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THE ROLE OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-6 IN THE DIFFERENTIATION OF PLACENTAL MESENCHYMAL STEM CELLS INTO SKELETAL MUSCLE

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ABSTRACT

Insulin-like growth factor binding protein-6 (IGFBP-6), a component of the stem cell niche involved in the differentiation of skeletal muscle myogenesis, is expressed in developing muscle cells and is the main regulator of IGF-II. In this study, I investigated the role of IGFBP-6 in the commitment of skeletal muscle derivation from placenta mesenchymal stem cells (PMSCs). I hypothesized that IGFBP-6 inhibits the temporal maintenance of PMSCs and promotes the differentiation of PMSCs into muscle via both extracellular and intracellular mechanisms. PMSCs can differentiate into muscle cells expressing the muscle markers Pax3/7, MyoD, and Myogenin with the formation of multi-nucleated fibers. Under differentiation conditions, silencing IGFBP-6 increased Pax3/7 and decreased OCT4 levels. In contrast, under non-differentiation conditions, there was a significant increase in Pax3/7 levels at day 7 with intracellular and extracellular increase of IGFBP-6, similarly silencing IGFBP-6 under non-differentiation conditions significantly increased Pax3/7 at 24 hours and decreased OCT4 levels over time same as in differentiation conditions. I concluded that increasing IGFBP-6 promotes PMSCs differentiation with more prominent effects at the beginning of the differentiation process, while silencing IGFBP-6 has more dramatic effects on PMSCs. Knowledge of the effects of IGFBP-6 on muscle differentiation will help improve strategies for skeletal muscle regeneration therapies using stem cells.

KEYWORDS

Placental mesenchymal stem cell, IGFBP-6, muscle differentiation, Pax3/7, OCT4, MyoD, Myogenin.

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TABLE OF CONTENTS

CERTIFICATE OF EXAMINATION ii
ABSTRACT iii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS v
LIST OF TABLES viii
LIST OF FIGURES ix
LIST OF ABBREVIATIONS xi

1.0 INTRODUCTION 1
1.1 Stem Cells 1
1.1.1 Overview 1
1.1.2 Embryonic Stem (ES) Cells 2
1.1.3 Induced Pluripotent Stem (iPS) Cells 2
1.1.3.1 Stem Cell Pluripotency 3
1.1.4 Adult Mesenchymal Stem Cells 3
1.1.4.1 Placental Mesenchymal Stem Cells 4
1.2 Stem Cell Microenvironment 5
1.2.1 Niche Components 6
1.3 Insulin-like Growth Factor (IGF) System 9
1.3.1 Insulin-like Growth Factor-I and –II 12
1.3.1.1 IGFs in Placental Development 12
1.3.2 Insulin-like Growth Factor Type-I and –II Receptors 13
1.3.3 Insulin-like Growth Factor Binding Proteins 17
1.3.4 Insulin-like Growth Factor Binding Protein-6 17
1.4 Myogenesis 23
1.4.1 Myogenic Regulatory Factors (MRFs) 23
1.4.2 Insulin-like Growth Factors and Myogenesis 28
1.4.3 Insulin-like Growth Factor Binding Protein-6 and Myogenesis 29

2.0 HYPOTHESIS 32

2.1 OBJECTIVES 32
2.2 RATIONALE 32

3.0 MATERIALS AND METHODS 33
3.1 Reagents 33
3.1.1 Cell Culture Reagents 33
3.1.2 Muscle Differentiation Reagents 33
3.1.3 Insulin-like Growth Factor Binding Protein-6 33
3.2 Placental Mesenchymal Stem Cell Culture in Muscle Differentiation Conditions 34
3.2.1 Placental Mesenchymal Stem Cell Freezing and Thawing 34
3.3 IGFBP-6 Protein Transport System (Chariot™) 35
3.4 Down-regulation of IGFBP-6 Expression by siRNA 40
3.5 Cell Lysate Preparation 40
3.6 Protein Assay 40
3.7 Immunocytochemistry Procedure 41
3.8 Immunoblotting Procedure 41
3.8.1 Sample Preparation 41
3.8.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis and Protein Transfer 42
3.8.3 Antibody Probing 42
3.8.4 Chemiluminescence, Imaging, and Densitometry 45
3.8.5 Stripping Western Blot Membranes 45
3.9 Quantification of the IGFBP-6 production by (ELISA) 45
3.10 Statistical Analysis 46

4.0 RESULTS 47
4.1 Placental Mesenchymal Stem Cell Differentiation into Skeletal Muscle 47
4.2 IGFBP-6 Intracellular Actions on Developing Muscle Cells 55
4.3 Extracellular Actions of IGFBP-6 on Developing Muscle Cells 84

5.0 DISCUSSION **94**
5.1 PMSC Differentiation into Skeletal Muscle 94
5.2 Increase in Intracellular IGFBP-6 in PMSCs using the Chariot™ Transport System 95
5.3 IGFBP-6 on Muscle Differentiation 96
5.4 Silencing IGFBP-6 under Non-differentiation and Differentiation Conditions 97
5.5 Increased IGFBP-6 in PMSC Microenvironment 98

6.0 SUMMARY AND CONCLUSIONS **100**
7.0 LIMITATIONS **101**
8.0 FUTURE STUDIES **102**
9.0 SIGNIFICANCE **113**
REFERENCES **104**
CURRICULUM VITAE **115**

LIST OF TABLES

Table 1. Primary antibodies for western blotting and/or immunocytochemistry.....43

Table 2. Secondary antibodies for western blotting.....44

Table 3. Secondary antibodies for immunocytochemistry.....44

LIST OF FIGURES

Figure 1. Schematic of the stem cell microenvironment....8

Figure 2. Insulin-like growth factor superfamily....11

Figure 3. Schematic of the IGF-IR signaling pathway....16

Figure 4. Schematic of IGF-dependent and -independent functions of insulin-like growth factor binding protein -6....22

Figure 5. Overview of muscle differentiation....27

Figure 6. Proposed model....31

Figure 7. Diagram of the Chariot™ Process....37

Figure 8. Chariot™ system efficiency....39

Figure 9. Placental mesenchymal stem cells have the ability to differentiate into Muscle....49

Figure 10. Placental mesenchymal stem cells show muscle-like morphology at 14 days post-differentiation....52

Figure 11. Placental mesenchymal stem cells grown in muscle differentiation media show increasing levels of IGFBP-6 by time with no change in OCT4....54

Figure 12. Time course experiment for increased intracellular IGFBP-6 by Chariot™ in PMSCs under non-differentiation conditions....59

Figure 13. ELISA for the time course experiment for increased intracellular IGFBP-6 by Chariot™ in PMSCs under non-differentiation Conditions....63

Figure 14. Time course experiment for increased intracellular IGFBP-6 by Chariot™ in PMSCs under differentiation conditions....65

Figure 15. ELISA for the time course experiment for increased intracellular IGFBP-6 by Chariot™ in PMSCs under differentiation conditions....69

Figure 16. Time course experiment for silencing IGFBP-6 expression in PMSCs under non-differentiation conditions.....71

Figure 17. Time course experiment for silencing IGFBP-6 expression in PMSCs under differentiation conditions.....76

Figure 18. ELISA for the time course experiment for silencing IGFBP-6 Expression in PMSCs under non-differentiation conditions.....81

Figure 19. Time course of changes in IGFBP-6 secreted to the media following silencing of IGFBP-6 expression in PMSCs under differentiation conditions.....83

Figure 20. Changes in IGFBP-6 levels when extracellular IGFBP-6 was increased in PMSCs under non-differentiation conditions.....87

Figure 21. Changes with time after addition of extracellular IGFBP-6 in PMSCs under differentiation conditions.....91

LIST OF ABBREVIATIONS

Abbreviation	Definition
°C	Degrees Celsius
α	Alpha
β	Beta
m	Milli
μ	Micro
n	Nano
λ	Wavelength
AKT	Serine/threonine protein kinase B
ALP	Alkaline phosphatase
ALS	Acid labile sub-unit
Amp	Amperes
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bFGF	Basic fibroblastic growth factor
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C terminal	Carboxy-terminal
CHO	Chinese Hamster ovary cell line
dH_2O	Distilled water
DMEM-F12	Dulbecco's modified eagle medium and nutrient mixture Ham's F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid

EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem cell
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
G	Gram
GH	Growth hormone
GRB-2	Growth factor receptor-bound protein 2
HCl	Hydrogen chloride
IGFBP	Insulin-like growth factor binding protein
IGFBP-6	Insulin-like growth factor binding protein-6
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGF-IR	Insulin-like growth factor receptor I
IGF-IIR	Insulin-like growth factor receptor II
IgG	Immunoglobulin G
IRS	Insulin receptor substrate
KCl	Potassium choride
kDa	Kilodalton
L domain	labile (or linker) domain
N	terminal amino terminal
MAPK	Mitogen-activated protein kinase
$MgCl_2$	Magnesium chloride
Min	Minutes
mL	Milliliter
mol	Mole
MSC	Mesenchymal stem cell

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NANOG	Homeobox protein Nanog
ng	nanogram
nM	nanomolar
nm	nanometre
OCT4	Octamer-binding transcription factor-3/4
p	Significance value
p85	Phosphatidyl-inositol-3-kinase regulatory subunit
Pax3/7	Paired box 3/7
pH	potential of hydrogen
PCR	polymerase chain reaction
PI3K	Phosphatidyl-inositol-3-kinase
PMSC	Placenta-derived mesenchymal stem cell
PVDF	Polyvinylidene fluoride
RD	Rhabdomyosarcoma derived
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel Electrophoresis
SHC	Src-homology 2 containing protein
SOX-2	SRY-related HMG box 2
SSEA	Stage specific embryonic antigen
STAT	Signal transducer and activator of transduction
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline supplemented with 0.05% Tween-20
TEMED	Tetramethylethylenediamine

Tra	Tumor rejection antigen
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
V	Volt
w/v	Weight per volume

1.0 INTRODUCTION

1.1 Stem Cells

1.1.1 Overview

Stem cells possess two distinctive characteristics, namely a) the ability to self-renew through mitotic divisions undergoing symmetric division, and b) the ability to differentiate into a diverse range of cells through asymmetric divisions (1). Pluripotency is the fundamental characteristic of stem cells, and based on its developmental potential, it can be categorized into three types - totipotent, pluripotent, or multipotent (2). Totipotent stem cells can give rise to all embryonic (including germ cells) as well as extraembryonic cell types. Pluripotent stem cells are descendants of totipotent cells and can give rise to cell types of all three germ layers (3). In contrast, multipotent stem cells can only produce a closely related family of cells or lineage, usually from one germ layer (4).

Research on stem cells has been performed mainly on two types - embryonic stem cells (ES) which are derived from the inner cell mass of early embryos at the blastocyst developmental stage (5), or adult stem cells which are derived from tissues from later fetal gestational stage or adult (4). The major difference between these two types is the ability to differentiate: Embryonic stem cells (ES) are pluripotent and can differentiate into most cell types in the body (3), whereas adult stem cells, sometimes called mesenchymal stem cells (MSCs), are multipotent cells and have more limited differentiation abilities (lineage specific tissue) (4).

Research on stem cells has progressed greatly in recent years and the hope of using stem cells in tissue regeneration and cellular therapies is becoming more possible due to the increased amount of knowledge (7, 8). However, before stem cells can be used reliably and safely in regenerative medicine, it is essential to understand how factors within the stem cell microenvironment influence stem cell differentiation and self-renewal as stem cell fate is easily altered by the culture conditions (9).

1.1.2 Embryonic Stem (ES) Cells

The first line of mouse ES cells was first reported in 1981 (10); however the derivation of human ES cell lines from blastocysts was not reported until 1998 (5). ES cells are unspecialized cells with nearly unlimited self-renewal capacity (11). Moreover, ES cells possess pluripotency which is an essential characteristic for the stem cell state (12). Furthermore, ES cells differentiate, *in vivo*, into cell types from all three germ layers (ectoderm, mesoderm, and endoderm) (13).

1.1.3 Induced Pluripotent Stem (iPS) Cells

Induced pluripotent stem cells (iPS) using transcription factor-based reprogramming demonstrated ES cell properties including ES cell morphology, expression of ES marker genes, high proliferation rate, and teratoma formation (14, 15).

In 2006, the transcription factor-based reprogramming began, when mouse fibroblasts were reprogrammed by retroviral introduction of transcription factors OCT-4, SOX-2, KLF-4, and c-MYC (16). Moreover, in a 2009 study by Kim et al, it was shown that the expression of OCT-4 alone could induce the reprogramming of human adult neural stem cells (17).

1.1.3.1 Stem Cell Pluripotency

The main transcription factors that regulate pluripotency are OCT4, SOX2, and NANOG (15, 16, 17). These factors either cooperate together or function alone to promote stem cell pluripotency and inhibit cell differentiation (18).

In stem cells, OCT4, a member of the POU homeodomain protein family, is a transcription factor that is integral for pluripotent ES cells and is used as a marker of pluripotency but is not sufficient to maintain ES cells in the undifferentiated state (19). OCT4 regulates pluripotent gene expression by repressing genes involved in differentiation and development (19). Moreover, the down-regulation of OCT4 is associated with differentiation, where terminally differentiated cells lose the ability to express OCT4 (20). Furthermore, OCT4 is considered the "master regulator" of pluripotency (17).OCT4 expression is shown in numerous human adult stem cell types, including hematopoietic (21), liver (22), pancreatic (22), gastric (22), and mesenchymal stem cells (22, 23).

