differentiation by PMSCs (Chapter Three) and that the effects of IGFBP-6 on muscle differentiation are both IGF-dependent and IGF-independent (Chapter Four). These findings provided basic insight into the role of IGFBP-6 and IGFs on PMSCs muscle differentiation. The aim of this study was to characterize the effects of IGF-1R and IR activation on the differentiation of PMSCs into skeletal muscle and to investigate IGFBP-6 role in this process.

In these studies, we showed that IGF-1R inhibition using PPP decreased cellular IGFBP-6, OCT4 and SOX2, and muscle differentiation markers MyoD, MyoG, and MHC levels. These results indicated the importance of IGF-1R in PMSCs for IGFBP-6 localization, maintaining a multipotent profile, and muscle differentiation. Importantly, IGFBP-6 supplementation stimulated PMSCs differentiation into muscle cells while IGF-1R was inhibited as seen with the increased levels of muscle differentiation markers. These data
indicate that IGFBP-6 was required for PMSCs muscle differentiation and its action may be IGF-independent.

Triggering downstream phosphorylation of AKT or ERK1/2 independent of IGF-1R activation by IGFBP-6 via an unknown mechanism could be responsible for IGFBP-6 impact on muscle cell differentiation. Inhibition of the PI3K pathway lowered IGFBP-6, and the addition of extracellular IGFBP-6 did not increase intracellular IGFBP-6. This indicates that the continuous inhibition of PI3K impacts muscle differentiation by decreasing intracellular IGFBP-6 levels. The addition of extracellular IGFBP-6 when PI3K was inhibited resulted in an earlier PMSC commitment to the muscle lineage shown by increased Pax3/7 levels and decreased in ALDH+ cells frequency. Also, IGFBP-6 combined treatment with PI3K-inhibition increased muscle differentiation, as shown by increased MHC levels. We concluded that PI3K pathway is essential for muscle differentiation, and when the pathway was inhibited, IGFBP-6 could overcome the impact by allowing the cells to commit earlier to the muscle lineage and enhancing late stage differentiation.

Blocking ERK1/2 phosphorylation with U0126 inhibited PMSCs differentiation into skeletal muscle and IGFBP-6 addition did not rescue the muscle differentiation process. These findings suggest that MAPK is a critical pathway for PMSCs skeletal muscle differentiation and cannot be substituted by an alternative pathway. The fact that IGFBP-6 did not accumulate in the intracellular environment when MAPK was inhibited shows that PMSCs differentiation by IGFBP-6 was mediated via the MAPK pathway. It was suggested previously that IGFBP-6 must be endogenously synthesized to have IGF-independent effects (32).
Blocking insulin receptor, IR, using HNMPA delayed muscle differentiation of PMSCs with no effect on pluripotency-associated markers. During IR-inhibition, IGFBP-6 addition improved the muscle differentiation and increased pluripotency-associated markers protein levels suggesting that insulin or IGFs could trigger myogenic differentiation; however, IGFBP-6 could also promote differentiation independent of insulin or IGFs. These results are in agreement with previous reports on the importance of IGF-1R, and its downstream pathways, and the IR in muscle development and differentiation. However, this study is the first to show these effects on human stem cells isolated from the placenta and that IGFBP-6 addition enhanced the muscle differentiation process of PMSCs when IGF-1R or IR were inhibited *in vitro*.

Different signaling pathways, including IGF-1R and IR, cross talk; and the complexity of signaling and its effects on PMSCs differentiation into muscle is beyond the scope of one study. The possibility that a different pathway, not examined in this study, is responsible for IGFBP-6 effects on PMSCs differentiation into skeletal muscle must be considered and further investigated to better understand IGFBP-6 role in this differentiation process. Moreover, to confirm the results from this study, increasing the sample number to have biological replicates is vital as experiments were performed from one preterm placental tissue (15 weeks).