1.1.4 Adult Mesenchymal Stem Cells (MSCs)

The first report of the presence of adult stem cells was in 1961 by Canadian scientists McCulloch and Till (24). Bone marrow MSCs were the first to be isolated in 1968 by Friedenstein (25). To define the MSC population *in vitro*, the International Society for Cellular Therapy established three criteria, namely that a MSC must be a) plastic-adherent when maintained in standard culture conditions, b) express CD105 (a stromal and vascular marker), CD73, and CD90 (fibroblast markers) and lack hematopoietic markers such as CD45 and CD34, and c) be capable of differentiation into osteoblasts, adipocytes, and chondroblasts (3).

Although traditionally isolated from bone marrow (26), more recent reports have described the isolation of cells with MSC characteristics from other mature organs and tissues such as skeletal muscle (27), adipose tissue (28), deciduous teeth (29), umbilical cord (30) and peripheral blood (31), fetal liver and lung, amniotic fluid, synovium and the circulatory system (32). In culture, MSCs are defined as plastic-adherent, fibroblast-like cells which are able to self-renew and differentiate into different mesodermal cell lineages including bone, adipose and cartilage tissue (3).

Moreover, MSCs are very important candidates for regenerative medicine because most adult tissues possess multipotent MSCs, which are crucial for tissue maintenance and repair (33). Also in contrast to ESC, human MSCs have been studied in phase 1 and 2 clinical trials with the possibility of being used in the clinic in the very near future (32).

1.1.4.1 Placental Mesenchymal Stem Cells (PMSCs)

The placenta is a vital organ for the embryo, acting as the interface between the fetal and maternal environments (34) unlike the early embryo from which embryonic stem cells are derived, the placenta is a non-controversial source of adult mesenchymal stem cells (PMSCs) and is a readily available source of MSCs for possible tissue regeneration therapy for human patients (5 36, 37).

The embryonic mesenchymal cells infiltrate the cytotrophoblast layer at 6-7 days post fertilization, where these cells in conjunction with trophoblast stem cells, invade the maternal endometrium for implantation and initiation of placental development (38, 39). The main functional units of the placenta are the chorionic villi, which carry the embryonic blood and allow the fetal-maternal exchange (40). Moreover, stem cells

derived from the chorionic villi have greater cell expansion compared to the adult bone marrow MSCs (41).

Previous studies from our laboratory have demonstrated that placental mesenchymal stem cells isolated from the chorionic villi express markers common to mesenchymal stem cells including CD105 (a stromal and vascular marker), CD73, and CD90 (fibroblast markers), and lack the hematopoietic markers CD34, CD45 and CD14 (42, 43). In addition, PMSCs exhibit ESC surface markers SSEA-4, TRA-1-61, and TRA-1-80 (44). Adipogenic, osteogenic, myogenic and neurogenic differentiation is achieved after culturing PMSCs under the appropriate culture or niche conditions (42, 44, 45). All of the above indicate that stem cells isolated from the placenta achieve MSC criteria. Therefore, PMSCs could provide an ethical and readily available source of multipotent stem cells for future experimental and clinical applications (46).

In this study, cells previously obtained from preterm placental tissue (13-weeks) and representative of late first trimester pregnancy, and characterized extensively for mesenchymal stem cell markers and differentiation ability in the Han laboratory, were used.

1.2 Stem Cell Microenvironment

The stem cell microenvironment or "niche" hypothesis was developed in 1978 by Schofield, who proposed that stem cells reside within fixed niches that maintain stem cell properties (47). The stem cell microenvironment is an anatomical structure, including cellular and non-cellular components which integrate local and systemic factors to regulate stem cell proliferation, differentiation, survival and localization (48).

Proliferation and differentiation of stem cells are maintained by the surrounding microenvironment, sustaining a healthy proliferative state via several clues including physical, structural, neural, paracrine, autocrine, and metabolic interactions (49). A combination of different micro-environmental signals would regulate the state of stem cells to either be proliferative or quiescencent depending on the active process of tissue regeneration (48).

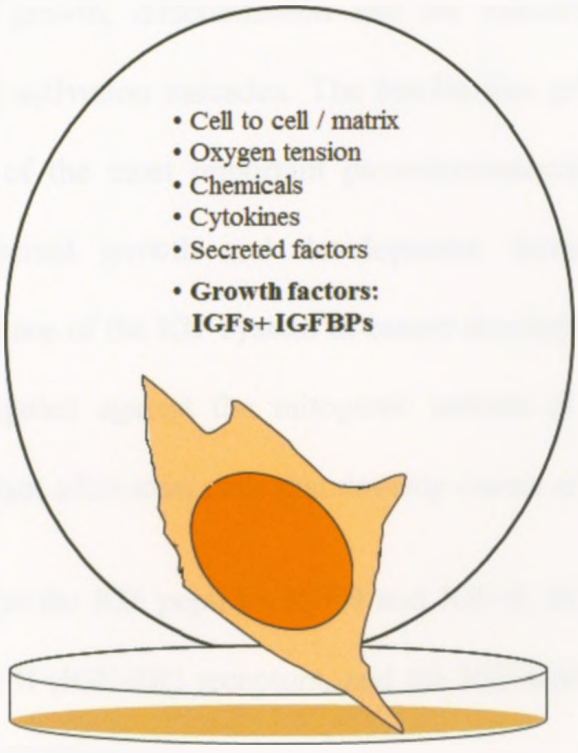
1.2.1 Niche Components

The components present in the microenvironment, such as growth factors, cytokines, extracellular matrices, chemokines and oxygen levels, are critical for limiting or facilitating MSC survival and propagation to self-like cells (symmetric division) or to more specialized committed cells (asymmetric division) (47). Therefore, regulation of such factors would change the proliferative state of stem cells. Moreover, stem cells grown in culture are also affected by cell plating density, passage number, and surface quality.

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).

Figure 1: Schematic of the stem cell microenvironment.

Cells grown in culture are influenced by many factors from the microenvironment; these components present in the microenvironment, such as growth factors, cytokines, extracellular matrices, chemokines and oxygen levels, are critical for limiting or enabling the MSCs proliferation and differentiation.


• Cell to cell / matrix
• Oxygen tension
• Chemicals
• Cytokines
• Secreted factors
• Growth factors:
IGFs+IGFBPs

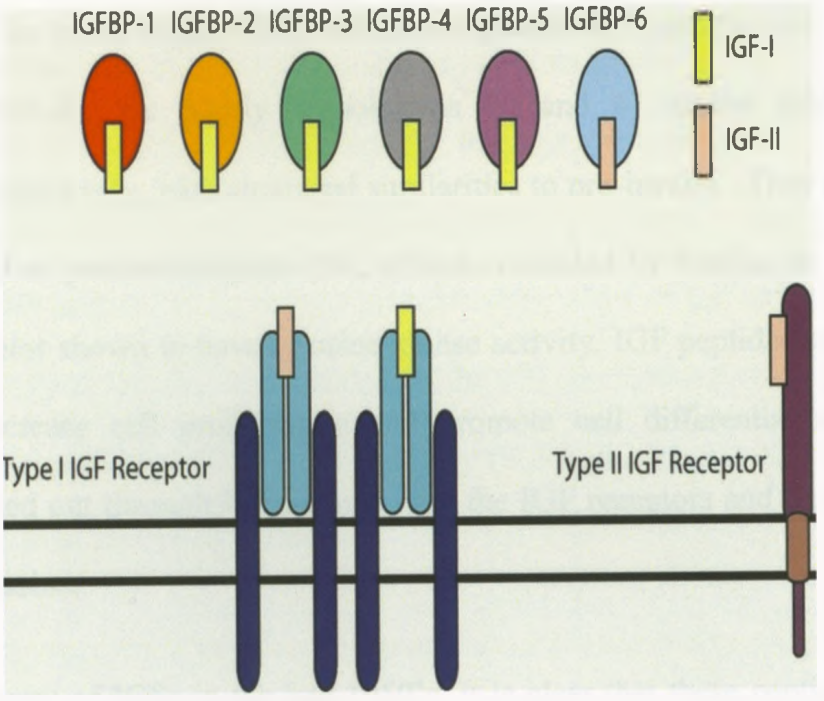
1.3 Insulin-like Growth Factor (IGF) System

The insulin-like growth factor (IGF) system is a complex super-family that has been shown to regulate cell growth, differentiation and the maintenance of cell survival through several mitogen activation cascades. The insulin-like growth factor family has been shown to be one of the most important paracrine/autocrine growth factors that regulate fetal and placental growth and development. Several studies have also demonstrated the importance of the IGF system in cancer development (50, 51) and some cancer therapies are targeted against the mitogenic actions of this system (52, 53). Whether the therapies target adult stem cells that develop cancer cells is unknown.

This super-family includes the IGF peptides IGF-I and IGF-II, the cell surface receptors type I (IGF-IR) and type II (IGF-IIR) receptors, and the IGF binding proteins (IGFBPs) IGFBP-1 through IGFBP-6 (Figure 2).

Figure 2: Insulin-like growth factor superfamily.

The IGF superfamily consists of IGF peptides IGF-I and IGF-II; the cell surface receptors IGF-IR and IGF-IIR; and the IGF binding proteins (IGFBPs).



Cell growth,
differentiation, survival

1.3.1 Insulin-like Growth Factor-I and –II

Insulin-like growth factors are critical for fetal and placental development (34), with important roles in tissue repair (54), and tumorigenesis (55).Insulin-like growth factor (IGF-I) and (IGF-II), are highly homologous 70 and 67 amino acid single-chain polypeptides, respectively, with structural similarities to pro-insulin . They are circulating peptides that act as potent mitogens (56), effects mediated by binding to the IGF-IR, a membrane receptor shown to have tyrosine kinase activity. IGF peptides stimulate amino acid uptake, increase cell proliferation and promote cell differentiation (57). These actions are carried out through interactions with the IGF receptors and the actions of the IGF binding proteins.

Since the discovery of IGFs in the late 1950's, it is clear that these peptides can induce and mediate a variety of cellular responses including survival, growth, proliferation, and differentiation (55, 58, 59). It is also known that IGFs are produced locally within almost all tissues for autocrine and paracrine effects, but circulating IGFs are mainly produced by the liver for endocrine functions (60). Although IGF levels decrease with age, the IGF system continues to regulate cells throughout adulthood (61).

1.3.1.1 IGFs in Placental Development

IGF-I and –II are both present in the human placenta as early as 6 weeks gestation (62). While IGF-I is involved in enabling nutrient exchange in the placenta, IGF-II increases the total surface area for gas exchange between the mother and the fetus in the placenta (63, 64, 65). Inhibition studies for IGF-I and IGF-II demonstrated that both peptides are important for placental development as they enhance placenta derived fibroblast proliferation and survival (66, 67). IGFs and the IGF system are vital for placental

development as they regulate the function and growth of placental cells, and therefore are important for regulating PMSC fate.

1.3.2 Insulin-like Growth Factor Type-I and –II Receptors

The biological actions of the IGFs are mediated through two types of IGF receptors (67). IGF-IR is a member of receptor tyrosine-kinases (RTKs) family of cell surface receptors and was originally isolated from human placenta (68). Most of the mitogenic actions of the insulin-like growth factors are mediated by the IGF-IR, which can bind IGF-I, IGF-II and insulin, but binds IGF-I with highest affinity (2-3 fold higher than IGF-II, and 100-1000 fold higher than insulin) (55, 60).

IGF-IR is a trans-membrane tetramer receptor that exists as dimers composed of two α- and two β-subunits linked by disulfide bonds. The extracellular α-subunits bind the ligand, whereas the two catalytic β-subunits possess tyrosine kinase activity (67). Upon activation of the extracellular α subunits of the IGF-IR by ligand binding, activation and deactivation of intracellular adaptor proteins (IRS-1), SHC (Src homology 2 domain-containing transforming protein C1), GRB2 (Growth factor receptor-bound protein 2), p85 (regulatory subunit for PI3K), and Src kinase (tyrosine kinase) proteins will take place (67, 68). IGF-IR initiates the activation of two main signal transduction pathways through IRS-1 and SHC proteins, which are: a) phosphatidylinositol 3-kinase (PI3K) that is activated by IRS-1 that activates p85 which targets AKT, a serine/threonine kinase, by phosphorylation at specific sites, to promote cell survival and proliferation (69, 70), and b) mitogen-activated protein kinases (MAPK) which are activated by phosphorylated SHC which activates GRB2 complexes to activate Ras GTP-binding protein that leads to

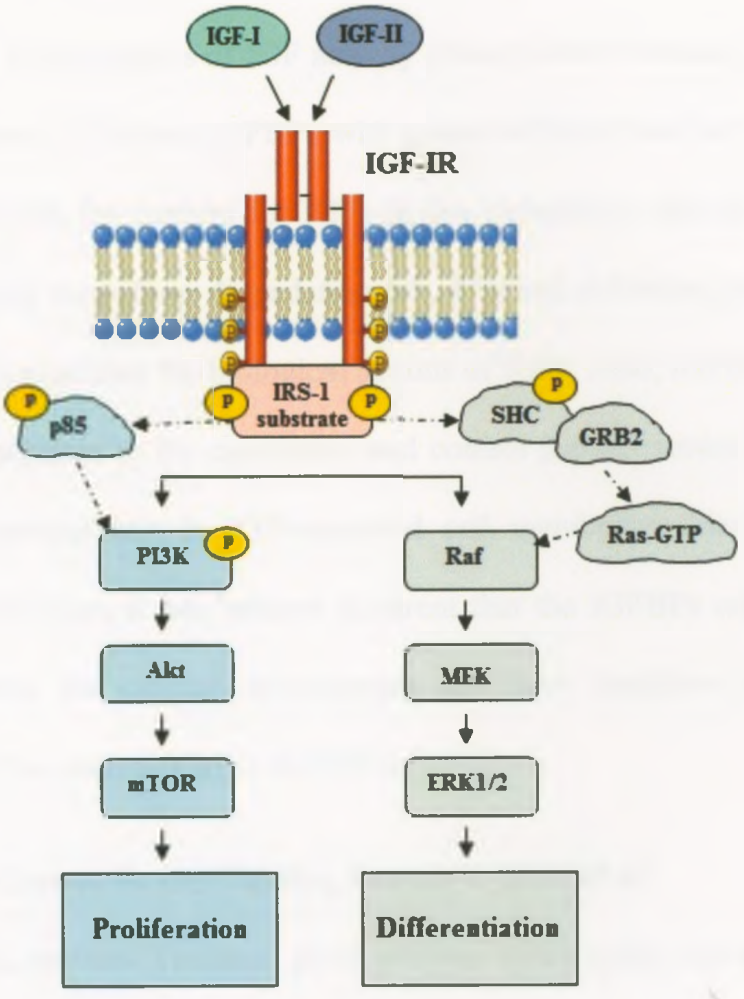
the recruitment of the extracellular signal-regulated kinase 1 and 2 (Erk1/2) resulting in differentiation (58, 69, 71) (Figure 3).

Therefore, a crosstalk between different RTKs and their ligands can lead to different responses and outcomes. Moreover, the availability of target effectors and the period of their activation are of high importance in determining cell fate decisions for committing either to proliferation or differentiation (72). It was reported by Marshall et al., that a prolonged activation of Erk1/2, which allows the translocation of Erk1/2 into the nucleus, leads to differentiation; whereas a transient activation of Erk1/2 leads to proliferation and is not sufficient to elevate the levels of nuclear Erk1/2 (73). Therefore, the type and the availability of growth factors in the microenvironment plays an important role in determining stem cell fate.

On the other hand, the IGF-IIR or cation-dependent mannose 6-phosphate (Man-6-P) receptor is a monomeric transmembrane receptor that is not mitogenic. The IGF-IIR does not bind insulin, but can bind man-6-P, IGF-II, and IGF-I, with a 500-fold higher affinity for IGF-II than IGF-I (74); and it does not have tyrosine kinase activity and does not transduce a signaling cascade but it can control IGF-II availability (75, 76).

Figure 3: Schematic of the IGF-IR signaling pathway.

Insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II) binding to the insulin-like growth factor receptor (IGF-IR) activate its intrinsic tyrosine kinase activity resulting in signaling through cellular pathways that stimulates proliferation and/or differentiation. The key downstream signaling pathways include PI3K-Akt-mTOR and the Raf-MEK-ERK (MAPK) pathway.


IGF-I
IGF-II
IGF-IR
IRS-1
substrate
p85
SHC
GRB2
PI3K
Raf
Ras-GTP
Akt
MEK
mTOR
ERK1/2
Proliferation
Differentiation

1.3.3 Insulin-like Growth Factor Binding Proteins

Six IGF specific binding proteins, IGFBP-1 through -6, are soluble ~30 kDa binding proteins which serve as modulators of IGF activity through direct binding. Under normal physiological conditions IGFs bind IGFBPs with greater affinity than they bind IGF-IRs (77, 78, 79). IGFBPs are the carriers for IGFs in the circulation, through non-covalent binding (80), protecting them from degradation (81, 82), and delivering them to specific tissues. Thus, IGFBPs modulate the biological actions of IGFs. Also, IGFBPs increase the half-life of the IGF peptides in the circulation and control peptide access to the IGF-IR, thus playing an important role in IGF-regulated cell metabolism, development, and growth (72). In recent years, it has become apparent that the IGFBPs can be expressed and maintained within the cellular environment and have functions independent of regulating IGF (83). One such IGFBP is IGFBP-6.

1.3.4 Insulin-like Growth Factor Binding Protein-6 (IGFBP-6)

IGFBP-6 is a 30 kDa, secreted, O-linked glyco protein, which unlike other IGFBPs has a significantly higher affinity (100 fold), for IGF-II than IGF-I (83, 84). 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-II where IGFBP-6 binds overlaps with that of the IGF-IR binding site, giving a structural basis for IGFBP-6's ability to inhibit IGF-II binding to the IGF-IR (85). 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 (86). This difference appears to be a contributing factor to the unique preference of IGFBP-6 for IGF-II (86).

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-II may prolong degradation rates (87). The balance between proteolysis and glycosylation of IGFBP-6 helps maintain stability in IGF-II binding and thus IGF-II function modulation. This equilibrium is due to the fact that non-glycosylated IGFBP-6 has a threefold increase in binding affinity for IGF-II, but proteolysis also decreases the ability of IGFBP-6 to bind IGF-II significantly (88). Therefore, the decrease in proteolysis of IGFBP-6 because of glycosylation leads to higher IGF-II binding rates (88).

IGFBP-1, IGFBP-3 and IGFBP-5 human proteins have been shown to be phosphorylated (89, 90); however, studies in CHO cells on human IGFBP-6 indicated that it is not phosphorylated (91). Moreover, there is only one IGFBP-6 gene identified in humans, while two IGFBP-6 genes have been identified in zebrafish (92).

The most commonly noted function of IGFBP-6 is the modulation of IGF-II activity. Like other binding proteins, it can bind IGF-II in the circulation and protect it, as well as itself, from proteolysis and thereby enhance IGF-II's action by increasing its half-life. IGFBP-6 can bind IGF-II at the cell surface and enhance the action of this peptide by sequestering a pool of IGF-II to the area and delivering to the IGF-IR (93). Alternatively, IGFBP-6 can inhibit the action of IGF-II by binding to the peptide in the extracellular environment and

preventing it from interacting with the IGF-IR; it is still not known how IGFBP-6 chooses which function to perform. IGFBP-6 has been shown to have an effect on cell function through its ability to sequester IGF peptides and prevent them from interacting with the IGF-IR receptor in several different cell types. Moreover, IGFBP-6 expression is also associated with non-proliferative states and inhibition of IGF-II-dependent tumor cell growth, such as rhabdomyosarcoma, neuroblastoma, and colon cancer (94). Neuroblastoma cells undergo a decrease both in cell proliferation and tumorigenic potency as a result of exogenous IGFBP-6 expression, as IGFBP-6 reduces the ability of IGF-II to stimulate a mitogenic response in the cells (95, 96). In osteoblast cells, IGFBP-6 has also been shown to bind IGF-II and modulate its effects on cell growth by reducing the bioavailability of IGF-II for its receptor in the bone microenvironment (97). In human bronchial epithelial cells, IGFBP-6 over-expression induces a proliferative arrest, with no evidence of apoptosis, suggesting an inhibition of IGF-II's ability to induce mitosis (98). Taken together, these examples show a strong role for IGFBP-6 in the modulation of IGF peptide activity.

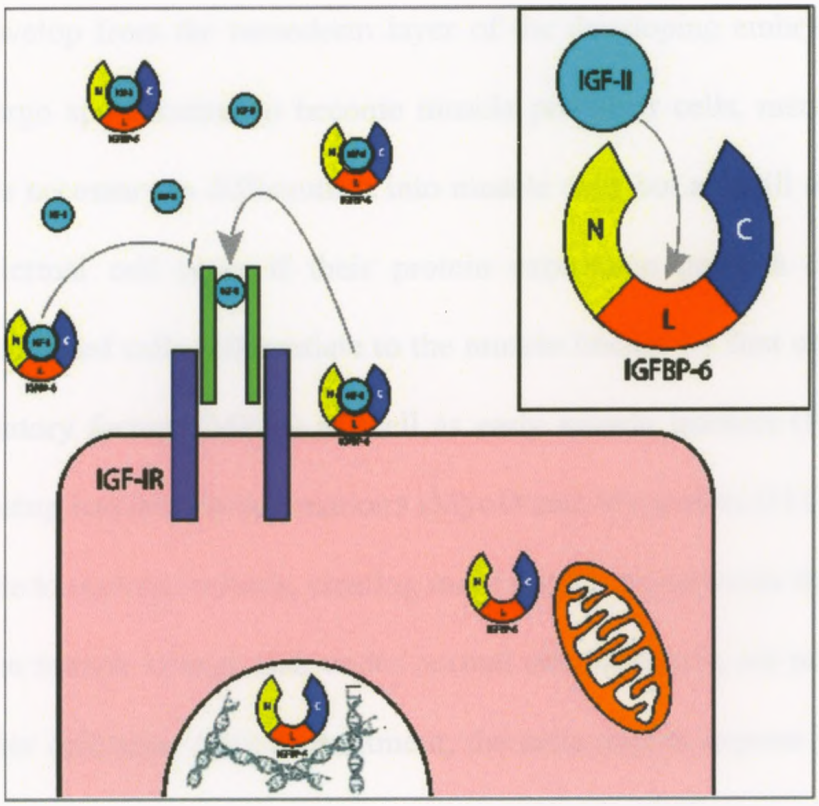
This ability to modulate IGF activity through binding the peptides and changing their interactions with the IGF receptors were long thought to be the only function of the binding proteins. However, recent evidence suggests that the IGF binding proteins, including IGFBP-6, also act independently of the IGF peptides to affect cellular functions. Binding proteins have been found to not only be secreted to the extracellular environment where they interact with IGF peptide, but also localize to the intracellular environment of the cell. Cytoplasmic and nuclear IGFBP-6 has been shown by Iosef et.al. (99, 100), (Figure 4); and cytoplasmic IGFBP-6 has been imaged in Malassez epithelial

cells and fibroblasts by Gotz et.al. (101). Due to the fact that the IGF peptides are rarely found in the intracellular environment, this suggests that the binding proteins may have a function independent of IGF-I and -II. Most commonly, the over-expression or ectopic expression of any of the IGF binding proteins has led to the induction of apoptosis (96, 102-107). Previous studies in our laboratory linked IGFBP-6 to Ku proteins in regulating cell fate (100). Moreover, the expression of IGFBP-6 is associated with non-proliferative states such as cell differentiation (108). Also, IGFBP-6 over-expression in cells has either a growth-promoting or a growth-inhibitory effect, depending on the cell type (109, 110).

To date, no previous studies have been performed on IGFBP-6 and PMSCs. Moreover, the conflicting data about IGFBP-6 in other cell lines raises many questions about how this binding protein will function in PMSCs and how it affects the fate of these cells. Therefore, in order to answer these questions and many more to come, we must first have a general understanding about IGFBP-6's role in PMSCs which is the focus of this study.

Figure 4: Schematic of IGF-dependent and -independent functions of insulin-like growth factor binding protein-6.

IGFBP-6 has three IGF dependent functions: a) to bind IGF-II and protect it from degradation in the extracellular environment, thereby increasing its half-life, b) to bind IGF-II and the extracellular matrix and help deliver it to the receptor, thereby enhancing its function, and c) to bind IGF-II and prevent it from accessing the receptor, thereby inhibiting its function. Recent studies have shown that IGFBP-6 is present in the intracellular environment, as well as in the nucleus, suggesting the IGFBP-6 has IGF independent functions, due to the fact that the IGF peptides are not found within the cell (99, 100, 101).


IGF-II
N
C
L
IGFBP-6
IGF-IR

these proteins indicates which stage the developing cell has passed. Before specification, mesoderm cells express the mesoderm identifying protein Brachyury (T). Once the cells become muscle precursors, after specification, the cells begin to express ß-catenin, Pax3/7, Mox1, Gli2 and Six1 (112, 113). The muscle precursor cells then undergo commitment to become myoblast cells. Myoblasts express the myogenic regulatory factors and the MEF2 proteins. Myoblasts are the last cell type in the differentiation process that is able to divide. Once they undergo terminal differentiation the cells become myocytes. Once myocytes are formed, the cells are no longer able to divide and start expressing muscle specific proteins such as the myosin heavy and light chains. Myf-5 is the first myogenic regulatory factor to be expressed in the developing embryo and continues to be expressed through the development of muscle (114, 115). Shortly after Myf-5 expression is detected, the expression of Myogenin is observed throughout the myotome (116, 117), followed by MRF4 and MyoD respectively (118, 119). The MRFs are able to trans-activate each other, and are able to positively regulate their own expression (120, 121, 122, 123).

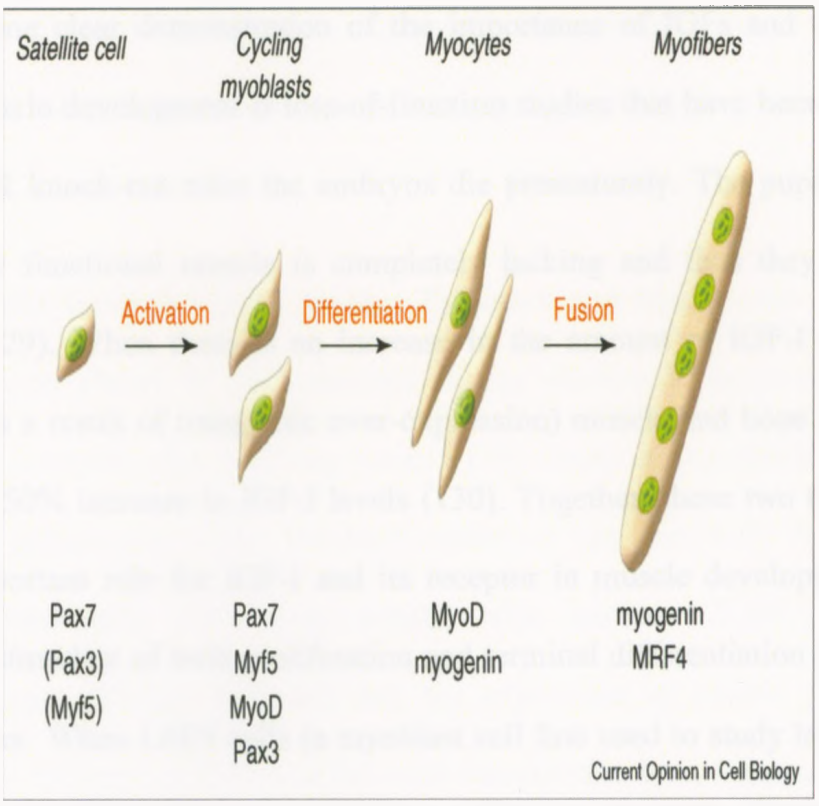
The myogenic regulatory factors each serve distinct but overlapping functions in the development of muscle. The use of knock-out experiments has helped elucidate the function of the MRF proteins. Mice develop skeletal muscle normally when either Myf-5 or MyoD are lacking. The loss of myf-5 results in an up-regulation of MyoD, or conversely the loss of MyoD results in an up-regulation of Myf-5 (124, 125). 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 (126). 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 it is lacking in mice, the mutations are also perinatal lethal, and the mice die shortly after birth due to severe muscle deficiency and the resulting respiratory failure (127).