In conclusion, data presented in this study, suggest that both the IGF-1R and IR signaling are important signaling pathways in PMSC differentiation towards skeletal muscle lineage. In addition, IGFBP-6 was also important for differentiation to occur, due to a combination of IGF-dependent and IGF-independent functions.
4.5 REFERENCES


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Appendix 3
Figure S4.1. PMSCs under muscle differentiation conditions treated with different concentrations of PI3K, MAPK, and IR inhibitors. Cells were treated with different concentrations of (A) LY294002 (PI3K inhibitor), (B) U0126 (MAPK inhibitor), or (C) HNMAP (insulin receptor inhibitor) for 3 days under muscle differentiation conditions to assess the optimal concentration to be used. The concentration of the inhibitors was selected based on maintaining low band intensity for the duration of the experiment (3 days) compared to muscle differentiation alone. 25 μM of the AKT inhibitor LY294002, 10 μM of the ERK1/2 inhibitor U0126, and 10 μM of the IR inhibitor HNMPA were selected. Protein levels were quantified by densitometry and normalized to total AKT, total ERK1/2, or β-Actin. Data is presented as the mean ± SEM of 3 independent experiments from one preterm placenta. Two-way ANOVA with Bonferroni’s multiple comparison test was performed to determine *P<0.05, **P<0.01, ***P<0.001 compared to muscle differentiation conditions.
Figure S4.2. Higher magnification of PMSCs treated with LY294002 or with IGFBP-6 supplementation with LY294002. PMSCs treated with LY294002, a PI3K inhibitor upstream of AKT, under muscle differentiation conditions showed less skeletal muscle compaction at 7 days compared to muscle differentiation alone; but the addition of IGFBP-6 with LY294002 delayed these changes (more compaction) as seen with the white arrows compared to the inhibitor alone (20X). Images are representative of 3 independent experiments from one preterm placenta.
Figure S4.3. Higher magnification of PMSCs treated with HNMPA or with IGFBP-6 supplementation with HNMPA. PMSCs treated with HNMPA under muscle differentiation conditions showed less skeletal muscle compaction and density at 14 days compared to muscle differentiation alone; but the addition of IGFBP-6 with HNMPA showed more muscle compaction as seen with the white arrows compared to HNMPA alone (20X). Images are representative of 3 independent experiments from one preterm placenta.
CHAPTER FIVE

DISCUSSION
5.1 Summary and Perspective

The aim of this study was to determine if adult mesenchymal stem cells isolated from the human placenta (PMSCs) have the capacity to differentiate into skeletal muscle, and to delineate the effects of the insulin-like growth factor system, specifically IGFBP-6, IGF-1, and IGF-2, on this differentiation process.

This study provides insights into the mechanisms of differentiation from PMSCs into skeletal muscle by IGFs and IGFBP-6 during development, and highlights a potential role for IGFBP-6 in regeneration therapy for skeletal muscle diseases. We found that IGFBP-6 could regulate PMSC fate (multipotency or differentiation) in the presence or absence of IGFs, particularly IGF-2. First, we validated that PMSCs possess multipotent stem cells characteristics including the ability to differentiate into multiple mesodermal lineages (Chapter Two). Second, we confirmed that PMSCs could also differentiate into skeletal muscle and that IGFBP-6 played a role in the maintenance of a multipotent profile and in the differentiation of PMSCs into skeletal muscle. Specifically, increased extracellular IGFBP-6 before commitment to the muscle lineage maintained PMSC multipotency alongside commitment to the muscle lineage, whereas a prolonged increase in IGFBP-6 delayed late stage differentiation (Chapter Three). Third, IGFs, specifically IGF-2, were required for PMSC muscle differentiation, and IGF-2 accelerated muscle commitment and differentiation. Moreover, extracellular IGFBP-6 enhanced late stage differentiation in the partial absence of IGF-2 before PMSCs muscle lineage commitment (Chapter Four). Finally, we showed that MAPK signaling through IGF-1R was required for muscle lineage differentiation. Also, IGFBP-6 promoted PMSCs differentiation into muscle partly independent of PI3K and IR signaling pathways (Chapter Four).
For use in regenerative therapies stem cells should be readily available, able to differentiate predictably under lineage-specific conditions, well-tolerated by the host without the induction of an immune response after transplantation, and will not promote tumor formation. Adult mesenchymal stem cells isolated from the human placenta possess all of these characteristics (1, 2). The PMSCs used for studies in this thesis were isolated from the chorionic villi of preterm human placentae (15-20 weeks). In Chapter 2, we confirmed that the PMSCs isolated from chorionic villi fulfill the minimal criteria for the derivation of MSC by the International Society for Cellular Therapy (3-6). They were plastic adherent, positive for mesenchymal markers CD73 and CD105, and differentiated into osteoblasts, adipocytes, and myocytes in vitro. Therefore, this study confirms previous reports that the chorionic villus of the human placenta is an ethical and readily available source of adult MSCs for use in regeneration therapies (7-9).