Figure 5: Overview of muscle differentiation.

Myogenesis can be divided into three distinct stages. After completing each of these stages the differentiating cell begins to express specific proteins which can be used to distinguish what point in the differentiation process the cells are at. Quiescent skeletal muscle satellite cell can become activated and start to proliferate into skeletal myoblasts that express the paired-box transcriptions factors Pax7 and Pax3, as well as the myogenic regulatory factors Myf5 and MyoD. Once committed to differentiation, myoblasts stop cycling and lose expression of Pax7, Pax3, and Myf5. Differentiating Myogenin positive myocytes will then align and fuse to form multinucleated myofibers. The MRFs are considered to be the master regulatory proteins of muscle development. Once the MRFs (myogenic regulatory factors) are expressed, cells are destined to become muscle cells and can no longer differentiate into other cells types.

(http://www.sciencedirect.com/science/article/pii/S0955067407001342)


Satellite cell
Cycling myoblasts
Myocytes
Myofibers
Activation
Differentiation
Fusion
Pax7
(Pax3)
(Myf5)
Pax7
Myf5
MyoD
Pax3
MyoD
myogenin
myogenin
MRF4
Current Opinion in Cell Biology

1.4.2 Insulin-like Growth Factors and Myogenesis

The insulin-like growth factor super-family has been shown to have a role in muscle development. One clear demonstration of the importance of IGFs and the type I IGF receptor for muscle development is loss-of-function studies that have been performed. In IGF-I or IGF-IR knock-out mice the embryos die prematurely. The pups are unable to survive because functional muscle is completely lacking and thus they are unable to breathe (128, 129). When there is an increase in the amount of IGF-I present during development (as a result of transgenic over-expression) muscle and bone were increased by 30% with a 50% increase in IGF-I levels (130). Together, these two types of studies point to an important role for IGF-I and its receptor in muscle development. The IGF peptides act to stimulate of both proliferation and terminal differentiation in a temporally separated manner. When L6E9 cells (a myoblast cell line used to study late myogenesis) are stimulated with IGF-I there is an initial proliferative response. During this time of rapid cell division the myogenic regulatory factors are inhibited. Approximately 30 hours later there is a stimulation of Myogenin expression and activity and the mitogenic factors are suppressed (131). These studies suggest an important role for IGF peptides and the type I receptor in the stimulation and regulation of myogenesis. However, the downstream factors involved in IGF stimulated differentiation still remain elusive.

Although the above factors have been shown to affect Myogenin expression and myogenesis in cell culture experiments, it is not known whether the same factors link IGF, Myogenin and myogenesis in the developing embryo in a similar manner. In addition to the well-studied effects of IGF-I and II on Myogenin expression, IGF-II has been shown to play a role in the regulation of MyoD function. When MyoD is expressed

in C3H 10T1/2 mouse embryonic fibroblast cells, there is an induction of IGF-II expression and subsequent activation of the IGF-IR and its downstream target Akt (132). When activation of either IGF-II target is inhibited, myogenesis does not occur (133). In fact, the IGF-II is required to allow continued recruitment of MyoD-associated proteins at the Myogenin promoter (134).

1.4.3 Insulin-like Growth Factor Binding Protein-6 and Myogenesis

IGFBP-6 has been shown to be expressed during embryonic development in many different tissues including the ossifying bones of the cranium, myoblasts and the motor neurons of the spinal cord (134). Previous studies in our laboratory have shown that rhabdomyosarcoma cells (RD cells) treated with mutant IGFBP-6 (non-nuclear) underwent terminal differentiation and that the expression of muscle specific myosin heavy chain was achieved in 0.05% of the cells compared to 0% of the untransfected RD cells or wild-type IGFBP-6 expressing cells. This percentage is significant due to the fact that untransfected RD cells, RD cells transfected with the empty vector or RD cells expressing the wildtype IGFBP-6 never underwent myogenesis.

In this study, we propose to culture skeletal muscle from placental mesenchymal stem cells and then test the effects of IGFBP-6 early before these cells commit to the muscle lineage (Figure 6).

Figure 6: Proposed model.

Growing muscle cells form placental mesenchymal stem cells and testing the effects of early insulin-like growth factor binding protein-6 on myogenesis before the cells commit to the muscle lineage.

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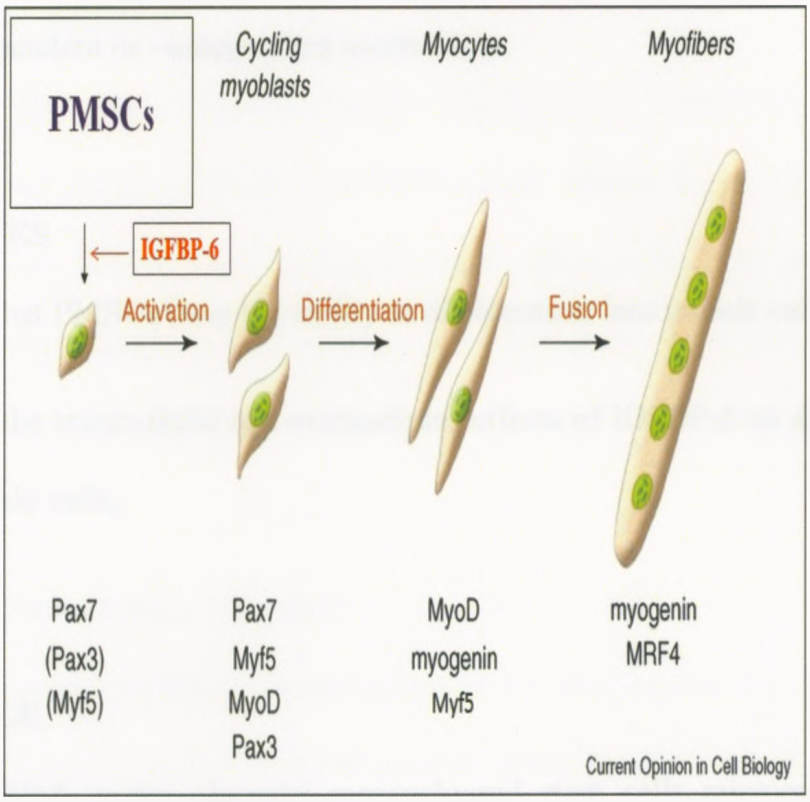

PMSCs
Cycling myoblasts
Myocytes
Myofibers
IGFBP-6
Activation
Differentiation
Fusion
Pax7
(Pax3)
(Myf5)
Pax7
Myf5
MyoD
Pax3
MyoD
myogenin
Myf5
myogenin
MRF4
Current Opinion in Cell Biology

2.0 HYPOTHESIS

IGFBP-6 inhibits the maintenance of pluripotency of placental mesenchymal stem cells and promotes their differentiation into muscle via intracellular or extracellular routes through IGF-dependent or –independent mechanism.

2.1 OBJECTIVES

(1) To confirm that PMSCs have the ability to differentiate into muscle cells.

(2) To evaluate the intracellular and extracellular effects of IGFBP-6 on differentiation of developing muscle cells.

2.2 RATIONALE

Increasing IGFBP-6 in the placental mesenchymal stem cells microenvironment was expected to deplete the system of IGF-II and therefore prevent it from interacting with the IGF receptor and negatively affect differentiation. Conversely, silencing of IGFBP-6 expression was expected to increase the differentiation of these cells, as a result of a greater availability of IGF-II (93, 97). In addition, IGFBP-6 is known to be present in the cytoplasm as well as in the nuclei (99, 100) suggesting that IGFBP-6 may have IGF-II independent function.

3.0 MATERIALS and METHODS

3.1 Reagents

3.1.1 Cell Culture Reagents

Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12), Dulbecco's Phosphate Buffered Saline (DPBS), ES-Quality Fetal Bovine Serum (FBS), 10x 0.5% Trypsin-EDTA and FGF-2 were purchased from Gibco-Invitrogen (Burlington, ON, CAN). Tissue culture flasks, 6 well plates, and 24 well plates were purchased from BD Falcon (Mississauga, ON, CAN), and filters were purchased from Nalgene (Rochester, NY, USA).

3.1.2 Muscle Differentiation Reagents

Skeletal muscle growth media (fetal calf serum 0.05 ml/ml, bovine 50 μg/ml, epidermal growth factor 10 ng/ml, basic fibroblast growth factor 1 ng/ml, insulin 10μg/ml, and dexamethasone 0.4 μg/ml); and skeletal muscle differentiation media which is serum-free medium containing only 10μg/ml insulin, were both purchased from Promocell (Heidelberg, Germany).

3.1.3 Insulin-Like Growth Factor Binding Protein-6

Recombinant Human IGFBP-6 was purchased from PROSPEC (Rehovot, Israel). The recombinant protein was resuspended in sterile MQ-H_2O and stored at -20°C. For use in the experiments, the vial was thawed and added to DMEM/F12 at a concentration of 750 ng/well.

3.2 Placental Mesenchymal Stem Cell Culture in Muscle Differentiation Conditions

Cells previously obtained from preterm placental tissue (13-weeks) were cultured and maintained using DMEM/F12 media supplemented with 10% ES-FBS serum and FGF-2 (25 ng/mL) (GIBCO/Invitrogen) in tissue culture flasks (BD Falcon). These cells have been characterized extensively and compared to several cell lines obtained from late first trimester placentae, and they are similar to others (36, 37, 43, 44). This cell line (the chorionic villi derived MSCs) is chosen as a representative first trimester PMSC to be studied. When cell cultures reached 90-100% confluency, the cells were passaged 1:2 approximately once per week using 0.05% Trypsin (GIBCO/Invitrogen) for 5-10 min at 37°C for dissociating cells and the medium was changed as needed approximately every two days. For treating cells under various conditions, 70-80% confluent cells in 6-well plates (BD Falcon) were grown in the presence of the muscle growth media (Promocell) for 24 hours before changing to the skeletal muscle differentiation media for muscle differentiation conditions. Finally, for IGFBP-6 treatment, cells were incubated with 0.75 µg/well of recombinant human IGFBP-6 protein (Prospec Protein Specialists) or silenced with IGFBP-6 siRNA (Santa Cruz, CA, USA). The cells were grown in a standard tissue culture incubator at 37°C in 5% CO_2.

3.2.1 Placental Mesenchymal Stem Cell Freezing and Thawing

1 mL of freezing media (30% FBS and 10% DMSO in DMEM/F12 media) was used to resuspend the pelleted PMSCs and the cells were frozen at -20°C for 30 minutes before transferring the cells to -80°C. Vials were thawed at room temperature when needed. The cells were resuspended in normal culture media (25 ng/ml FGF-2 and 10% ES-FBS in

DMEM/F12 media) for seeding after they were pelleted by centrifugation at 1000 rpm for 5 minutes.

3.3 IGFBP-6 Protein Transport System (Chariot™)

The Chariot™system was purchased from Active Motif (Carlsbad, CA, USA). It is a delivery reagent that quickly and efficiently transports biologically active proteins, peptides and antibodies directly inside the cells. We used Chariot ™ to deliver the intracellular recombinant IGFBP-6 protein into PMSCs. Chariot ™ was prepared as stated by the company protocol. Following 24 hour of incubation in serum free medium, cells were washed with DPBS (1x) and a mixture of Chariot ™ and IGFBP-6 was added to cells in 6-well plates for 1 hour in 400 µL of serum free media (DMEM/F12) in a standard tissue culture incubator at 37°C in 5% CO_2. Afterwards, another 400 µL of DMEM/F12 media was added in each well for an additional 2 hours in a tissue culture incubator at 37°C in 5% CO_2 (Figure 7). Then the Chariot™ complex was washed from the cells with DPBS (1x) and the muscle growth media was added for non-differentiated conditions and muscle differentiation media for the differentiated conditions. Cells were lysed after 0, 12, 24, 48, 72 hours and 7 days following removal of the Chariot™ complex.

β-galactosidase staining was used in order to test efficiency of the Chariot™ delivery system. When β-galactosidase hydrolyses X-gal, it produces a blue color that can be visualized under a bright field microscope. The β-galactosidase was prepared as stated in the Chariot™ manual (Active Motif) and then added to the cells grown in a 6-well plate for 2 hours in a standard tissue culture incubator at 37°C in 5% CO_2. Finally, the cells were studied under a microscope (Figure 8).

Figure 7: Diagram of the Chariot™ Process.

A mixture of Chariot™ and IGFBP-6 was added to the cells after washing with DPBS (1x) in 6-well plates for 1 hour in 400 µL of serum free media (DMEM/F12) in a standard tissue culture incubator at 37°C in 5% CO_2; cells were serum starved for 24 hours prior to treatment. After that, another 400 µL of DMEM/F12 media was added in each well for an additional 2 hours in a tissue culture incubator at 37°C in 5% CO_2.

(www.activemotif.com)

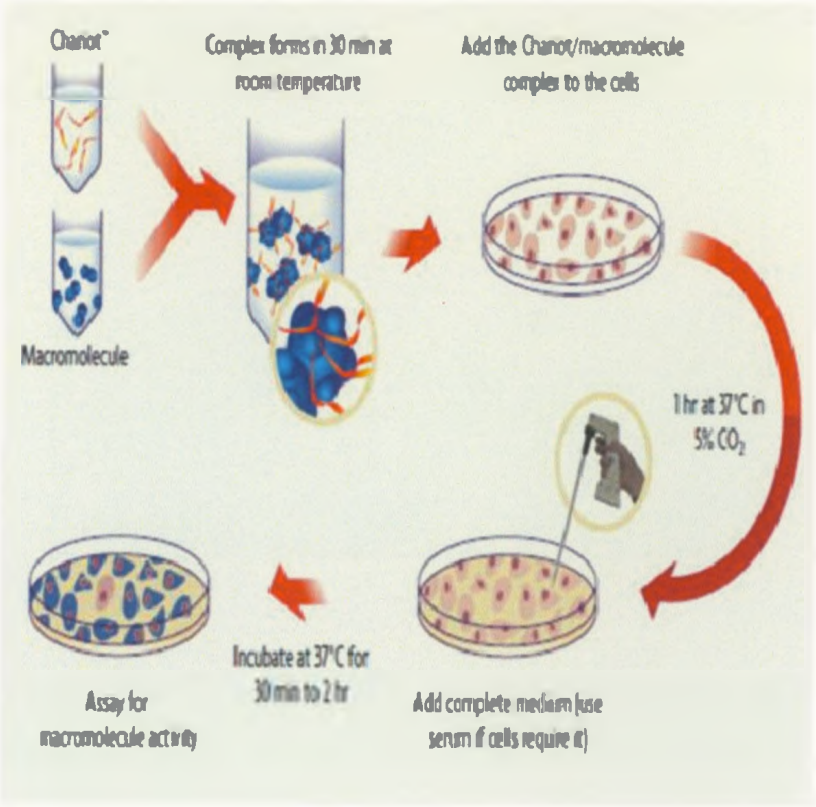
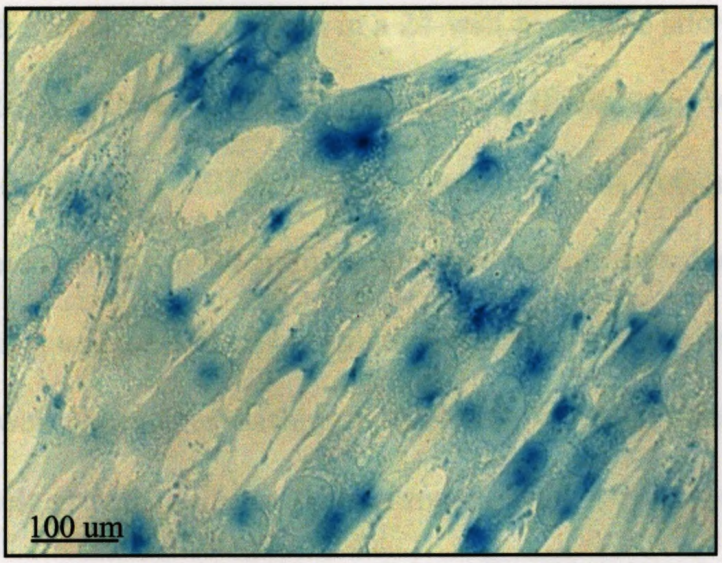

Chariot™
Macromolecule
Complex forms in 30 min at room temperature
Add the Chariot/macromolecule complex to the cells
1 hr at 37°C in 5% CO_2
Add complete medium (use serum if cells require it)
Incubate at 37°C for 30 min to 2 hr
Assay for macromolecule activity

Figure 8: Chariot™ system efficiency.

Chariot™ transport system introduced indicator protein inside the PMSCs using X-gal detection under bright field microscopy by β-galactosidase transfected into placental mesenchymal stem cells grown in muscle growth media; (~90% of cells were positively stained).


100 um

3.4 Down-regulation of IGFBP-6 Expression by siRNA

In order to silence the endogenous IGFBP-6, siRNA against IGFBP-6 (Santa Cruz, CA, USA) was used to transfect PMSCs grown in a 24-well plate. The siRNA has a pool of 3 target-specific 20-25 nt siRNAs. 8 μL of Lipofectamine (Invitrogen) with either 8 μL of scrambled siRNA (control) or siRNA-IGFBP-6 was added to 100 μL of DMEM/F12 media (transfection solution) for 40 minutes at room temperature; the concentration of siRNA in the solution was 80 nM. This solution was then added to the 80% confluent cells cultured in 6-well plates after washing with DPBS (2x) and incubated for 7 hours at 37°C; 1 mL of muscle growth media or skeletal muscle differentiation media (Promocell) was added to the cells depending on the condition after the removal of the transfection solution. The cells were then lysed after 24, 48, 72 hours, and 7 days.

3.5 Cell Lysate Preparation

After specific treatments and incubation time at 37°C, cells were washed with ice-cold DPBS while on ice. 200 μL of (1x) cell lysis buffer purchased (Cell Signaling Technologies.Danvers, MA) mixed with protease inhibitor (1:100) as stated in the company protocol, was added to the cells. Cells were detached using a cell scraper; the lysate was then sonicated for 15 seconds, and then placed on ice for 10 minutes. Finally, the samples were centrifuged for 15 min at 14,000 rpm at 4°C, and the supernatant was collected and stored at -80°C.

3.6 Protein Assay

The Bio-Rad protein assay was purchased from Bio-Rad (Mississauga, Ontario) and used to quantify the protein concentration of cell lysates and Bovine Serum Albumin (BSA) standards (concentrations of 750, 500, 250, 125, 25, 0 μg/mL) purchased from Thermo

Fisher Scientific (Rockford, IL); samples were either diluted to 1:10 or 1:5. Duplicate 10 µL aliquots of each sample and standard were pipetted into a 96-well plate (Thermo LabSystems, Milford MA, USA) containing 200 µL of diluted (1:4) Bio-Rad protein assay reagent in dH_2O. The plate was then placed in Thermo LabSystems Multiskan Ascent Plate Reader (Thermo LabSystems) to read protein concentration at absorbance of 595 nm. Standard curve and protein concentrations were analyzed using Multiskan Ascent analysis software (Thermo LabSystems).

3.7 Immunocytochemistry Procedure

Placental mesenchymal stem cells were grown in 6-well plates on glass slips and incubated with primary antibodies at 4°C overnight after blocking in 2% BSA for an hour. The primary antibodies (Table 1) were then washed away using PBS (3x for 10 min), cells were then incubated with the secondary antibody for an hour at room temperature while covered with aluminium foil. The secondary antibody (Table 3) was washed using PBS (3x for 10 min). Cells were rinsed with water for 5 minutes and the nuclear stain (Hoechst dye) was added for 7 minutes, and then rinsed out using water for 10 minutes. Finally, the slides were mounted with 70% glycerol: 30% PBS cover slipped and then stored over night at 4°C. The images were taken using either a Leica inverted microscope or an Olympus fluoview confocal microscope.

3.8 Immunoblotting Procedure

3.8.1 Sample Preparation

20µg of each protein sample was 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° and then placed on ice for 3 minutes. Finally, the samples were briefly centrifuged at 3000 rpm for 20 seconds before loading.

3.8.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis and Protein Transfer

Protein samples were resolved by molecular weight using 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) gels made of 0.375 M Tris HCl pH 8.8, 0.125 M Tris HCl pH 6.8, 30% acrylamide/bis solution (29:1), 10% ammonium persulfate, and 0.01% TEMED . Protein samples were loaded next to one well containing 12 µL of BenchMark Prestained Standard mixed with 2.5 µL of MagicMark XP (Invitrogen, Mississauga, ON). Samples were separated at U=80 V until the dye front passed through the stacking gel, and the voltage was raised to 120 V for the remainder of the electrophoresis. Gels were transferred onto poly-vinyl-dene-fluoride (PVDF) membranes (Millipore) in transfer buffer for 2 hours at 700 constant mA.

3.8.3 Antibody Probing

After transfer, the 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 1x TBS+ 0.1 % Tween 20 (3x for 5 min) followed by incubation at 4°C overnight on the shaker with specific primary antibodies in 5% BSA or 5% non-fat-dry milk in TBS-T following the manufacturer's protocol (See Table 1). Then membranes were washed with 1x TBS-T (3x for 10 min), blots were then incubated for an hour at room temperature with the corresponding secondary HRP antibody (See Table 2). The unbound antibody was washed with TBS-T (3x for10 min).

Table 1. Primary antibodies for western blotting and/or immunocytochemistry.

Name	Origin	Dilution and Diluent	Company and Catalogue Number
IGFBP-6	Rabbit polyclonal	1:1000 for western blots in 5% milk, TBS-T 1:50 for immunocytochemistry	Santa Cruz, Cat# sc- 13094
Pax3/7	Rabbit polyclonal	1:300 for western blots in 5% milk, TBS-T 1:50 for immunocytochemistry	Santa Cruz, Cat# sc- 25409
Oct3/4	Goat Polyclonal	1:300 for western blots in 5% BSA, TBS-T 1:100 for immunocytochemistry	Santa Cruz, Cat# sc- 8628
Pan Actin	Mouse monoclonal	1: 2000 for western blots in 5% milk, TBS-T	Neo markers, Cat# MS-1295-P
MyoD	Mouse monoclonal	1:100 for immunocytochemistry	Santa Cruz, Cat# sc- 32758
Myogenin	Mouse monoclonal	1:100 for immunocytochemistry	Santa Cruz, Cat# sc- 12732

Table 2. Secondary antibodies for western blots.

Name	Origin	Dilution	Company and Catalogue Number
Anti-goat IgG HRP	Donkey	1:15,000	Santa Cruz, Cat# sc-2020
Anti-rabbit IgG HRP	Goat	1:10,000	Bio-Rad, Cat # 170-6515
Anti-mouse IgG HRP	Goat	1:10,000	Bio-Rad, Cat # 170-6516

Table 3. Secondary antibodies for immunocytochemistry.

Name	Origin	Dilution	Company and Catalogue Number
Alexa Fluor® 568 goat anti-rabbit IgG	Goat	1:50	Invitrogen, Cat# 835724
Alexa Fluor® 488 donkey anti-goat IgG	Donkey	1:50	Invitrogen, Cat# A-11055
Alexa Fluor® 568 goat anti-mouse IgG	Goat	1:50	Invitrogen, Cat# A-11031

3.8.4 Chemiluminescence, Imaging, and Densitometry

Resolved protein bands were detected using chemiluminescence (ECL) by incubating the membranes for 2-4 minutes according to the manufacturer's instruction (Amersham). Images were taken using the VersaDoc Imager (Bio-Rad). Densitometry was performed using Image lab software (Bio-Rad). The band intensities were normalized to loading control (pan-Actin).

3.8.5 Stripping Western Blot Membranes

After detection, membranes were rinsed in distilled water and then were stripped using NaOH (0.5M solution) for 15 min on a platform shaker at room temperature. The membranes were washed with distilled H_2O for 10 minutes on the shaker. Membranes were re-blocked in TBS-T containing 5% non-fat dried milk in preparation for the next immunoblotting experiment.

3.9 Quantification of the IGFBP-6 production by Enzyme-Linked Immunosorbent Assay (ELISA)

Human IGFBP-6 ELISA kit (RayBio®) was used to measure the amount of IGFBP-6 secreted into the media of different treatment conditions. The media was collected at 0, 12, 24, 48, 72 hours and 7 days post-treatment. This is an assay that employs an antibody specific for human IGFBP-6 coated on a 96-well plate. Standards and samples were loaded into the wells and IGFBP-6 present in the sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human IGFBP-6 antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was added. After washing again, a TMB substrate solution was added and a blue color developed in proportion to the amount of IGFBP-6 bound. The

Stop Solution changes the color from blue to yellow, and the intensity of the color was measured at 450 nm (RayBio®). Results were analyzed using Multiskan Ascent analysis software (Thermo LabSystems).

3.10 Statistical Analysis

For data analysis GraphPad Prism 5 analysis software (La Jolla, CA) was used. Three independent experiments were performed; within each experiment, some replicates were performed (depending on the amount of cell lysate available). Comparisons between groups were analyzed by either One-way or Two-way ANOVA followed by a t-test at each time point and significant difference was considered when $p<0.05$. Graphic representation values are presented as mean ± (SEM shown as variance bars).

4.0 RESULTS

4.1 Placental Mesenchymal Stem Cell Differentiation into Skeletal Muscle

Placental mesenchymal stem cells (PMSCs) have the ability to differentiate into a diverse range of tissues, including muscle. Therefore, PMSCs cultured in the laboratory under specific conditions can differentiate into skeletal muscle cells (3).

To determine if PMSCs have the ability to differentiate into skeletal muscle cells, cells were grown in 6-well plates on glass slips in muscle differentiation media (serum free with 10μg/ml insulin), and stained for the early muscle marker Pax3/7 at day 3 post-differentiation, and for late muscle markers Myogenin and MyoD at 14 days post-differentiation. Fluorescent microscopy shows that placental mesenchymal stem cells do differentiate into muscle committed cells as they express Pax3/7 (Figure 9A) and show multi-nucleation (Figure 9B) at day 3. Moreover, at the protein level, Pax3/7 levels increased over time until they reached a two-fold increase at 72 hours (Figure 9C). PMSCs also express Myogenin (Figure 9D) and MyoD (Figure 9E) at day 14. Also at day 14, with phase contrast microscopy, PMSCs show the formation of multi-nucleated fibers (Figure 10).