We have shown that the IGF system is a central component of the stem cell niche controlling both multipotency and muscle lineage differentiation in vitro. We believe that modulation of this system during expansion in vitro may provide a novel therapeutic strategy to generate progenitor cells suitable for use in skeletal muscle regenerative therapies. In this thesis we focused on combined effects of ligands, receptors, and binding-proteins (specifically IGFBP-6) of the IGF system, to direct the differentiation of mesenchymal stem cells from preterm placenta (15 weeks). However, previous studies in our lab showed that PMSCs from different gestational ages respond differently to IGFs (10). Therefore, future studies are warranted to directly compare MSCs from the chorionic villi of different gestations (preterm and full term human placentae), which will further improve our understanding of skeletal muscle differentiation and the effects of the
IGF system based on ontogeny, and will help in choosing the best gestation age PMSC for skeletal muscle differentiation.

5.1.1 Role of the IGF System in PMSCs Multipotency and Muscle Differentiation

In this thesis, we manipulated several components of the IGF system during skeletal muscle differentiation, in order to delineate the roles of specific components in muscle differentiation. Our findings helped define the microenvironment needed by adult mesenchymal stem cells to achieve and maintain skeletal muscle differentiation in vitro and identify molecular mechanisms controlling IGF-signaling. We have demonstrated that the IGF system plays an important role in determining adult MSCs fate, either maintaining multipotency or progressing to differentiation in vitro.

Understanding the multipotency and differentiation processes, as well as the effects of niche factors, are fundamental in stem cell biology and the utilization of stem cells in regeneration therapies. Because the IGF system is expressed in the very early stages of embryogenesis, at the pre-implantation stage, the IGF system is believed to be one of the most important stem cell niche factors in embryonic or mesenchymal (adult) stem cell development. As shown in previous studies, in human pregnancies, IGFs play an early role in promoting proliferation and differentiation of placental cells (11), and reduced levels of circulating IGFs during development lead to fetal growth restriction (12). Furthermore, IGF-1R knockout mice have restricted growth and die prematurely due to the lack of functional respiratory muscle (13-15).
5.1.1.1 Roles of IGFBP-6

To date, no previous studies have reported the role of IGFBP-6 on the maintenance of multipotency within adult mesenchymal stem cells or on the role of IGFBP-6 on the differentiation of PMSCs into skeletal muscle. To achieve this goal we developed a surrogate system to study the stages of muscle differentiation by PMSCs through detection of the muscle commitment marker (Pax3/7) and myotubular proteins (MyoD, MyoG, and MHC) to mark different stages of muscle development. We showed that as PMSCs differentiate into skeletal muscle, they expressed greater levels of IGFBP-6 both intracellularly (whole cell protein) and extracellularly (secreted to the media).

Overall, increased extracellular IGFBP-6 before muscle commitment enhanced differentiation, whereas the continued expression of IGFBP-6 at later time points delayed full myogenesis. Interestingly, pluripotency-associated markers were increased as IGFBP-6 levels increased. In contrast, silencing IGFBP-6 using siRNA, which decreased intracellular and extracellular IGFBP-6, significantly reduced pluripotency-associated markers and delayed the expression of muscle differentiation markers at the early stages of PMSCs differentiation into muscle. These findings suggested that extracellular IGFBP-6 stimulates muscle lineage commitment while a prolonged exposure inhibits late stage differentiation. In contrast, intracellular IGFBP-6 helped maintain OCT4 and SOX2, delayed commitment, and enhanced differentiation. In summary, we suggest that IGFBP-6 plays a dual role in both PMSC multipotency and in differentiation towards muscle, with greater impact during the early stages of the differentiation process, before muscle commitment has occurred. Also, the timing of the biologic actions of IGFBP-6 and the balance between intracellular and extracellular protein concentrations may
represent an important regulatory mechanism in the differentiation of PMSCs into skeletal muscle. Although *in vivo* studies are required to validate our findings, our study is one of the first to show the role of IGFBP-6 on skeletal muscle lineage differentiation in PMSCs.

These results support previous reports that IGFBP-6 is highly expressed in developing muscle cells and is also abundant in differentiated skeletal muscle (16-18). However, the role of IGFBP-6 in muscle development is unclear and IGFBP-6 was mostly investigated in cancer cell lines. IGFBP-6 expression is associated with non-proliferative states and inhibition of IGF-2-dependent tumor cell growth, such as rhabdomyosarcoma, neuroblastoma, and colon cancer (19-21). Conversely, due to the fact that IGFs are rarely found in the intracellular microenvironment, intracellular IGFBP-6 has IGF-independent functions (22, 23). Therefore, IGFBP-6 was either secreted into the extracellular microenvironment and interacted with the IGF peptides or IGFBP-6 was localized to the intracellular microenvironment and functioned in an IGF-independent manner (22-24).