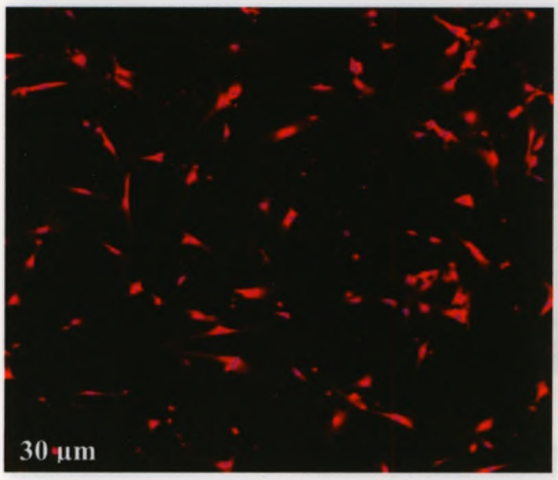
The expression of OCT4, 3days post-differentiation under muscle differentiation conditions (Figure 11A), had no significant change at the protein level over time (Figure 11B), which suggests that placental mesenchymal stem cell differentiation into muscle has no effect on OCT4 expression. On the other hand, IGFBP-6 expression was also observed in these cells (Figure 11C) with an increase in protein level over time (1-fold) at day 3 (Figure 11D), confirming that developing muscle cells express IGFBP-6.

Figure 9: Placental mesenchymal stem cells have the ability to differentiate into muscle.

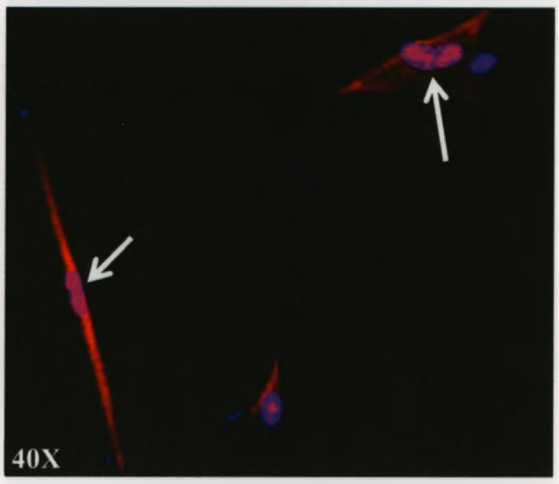
Using Olympus fluoview confocal microscope, PMSCS grown in muscle differentiation media (serum free with 10μg/ml insulin) are immunoreactive for, **(A)** early muscle marker Pax3/7 (red-Alexa 568, λ-568 nm) **(B)** showed multi-nucleation process [40X] with positive staining for Pax3/7 and **(C)** increased protein levels of Pax3/7 at 72 hours (2-folds) at days 3 post-differentiation. At 2 weeks post-differentiation, PMSCs are immunoreactive for **(D)** Myogenin (cytoplasmic localization), and **(E)** MyoD (intra-nuclear localization). Lower row shows magnification of the boxes in the upper row. Nuclei were stained with Hoechst dye (blue, λ=340 nm). The arrows show the multi-nucleation formation.

For western blot, the top panel shows a representative western blot of three independent experiments and the lower panel shows the quantification and statistical analysis of replicate experiments. Error bars represent the standard error of the mean (SEM). (One-way ANOVA followed by t-test, ***=P<0.001).

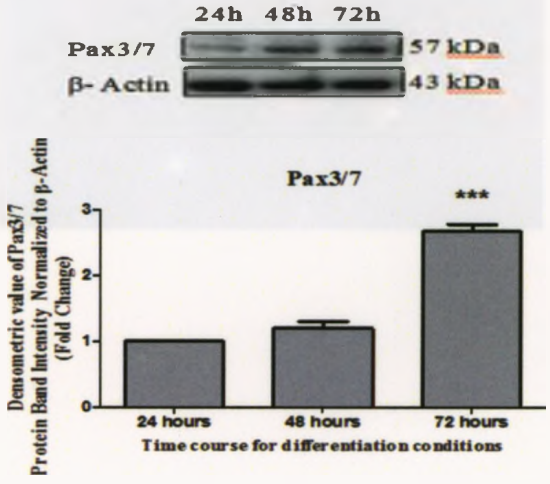
(A) Pax3/7



(B) Pax3/7



(C)



(D) Myogenin

(E) MyoD

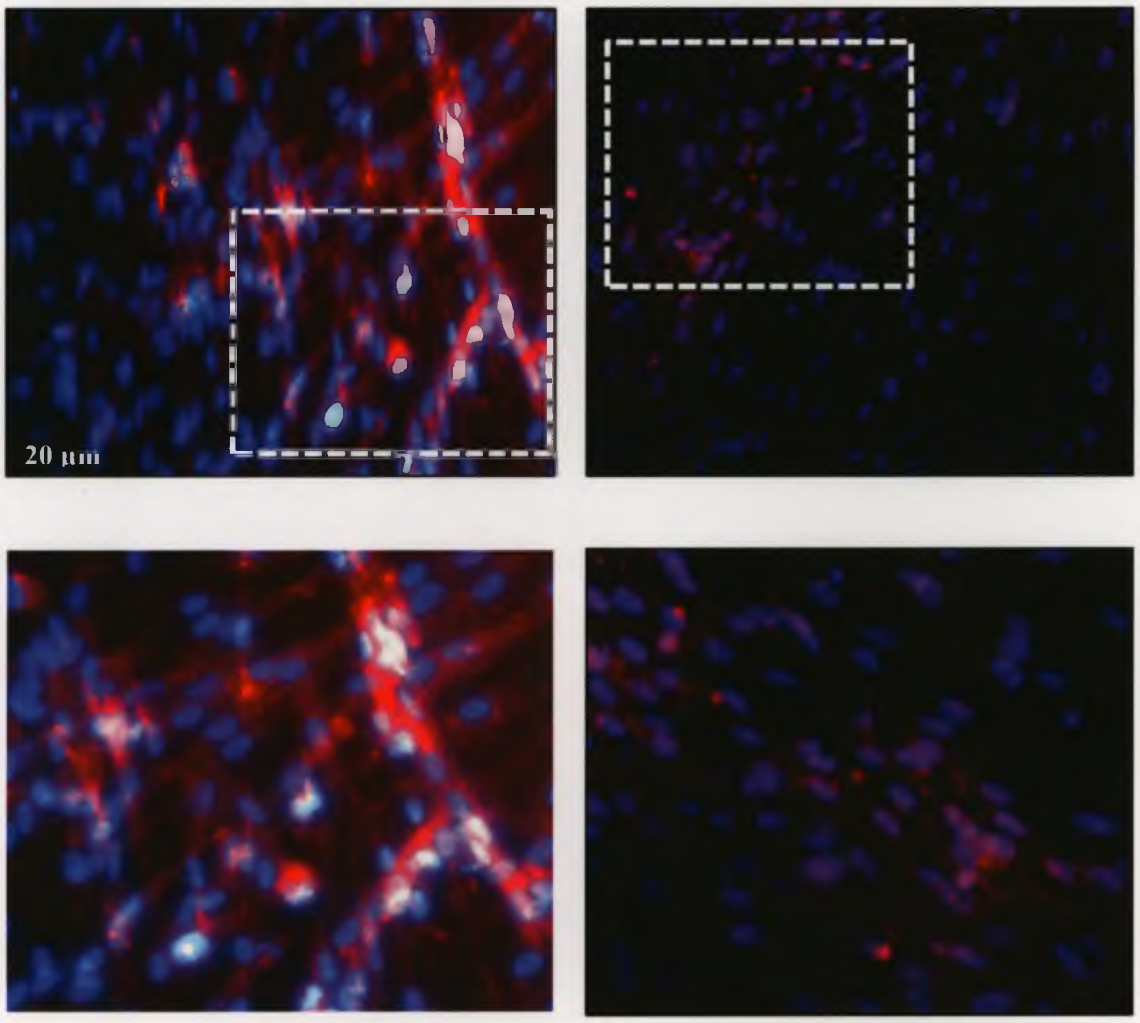
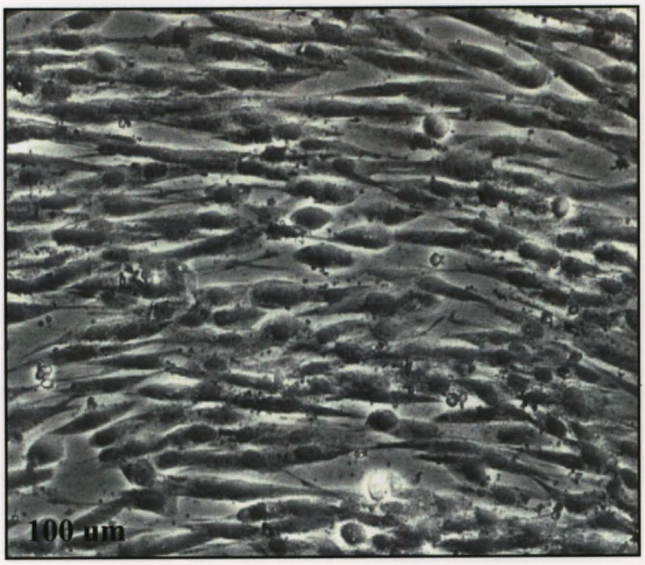


Figure 10: Placental mesenchymal stem cells show muscle-like morphology at 14-days post-differentiation.

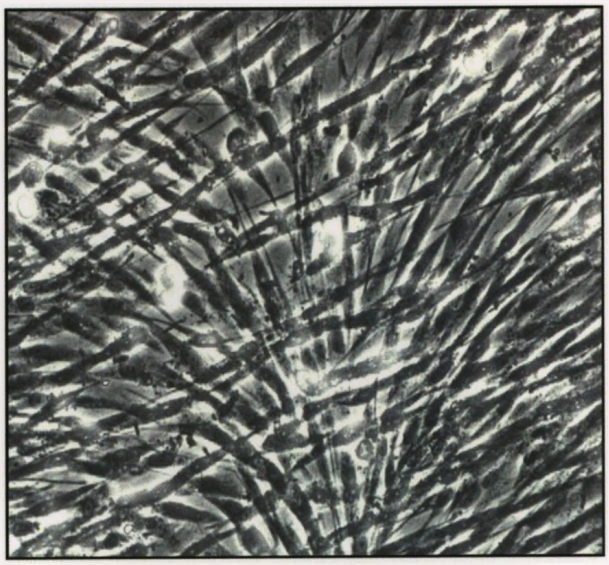
(A) PMSCs grown in normal culture media (25 ng/ml FGF-2 and 10% ES-FBS in DMEM/F12 media) did not show muscle formation compared with **(B)** PMSCs grown in muscle differentiation media (serum free with 10μg/ml insulin) showed skeletal muscle morphology at day 14. **(C)** Multi-nucleated skeletal muscle cells were derived from placental MSCs grown under muscle differentiation conditions (contrast phase imaging). Arrows show the multi-nucleated process.

(A)



Control

(B)



Skeletal muscle differentiation

(C)

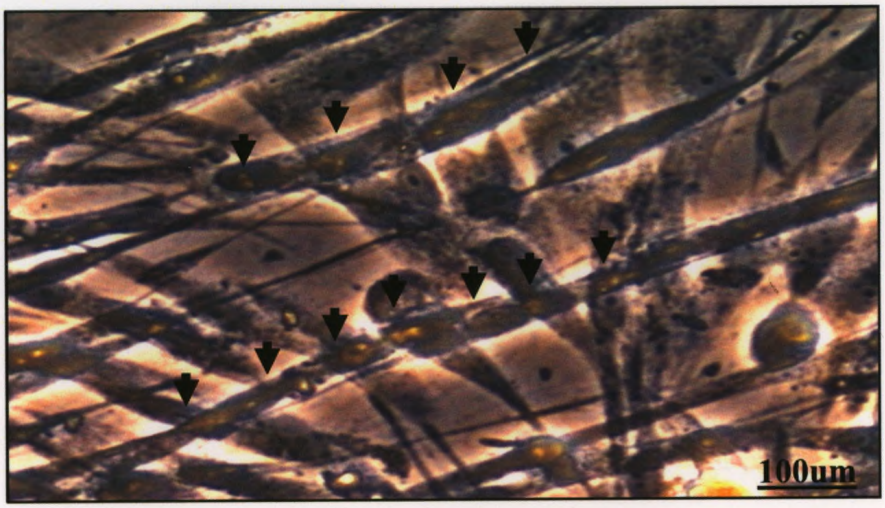
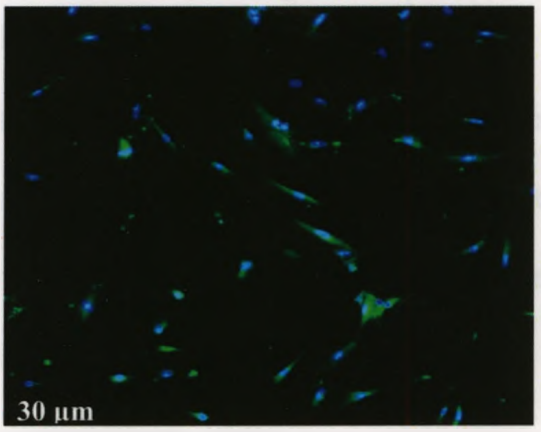


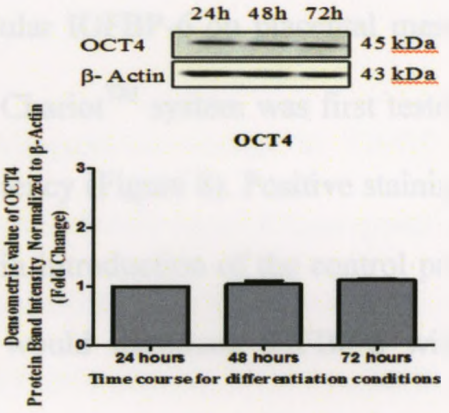
Figure 11: Placental mesenchymal stem cells grown in muscle differentiation media show increasing levels of IGFBP-6 by time with no change in OCT4.