### 5.1.1.2 IGF-Dependent Functions of IGFBP-6

Our findings also suggest that IGF-1 and IGF-2 play different roles during PMSC differentiation into skeletal muscle. We demonstrated that IGF-1 supplementation increased IGFBP-6 protein levels within 24 hours but the effect was short-lived and after 3 days, the levels decreased. In contrast, IGF-2 supplementation increased IGFBP-6 levels more gradually throughout the 14-day time course. Therefore, by promoting an immediate increase in intracellular IGFBP-6, IGF-1 delayed PMSC muscle commitment as shown with the increased Pax3/7 protein levels. In contrast, IGF-2 by increasing
IGFBP-6 levels gradually for a longer duration, promoted muscle differentiation. These results suggested that IGF-1 and IGF-2 have direct effects on IGFBP-6 expression in PMSCs during muscle differentiation. Although IGF-1 generally delayed PMSCs muscle lineage commitment, IGF-2 acted to enhance muscle differentiation. IGFs may affect differentiating PMSCs IGF-signaling directly or indirectly via IGFBP-6.

IGF-1 and IGF-2 stimulate both proliferation and terminal differentiation of many tissues in developing embryos and adults. IGFBP-6 has a significantly higher affinity (~70-100 fold) for IGF-2 than IGF-1 (25-27). Interestingly, the site on IGF-2 where IGFBP-6 binds overlaps with IGF-1R binding site, giving a structural basis for IGFBP-6 to inhibit IGF-2 binding to the IGF-1R (28). Studies in various cell lines have shown mostly an inhibitory action of IGFBP-6 mainly via IGF-2-dependent actions. In L6A1 myoblast, IGFBP-6 inhibited muscle differentiation induced by IGF-2 but not IGF-1 (29). Previous reports on the effects of IGFs on muscle differentiation were using mouse cell lines (30-36), thus our study is one of the first to show the effects of IGF-dependent functions of IGFBP-6 on human mesenchymal stem cells differentiation into skeletal muscle in vitro.

5.1.1.3 IGF-Independent Functions of IGFBP-6

Our results also demonstrated that although IGF-2 was important for PMSCs differentiation into skeletal muscle, IGFBP-6 extracellular supplementation with IGF-2 knockdown could compensate for IGF-2 loss and could enhance the differentiation process independent of IGF-2 as seen with the increased levels of MyoD and MHC.

Our findings confirm previous reports that IGF-2 is required for myogenesis (37, 38). Previous studies linked IGFBP-6 independent functions to regulate cell fate through the
Ku proteins (23) and affect cancer cell migration and proliferation (25-27). This study is the first to investigate the interaction between IGF-2 and IGFBP-6 on the early differentiation stages of PMSCs into skeletal muscle.

In this thesis, we focused on the effects of IGFBP-6 on IGF-2, due to its higher affinity, and how IGFBP-6 compensated for IGF-2 reduction under muscle differentiation conditions. However, IGFBP-6 may also regulate IGF-1 availability, so the effects of IGFBP-6 on IGF-1 need to be further delineated. For future studies, IGF-1 knockdown combined with IGFBP-6 addition is necessary to compare to IGF-2 results. Moreover, we showed that IGFBP-6 impacts on PMSCs muscle differentiation in both IGF-dependent and IGF-independent manner. In future studies, use of a mutant IGFBP-6, that does not bind IGF-2 and IGF-1 (18), would provide further evidence on the relative roles of IGF-dependent and IGF-independent actions on skeletal muscle differentiation.

5.1.1.4 IGFBP-6 has IGF-1R-Dependent and –Independent Functions, but only IR-Independent Functions

In these studies, we also demonstrated that IGF-1R and its downstream signaling pathways (PI3K-AKT and MAPK pathways) were required for PMSCs muscle differentiation. We also showed that when the PI3K pathway was inhibited, increased extracellular IGFBP-6 improved PMSC differentiation into skeletal muscle as seen with the increased protein levels of MyoD and MHC. In contrast, MAPK pathway inhibition could not be rescued by increased extracellular IGFBP-6 as seen with the unchanged protein levels of muscle lineage differentiation markers. MAPK inhibition also caused a significant decrease in intracellular IGFBP-6 concentrations, which could not be reversed
by addition of extracellular IGFBP-6 as seen with the unaffected IGFBP-6 protein levels. These studies suggested that MAPK signaling was an important pathway for PMSCs differentiation into skeletal muscle, and that intracellular IGFBP-6 compliments this process. Therefore, we suggest that in PMSCs, IGFBP-6 acts in an IGF-1R dependent manner predominantly through the MAPK signaling pathway and not through PI3K to achieve skeletal muscle differentiation. We further verified the importance of the insulin receptor (IR) in PMSCs differentiation into muscle, and the interaction with IGFBP-6.

In this thesis, we also demonstrated that IR plays an important role in PMSC muscle differentiation in addition to IGF-1R. We showed that inhibiting IR signaling delayed PMSCs differentiation into skeletal muscle but did not completely block the process as IGF-1R signaling was still active and most likely mediated the differentiation process. These observations also suggested that the induction of muscle differentiation by the high concentration of insulin (10 μg/mL) in the media is likely exerted by insulin binding to the IGF-1R, to which it has low affinity binding capacity. The fact that IGFBP-6 enhanced muscle differentiation when IR was inhibited suggests that IGFBP-6 induced PMSC differentiation into muscle could occur independent of IR signaling.

The IGF-1R and IR are both receptor tyrosine kinases that activate several signaling transduction pathways (39, 40). IGFs and insulin both promote cell proliferation and differentiation (37, 39-42), and IGFs also possess insulin-like metabolic effects, including increased glucose uptake in skeletal muscle, mediated by either IGF-1R or IR (43). Previous reports show that high concentrations of insulin activates both IGF-1R and IR (30, 44), however, not much attention is given to IGF-1R binding affinity and effects versus IR when insulin is used.
Therefore, in future studies, a phospho-kinase array may be used to specify interacting adaptors and signaling proteins within complementary IGF-1R and IR signaling pathways. Also, alterations in the PMSCs microenvironment can cause epigenetic changes; and it will be interesting to understand whether the IGF system affects multipotency and myogenesis through epigenetic modulation of promoter regions.

5.2 Overall Conclusions

To date, previous studies on the role of the IGF family, specifically IGFBP-6, have not been reported during the differentiation of PMSCs towards the skeletal muscle lineage. The data presented in this thesis, gives insights into the effects of the IGF system on PMSCs differentiation into skeletal muscle. By controlling the surrounding microenvironment in vitro, PMSCs can be directed to proliferate or differentiate into skeletal muscle in vitro. We have shown that the IGF family represents an early important niche factors that regulates PMSCs stemness and differentiation.

PMSCs possess the ability to achieve terminal differentiation into skeletal muscle under appropriate conditions. As summarized in Figure 5.1, this differentiation process is regulated by the IGF family. During early myogenesis, PMSCs lose pluripotency-associated markers (OCT4 and SOX2) and activate muscle commitment marker (Pax3/7). During later stages of myogenesis, Pax3/7 was decreased as muscle differentiation markers (MyoG, MyoD, and MHC) increased. A balance between IGF-1, IGF-2, IGFBP-6, IGF-1R, and IR was required for complete muscle differentiation by PMSCs. Extracellular and intracellular IGFBP-6 was expressed in PMSCs during differentiation, and promoted PMSCs muscle differentiation in both an IGF-1R dependent and
independent manner. The timing of manipulating the IGF system was crucial, as the outcomes were different if performed before or after PMSCs commitment to the muscle lineage. Thus, our findings indicated that IGFBP-6 regulates PMSC multipotency and differentiation into muscle in a time-dependent manner, with more prominent effects observed at the beginning of the differentiation process, before muscle lineage commitment.

Overall, manipulating the PMSCs microenvironment using the IGF system, particularly IGFBP-6, can improve PMSC myogenic differentiation, a first step towards PMSC use for muscle regeneration therapies.