Using Olympus fluoview confocal microscope, PMSCS grown in muscle differentiation media (serum free with 10μg/ml insulin) are immunoreactive for, **(A)** stem cell marker OCT4 (green-Alexa 488, λ=488 nm), with **(B)** no significant changes in OCT4 protein levels using Western blots. **(C)** IGFBP-6 (red-Alexa 568, λ-568 nm) was observed **(D)** with a one-fold increase in the protein level at day 3 in PMSCs under muscle differentiation conditions. By western blot, OCT4 and IGFBP-6 levels were quantified and normalized to β-Actin and then normalized to 24 hours using Versadoc software (Bio-Rad). The top panel shows a representative western blot of three independent experiments and the lower panel shows the quantification and statistical analysis of replicate experiments. Error bars represent the standard error of the mean (SEM). (One-way ANOVA followed by t-test, *= $P<0.05$). For confocal images, Nuclei were stained with Hoechst dye (blue, λ=340 nm) for confocal images.

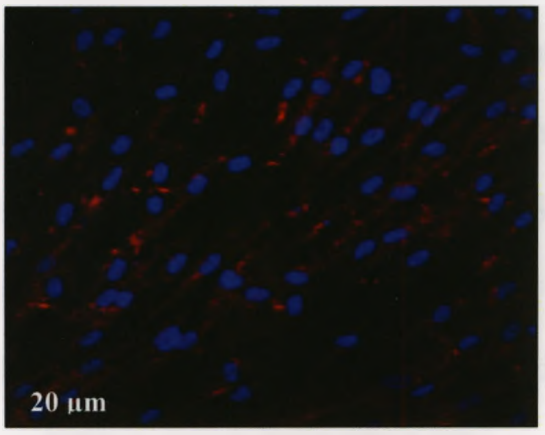
(A) OCT4



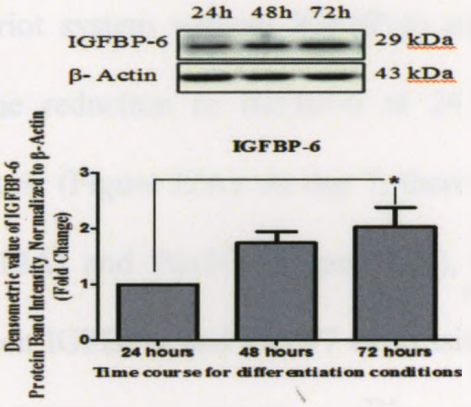
(B)



(C) IGFBP-6



(D)



4.2 IGFBP-6 Intracellular Actions on Developing Muscle Cells

To determine the effects of increased intracellular IGFBP-6 on placental mesenchymal stem cells grown in muscle growth media, the Chariot™ system was first tested using a staining kit in order to evaluate the system efficiency (Figure 8). Positive staining in 90% of cells indicates that the system was efficient in introduction of the control protein into the PMSCs. It is expected that the system would introduce IGFBP-6 with similar efficiency into PMSCs.

Under non-differentiation conditions, IGFBP-6 was elevated in PMSCs when IGFBP-6 was introduced using the Chariot™ system as shown with the significant increase at 12 hours compared to the Chariot™ control (Chariot system without IGFBP-6) and this over-expression was transient as seen with the reduction in IGFBP-6 at 24 hours compared to the Chariot™ (+)IGFBP-6 at 12 hours (Figure 12A). At day 7, there was a significant increase in both IGFBP-6 (Figure 12A) and Pax3/7 (Figure 12B), which indicates that there may be an association between IGFBP-6 and Pax3/7 expression that could have been set by the initial elevation of IGFBP-6 by the Chariot™ system. In contrast, there was no significant changes in OCT4 levels at any of the time points (Figure 12C), suggesting that OCT4 expression was not affected by the increase in IGFBP-6 under non-differentiation conditions.

Also, using ELISA, there was a significant increase in IGFBP-6 levels secreted into the media at both 24 and 48 hours with the intracellular increase of IGFBP-6 using Chariot™ with a significant reduction at day 7 (Figure 13),compared to both PMSCs alone and PMSCs with only Chariot™, indicating that adding intracellular IGFBP-6 increased IGFBP-6 secretion; and as the levels of IGFBP-6 increased, as seen using western blots,

at day 7 (Figure 12A) the amount secreted to the media is reduced at the same time point (Figure 13).

Under differentiation conditions, there was no significant change in IGFBP-6 (Figure 14A), OCT4 (Figure 14B), or Pax3/7 (Figure 14C) levels at each time point indicating that the changes caused by an increase in intracellular IGFBP-6 was more prominent under non-differentiation conditions before the PMSCs commit to the muscle lineage.

In contrast, by ELISA, there was a significant increase in the levels of IGFBP-6 secreted into the media with the intracellular increase (Figure 15), thus, increasing intracellular IGFBP-6 increased the amount of IGFBP-6 secreted into the media.

To evaluate the effects of IGFBP-6 silencing using siRNA on PMSCs grown in either muscle growth media or muscle differentiation media, a time course experiment was conducted to determine IGFBP-6, OCT4, and Pax3/7 levels 24, 48, 72 hours and 7 days after silencing.

Under non-differentiation conditions, there was a significant reduction in IGFBP-6 levels at 24 hours with IGFBP-6 silencing compared to the scrambled siRNA (used as control) (Figure 16A). This reduction was temporary and the IGFBP-6 levels returned to control levels by day 7 (Figure 16B). There was also a significant decrease in OCT4 levels at each time point with IGFBP-6 silencing (Figure 16C), which suggests that the IGFBP-6 silencing had an effect on OCT4. In contrast, there was a significant increase in Pax3/7 level at 24 hours with IGFBP-6 silencing (Figure 16D) that remained constant thereafter, suggesting that with IGFBP-6 silencing under non-differentiation conditions, PMSCs

responded by compensating for IGFBP-6 silencing by promoting and enhancing the differentiation process.

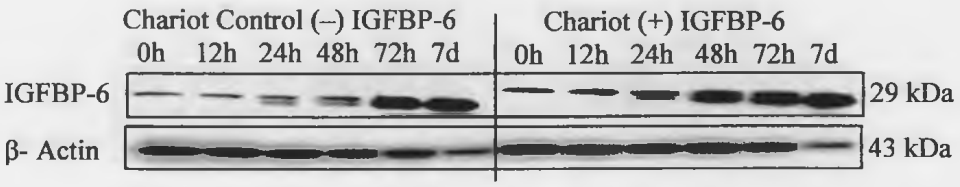
Under differentiation conditions, IGFBP-6 levels were restored at day 7 after IGFBP-6 silencing indicating that the siRNA effect was short term (Figure 17A). Interestingly, the cells that were treated by silencing IGFBP-6 had more significant increase in IGFBP-6 levels compared to the cells treated with the scrambled siRNA at day 7 (Figure 17A). OCT4 levels significantly decreased over time with IGFBP-6 silencing (Figure 17B). There was a significant increase in Pax3/7 levels at each time point compared to the scrambled siRNA but overall it seems that both treatments are going down with time, but the cells with the IGFBP-6 silencing had Pax3/7 decreased less (Figure 17C). Therefore, IGFBP-6 silencing may allow PMSCs to differentiate faster than the control cells.

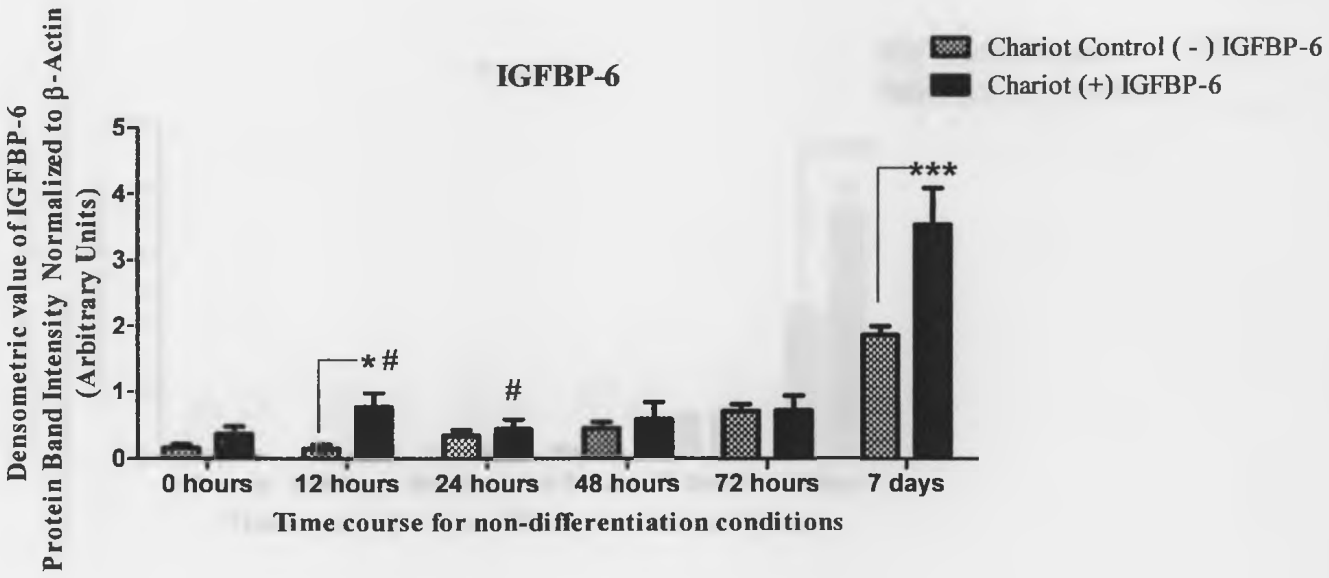
IGFBP-6 levels in the media as determined by ELISA showed that there was a significant decrease at each time point in IGFBP-6 levels secreted into the media with IGFBP-6 siRNA compared to the scrambled siRNA under both non-differentiation (Figure 18) and differentiation conditions (Figure 19), indicating that IGFBP-6 silencing using siRNA also decreased the amount of IGFBP-6 secreted to the media.

Figure 12: Time course experiment for increased intracellular IGFBP-6 by Chariot™ in PMSCs under non-differentiation conditions.

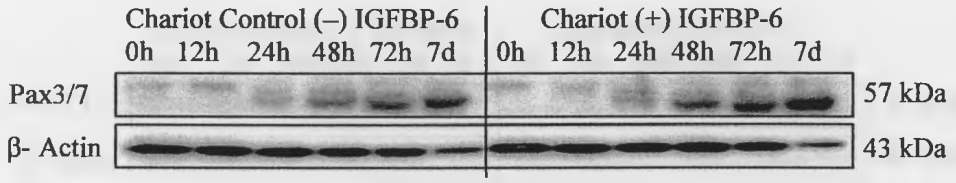
IGFBP-6, OCT4, and Pax3/7 levels as determined by Western blotting over time in PMSCs grown in muscle growth media after using the Chariot™ system to increase intracellular IGFBP-6 (0.75 μg/ well of recombinant human IGFBP-6 protein); the top panel shows a representative western blot of four independent experiments and the lower panel shows quantitative analysis of the densitometry. **(A) IGFBP-6** levels significantly increase at both 12 hours and 7 days compared to Chariot™ control; at 24 hours there is significant reduction in IGFBP-6 levels with intracellular IGFBP-6. **(B)** Intracellular IGFBP-6 increases **Pax3/7** levels significantly at day7. **(C)** There is no significant effect on **OCT4** levels at any of the time points in non-differentiation conditions compared to Chariot™ control (-) IGFBP-6. Protein levels were quantified and normalized to β-Actin using Versadoc software (Bio-Rad). Error bars represent the standard error of the mean (SEM). (*) indicates comparison between the different conditions, while (#) indicates comparison within the same treatment over time. (Two-way ANOVA followed by a t-test, *= $P<0.05$, ***=$P<0.001$).