5.3 Limitations and Future Studies

This study is the first to provide fundamental insight into the effects of the IGF system on PMSCs differentiation into skeletal muscle. Therefore, further experiments are required to address the limitations and to further delineate mechanistic interactions. Based on data presented throughout this study, future studies can be planned as follows:

5.3.1 IGFBP-6 and IGF-2 Silencing

When siRNA was used to knockdown IGFBP-6 and IGF-2 expression, only partial knockdown was achieved, as the protein levels of IGFBP-6 and IGF-2 were not completely diminished. Also, expression of each protein was restored back to control levels even with the continuous addition of siRNA every 3 days. Therefore, alternate methods to knockdown IGFBP-6 and IGF-2 expression is needed to improve gene silencing. For example, the use of IGFBP-6- or IGF-2-specific shRNAs, or CRISP/Cas9
gene editing technologies would improve the interpretation of data generated using our *in vitro* muscle differentiation system. The use of IGFBP-6 and IGF-2 shRNAs has previously been shown to induce a significant and stable down-regulation of mRNA and protein in primary human cells (45, 46).

### 5.3.2 The Role of OCT4 in PMSCs Muscle Differentiation

In our study, we found that OCT4 behaved differently than other pluripotency-associated markers. OCT4 expression was positively affected by IGFBP-6 and increased alongside muscle differentiation markers with IGFBP-6 treatment. Therefore, inhibiting or overexpressing OCT4 would further delineate its role in response to IGFs and IGFBP-6 in PMSCs under muscle differentiation conditions (47, 48). OCT4 localization and dose is important for its function (49); thus nuclear versus cytoplasmic fractionation experiments would be required to show if continued OCT4 expression during PMSCs muscle differentiation was relevant to OCT4 associated transcriptional activities.

### 5.3.3 PMSCs and IGFBP-6 Therapeutic Potential *in vivo*

In future experiments, mouse models with muscle injury can be used to test PMSCs and IGFBP-6 affects *in vivo*. We can test the effects of priming PMSCs with IGFBP-6 during their differentiation into skeletal muscle for engraftment or IGFBP-6 injections.
Figure 5.1. PMSCs differentiation into skeletal muscle using the insulin-like growth factor system. PMSCs isolated from the chorionic villus of preterm human placenta (15 weeks) expressed high levels of pluripotency-associated markers (OCT4 and SOX2) under normal growth conditions (10% FBS). As these cells differentiated into skeletal muscle, the levels of these markers decreased and the cells committed to the muscle lineage, indicated by Pax3/7 expression. Once committed to differentiation, PMSCs subsequently decreased Pax3/7 expression and increased muscle differentiation markers (MyoG, MyoD, and MHC) as myoblasts aligned and fuse to form multinucleated myofibers. IGF-1 and IGF-2 binds to the IGF-1R and activates its intrinsic tyrosine kinase activity resulting in signaling that accelerated muscle differentiation via downstream signals including PI3K-AKT-mTOR and the RAF-MEK-ERK (MAPK) pathway. Due to IGFBP-6 intracellular and extracellular locations, IGFBP-6 demonstrated both IGF-dependent and IGF-independent effects on PMSCs muscle differentiation. Extracellular IGFBP-6 binds IGFs and enhances the muscle differentiation process through the IGF-1R, while intracellular IGFBP-6 directly impacted PMSCs muscle differentiation through an unknown mechanism.
5.4 Significance

Knowledge on how myogenesis can be manipulated through IGFs and IGFBP-6 will help elucidate the mechanisms controlling MSC fate and thus will help us improve strategies for skeletal muscle regeneration therapies using stem cells from human placenta in myogenic diseases to rescue muscle loss.

5.5 References


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Appendix 4

Characterization of Placental Mesenchymal Stem Cells
MATERIALS AND METHODS

Isolation of PMSCs

PMSC isolation and experiments were conducted with the approval from the Health Sciences Research Ethics Board of Western University (REB# 12154). Informed consent was obtained from healthy women undergoing therapeutic termination of pregnancy, and the PMSCs used in this study were isolated from one 15 week preterm placental tissue. After surgery, chorionic villi were dissected, washed, and minced with surgical scissors and forceps, and then subjected to enzymatic digestion with collagenase IV (369 IU/mg), hyaluronidase (999 IU/mg) (Sigma-Aldrich, Oakville, ON), and DNase I (2,000 IU/mg) (Hoffmann-LaRoche, Mississauga, ON) for 10 minutes at room temperature, followed by 0.05% trypsin (Gibco/Invitrogen, Mississauga, ON) for 5 minutes at room temperature. The sample was then washed for 10 minutes with 10% FBS in DMEM/F12 medium and the resulting single cell suspension was separated by density centrifugation over a Percoll (Sigma-Aldrich, Oakville, ON) discontinuous gradient using a modified protocol by Worton et al. Cells from Percoll gradient fractions #3, #4, and #5 were plated on to T75 flasks, cultured, and maintained using DMEM/F12 media supplemented with 15% FBS serum (Gibco/Invitrogen, Mississauga, ON) and FGF-2 (50 ng/mL) (Sigma-Aldrich, Oakville, ON) containing 100 U/mL Penicillin, 100 μg/mL Streptomycin, and 0.25 μg/mL Amphotericin-B. The non-adherent cells were discarded at the time of media change, which was performed every 72 hours. The adherent cells were cultured until they reach 90% confluence. Cells were then passaged 1:2 approximately once per week using 0.05% Trypsin for 10 min at 37°C for 3 passages. Passaged cells were stored at -80°C in 1 mL of freezing media (30% FBS and 10% DMSO in DMEM/F12 media). When
needed, vials were thawed and cells were resuspended in normal culture media (25 ng/mL FGF-2 and 15% FBS in DMEM/F12).

**Osteogenic differentiation**

Cells were grown in 6-well plates (90% confluent) in osteogenic differentiation media (MesenCult basal medium, osteogenic differentiation promoting supplements, ascorbic acid, β-glycerophosphate and dexamethasone) purchased from Stem Cell Technologies (Vancouver, BC). Media was changed every 3 days. After three weeks, cells were fixed using 100% ice cold ethanol for 30 minutes, then dried at room temperature. The wells were stained with 1 mL of 0.01% of alizarin red for 40 minutes at room temperature. The staining was observed under an inverted light microscope (Zeiss, Germany). Cells grown in 10% FBS media (Gibco-Invitrogen, Mississauga, ON) were used as negative controls. The staining of the cells was solubilized using 1mL of 10% cetylpyridium phosphate in 10 nM sodium phosphate pH 7.0 and the absorbance of a 200 μL reaction volume was read at λ=570 nm using a plate reader-spectrophotometer.

**Adipogenic Differentiation**

Cells were grown in 6-well plates (90% confluent) in adipogenic differentiation media (StemPro basal medium, adipogenesis supplements, Gentamicin reagent) from Gibco-Invitrogen (Mississauga, ON) for three weeks. Media was changed every 3 days. Cells were fixed with 100% ice cold ethanol for an hour, then 60% Isopropanol was added for 5 minutes before staining with oil red O for an hour. Finally, hematoxylin stain was
added for one minute to stain nuclei. Cells grown in 10% FBS (Gibco-Invitrogen, Mississauga, ON) media were used as negative controls.

**Flow Cytometry Analysis**

Cells were trypsinized for 10 minutes using recombinant trypsin (TripleXExpress, Gibco-Invitrogen, Mississauga, ON) diluted 1:1 in PBS, at 37°C. After the cells were detached from the flask, trypsin was neutralized with 10% FBS in DMEMF/12 medium, cells were washed and incubated for one hour with fluorochrome labeled primary antibody against MSCs markers. CD73 (#550256) (BD Pharmingen, San Jose, CA), PE conjugated CD105 (#12-1057-73) (eBioscience, San Diego, CA), and CD-117/c-Kit (sc-13508) (Santa Cruz Biotechnology, Dallas, TX) were used.

**Statistical Analysis**

All experiments were performed in triplicate from two 15 or 20 weeks placental tissue (technical and biological replicates). GraphPad Prism Software 5.0 was used to generate all graphs and analyses. A two-way ANOVA followed by a Bonferroni’s multiple comparison test was used to calculate significant differences when p<0.05. Graphic representation values are presented as mean ± SEM (shown as variance bars).
RESULTS

PMSCs Isolated from Placental Tissue Showed Stem Cell Characteristics

Mesenchymal stem cells from preterm placenta (15-20 weeks) were isolated and characterized. Following cell expansion on tissue culture plastic, cell identity was verified by flow cytometry for the MSCs multipotency markers CD105 (>95%), CD73 (>95%), and CD117 (c-Kit) (<10%).

Mesenchymal stem cells from three preterm placentae were isolated; two from the late first trimester of pregnancy (15 weeks) (Figure 6.1) and one from the second trimester (20 weeks) (Figure 6.2). Cells were collected from layers 3, 4, and 5 based on the Percoll gradient density (Figures 6.1A and 6.2A). In vitro, the cells isolated were plastic adherent (Figure 6.1B and 6.2B). Stem cell markers CD73, CD105, and CD117 were measured using flow cytometry (Figures 6.1C and 6.2C).
Figure 6.1. PMSC Isolation from 15 weeks preterm placenta. (A) The dissected villous tissue was digested enzymatically and cells were separated using a discontinuous Percoll gradient. Five cell fractions were typically obtained corresponding to five different densities and cells were isolated from layers 3, 4, and 5. (B) Phase contrast images of the isolated PMSCs, from all three layers, grown in culture after 4 weeks. (C) PMSCs from passage 4 of all three layers were positive for CD73 and CD105 (>98%), and were negative for CD117 (<1%) (measured by flow cytometry). Flow cytometry histograms are representative of all 3 layers from one placental tissue as they showed the same results.
Figure 6.2. PMSC Isolation from 20 weeks preterm placenta. (A) Successful cell separation using Percoll gradient. Five cell fractions were obtained and cells were isolated from layers 3, 4, and 5. (B) Phase contrast images of the isolated PMSCs, from all three layers, grown in culture after 4 weeks. (C) PMSCs from passage 4 of all three layers were positive for CD73 and CD105 (>98%), and were negative for CD117 (<1%) (measured by flow cytometry). Flow cytometry histograms are representative of all 3 layers from one placental tissue as they showed the same results.
PMSCs Demonstrate Osteogenic and Adipogenic Differentiation Competence

PMSCs isolated from preterm placentae (15-20 weeks) tissue were cultured and maintained in a standard tissue culture incubator using DMEM/F12 media supplemented with 10% fetal bovine serum (FBS) in tissue culture flasks until they reached 60% confluency. Cells were then cultured in 6-well plates in either osteogenic or adipogenic differentiation media. Cells grown in 10% FBS media were used as negative controls. After 20 days in culture, cells were fixed and stained for differentiation markers. Alizarin red was used for osteogenic differentiation and oil red O for adipogenic differentiation.

All three layers of isolated PMSCs formed multilayered structures in which calcium deposits were detected by alizarin red staining from both 15 weeks (Figure 6.3A) and 20 weeks PMSCs (Figure 6.3B). Adipogenic differentiation was also achieved for all three layers, as seen with oil red O staining and the formation of round adipocytes for both gestational age PMSCs (Figure 6.4A and 6.4B).
Figure 6.4. PMSCs from different gestational ages differentiate towards the osteogenic lineage. PMSCs isolated from all three layers showed osteogenic differentiation when cultured in osteogenic medium, as measured by the accumulation of calcium deposits shown with alizarin red staining in cells isolated from (A) 15 weeks and (B) 20 weeks placenta samples compared to untreated control at 3 weeks (10x magnification). The lower panel shows alizarin red staining quantification. Data are representative of three independent experiments from one placenta per gestational age. (Two-way ANOVA followed by a Bonferroni’s multiple comparison test, ***=P<0.001).
Figure 6.5. PMSCs isolated from different gestational ages differentiate towards the adipogenic lineage. Cells isolated from 15 week (A) and 20 week (B) placentae, were cultured in adipogenic medium or left untreated (control) for 21 days. All three layers showed the ability to differentiate into adipocytes shown by the accumulation of lipid droplets within the cells with the oil red O staining. Results are representative of three independent experiments from one placenta per gestational age.
Conclusion

We isolated PMSCs based on density gradient separation and growth adherence to tissue-culture plastic. Moreover, cultured PMSCs were positive for the mesenchymal markers CD73 and CD105 (>95%) and did not express the hematopoietic marker c-kit (<10%).

Furthermore, PMSCs isolated from the chorionic villi of preterm placental tissue (at 15 weeks and 20 weeks) differentiated into osteoblasts and adipocytes; confirming multipotent differentiation of PMSCs. Thus, PMSCs represent a promising cell type for the development of tissue regeneration therapy, due to early ontogeny and multipotent potential.
Appendix 5
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Appendix 6

**Western Research**
**Western University Health Science Research Ethics Board**
**HSREB Annual Continuing Ethics Approval Notice**

**Date:** March 21, 2016  
**Principal Investigator:** Dr. Victor Han  
**Department & Institution:** Schulich School of Medicine and Dentistry/Anatomy & Cell Biology, Western University

**Review Type:** Full Board  
**HSREB File Number:** 101556  
**Study Title:** The Role of Insulin-Like Growth Factors in Human Stem Cells Development and Motility (REB #12154)

**HSREB Renewal Due Date & HSREB Expiry Date:**  
Renewal Due -2017/03/31  
Expiry Date -2017/04/25

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

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

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