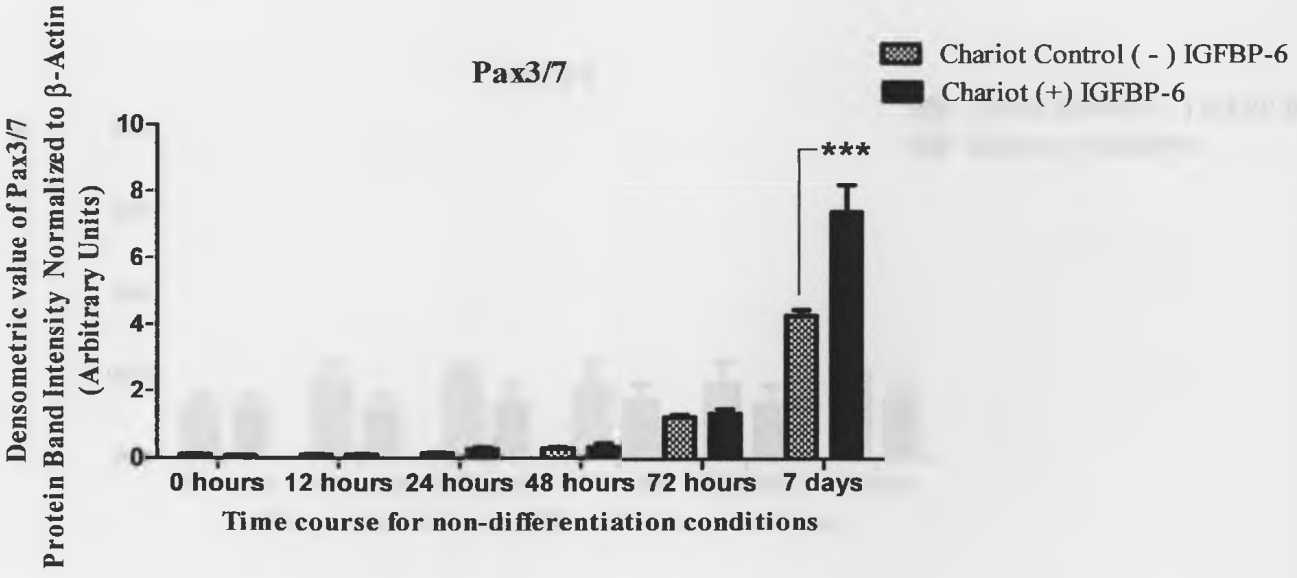
(A)


Chariot Control (–) IGFBP-6
0h 12h 24h 48h 72h 7d
Chariot (+) IGFBP-6
0h 12h 24h 48h 72h 7d
IGFBP-6
29 kDa
β- Actin
43 kDa

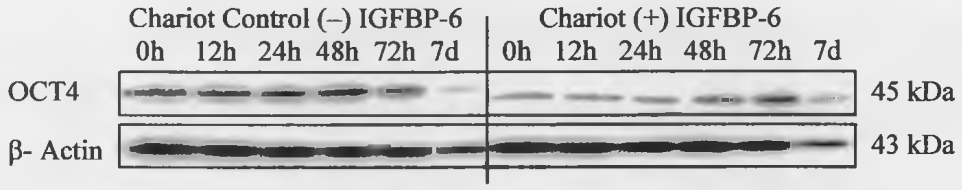

IGFBP-6
Chariot Control (-) IGFBP-6
Chariot (+) IGFBP-6
Densometric value of IGFBP-6
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
5
4
3
2
1
0
*#
#
***
0 hours
12 hours
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions

(B)


Chariot Control (–) IGFBP-6
0h 12h 24h 48h 72h 7d
Chariot (+) IGFBP-6
0h 12h 24h 48h 72h 7d
Pax3/7
57 kDa
β- Actin
43 kDa


Pax3/7
Chariot Control (-) IGFBP-6
Chariot (+) IGFBP-6
Densometric value of Pax3/7
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
10
8
6
4
2
0
***
0 hours
12 hours
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions

(C)


Chariot Control (–) IGFBP-6
0h 12h 24h 48h 72h 7d
Chariot (+) IGFBP-6
0h 12h 24h 48h 72h 7d
OCT4
45 kDa
β- Actin
43 kDa

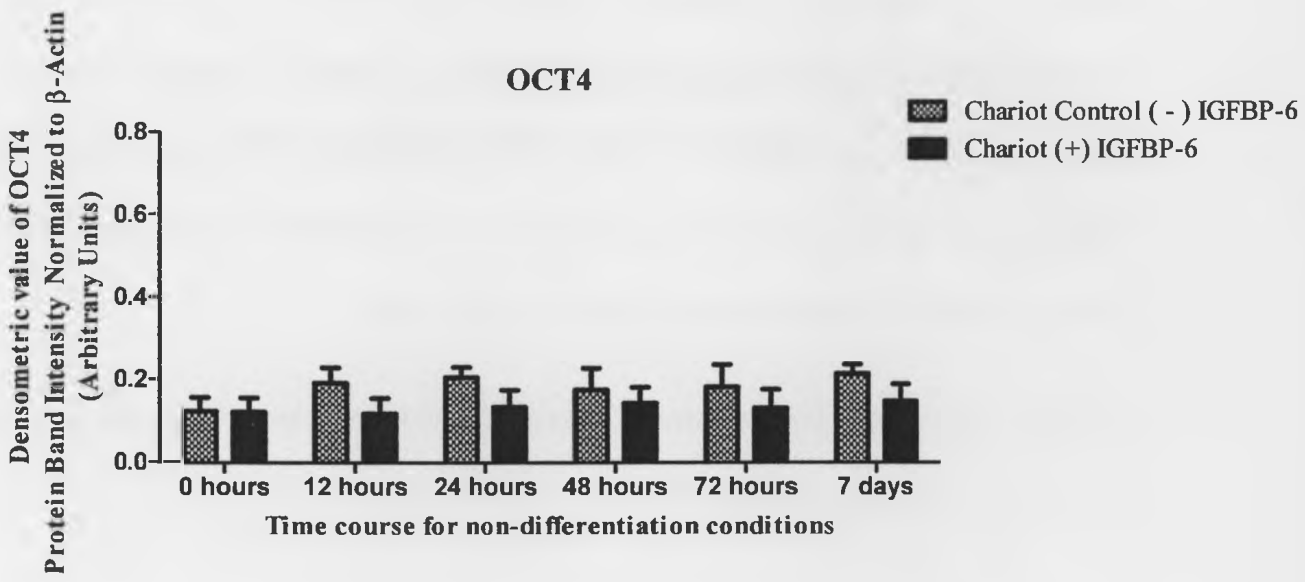

OCT4
Chariot Control (-) IGFBP-6
Chariot (+) IGFBP-6
Densometric value of OCT4
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0.8
0.6
0.4
0.2
0.0
0 hours
12 hours
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions

Figure 13: ELISA for the time course experiment for increased intracellular IGFBP-6 by Chariot™ in PMSCs under non-differentiation conditions.

IGFBP-6 levels secreted to the media increased over time after using Chariot™ to increase intracellular IGFBP-6 (0.75 μg/ well of recombinant human IGFBP-6 protein); significant increase at 24 hours and 48 hours in non-differentiation conditions. At day 7, there was a significant decrease in intracellular IGFBP-6 compared to the controls (control= PMSCs in muscle growth media alone, Chariot™ control= Chariot complex only); Chariot™ was applied only once at the beginning of the experiment. Error bars represent the standard error of the mean (SEM) as calculated from triplicate experiments. (Two-way ANOVA followed by a t-test, $P<0.001$, $n=3$).

ELISA sensitivity: Highest amount detectable 60,000 pg/ml; lowest amount detectable 82.3 pg/ml. Standard curve $R^2 = 0.99$

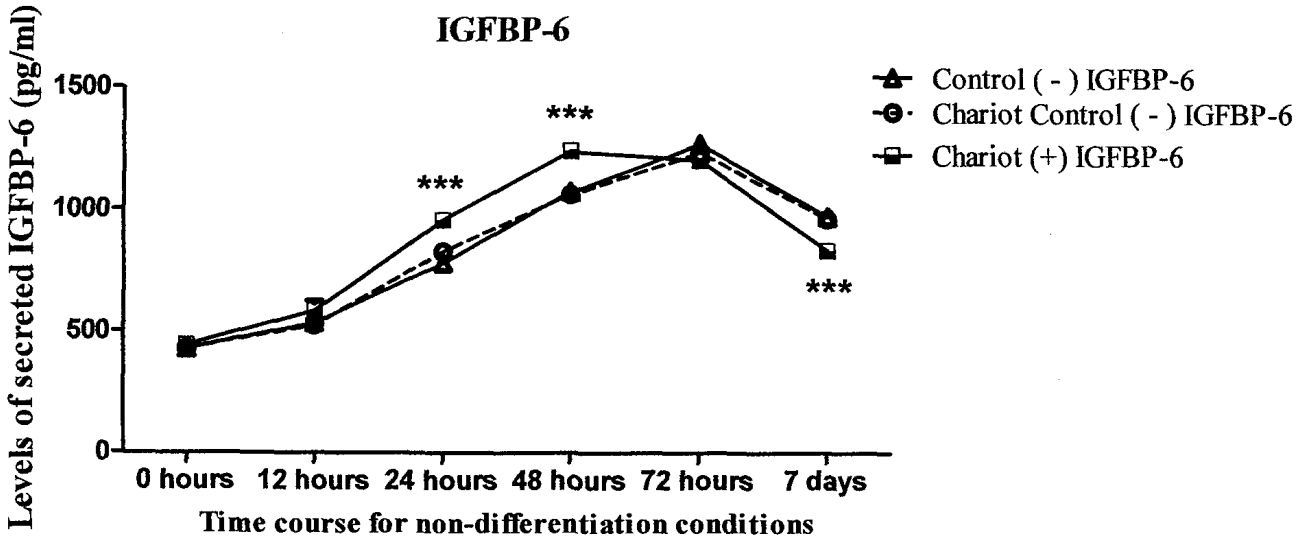
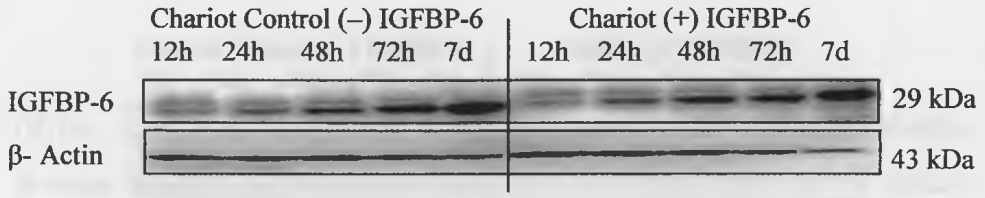

IGFBP-6
Levels of secreted IGFBP-6 (pg/ml)
1500
1000
500
0
***
***
***
0 hours
12 hours
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions
Control (-) IGFBP-6
Chariot Control (-) IGFBP-6
Chariot (+) IGFBP-6

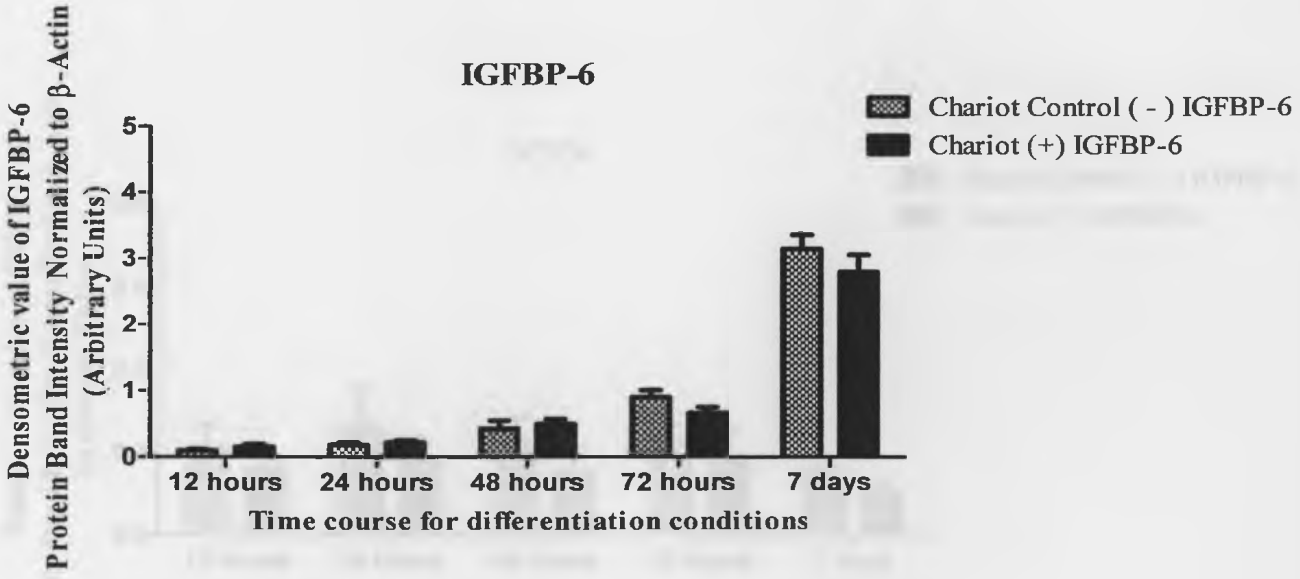
Figure 14: Time course experiment for increased intracellular IGFBP-6 by Chariot™ in PMSCs under differentiation conditions.

Images show Western blot analysis of lysates from PMSCs grown in muscle differentiation media with Chariot™ to increase the intracellular IGFBP-6 (0.75 μg/well of recombinant human IGFBP-6 protein); the top panel shows a representative western blot of four independent experiments and the lower panel shows quantification and statistical analysis. Intracellular increase of IGFBP-6 by Chariot™ had **no significant** effects on **(A) IGFBP-6** levels, **(B) OCT4** levels, or **(C) Pax3/7** levels in differentiation conditions compared to Chariot™ control.

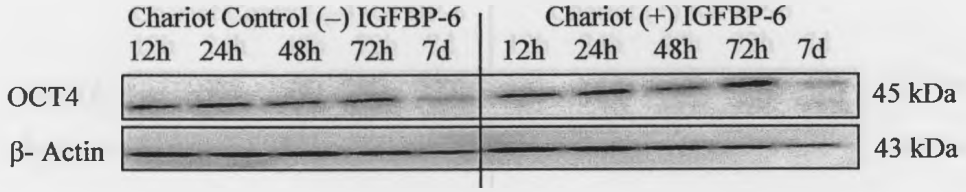
By western blot, protein levels were quantified and normalized to β-Actin using Versadoc software (Bio-Rad) and levels presented as means and standard error of the mean (SEM). (Two-way ANOVA followed by a t-test, $P<0.05$, $n=4$).

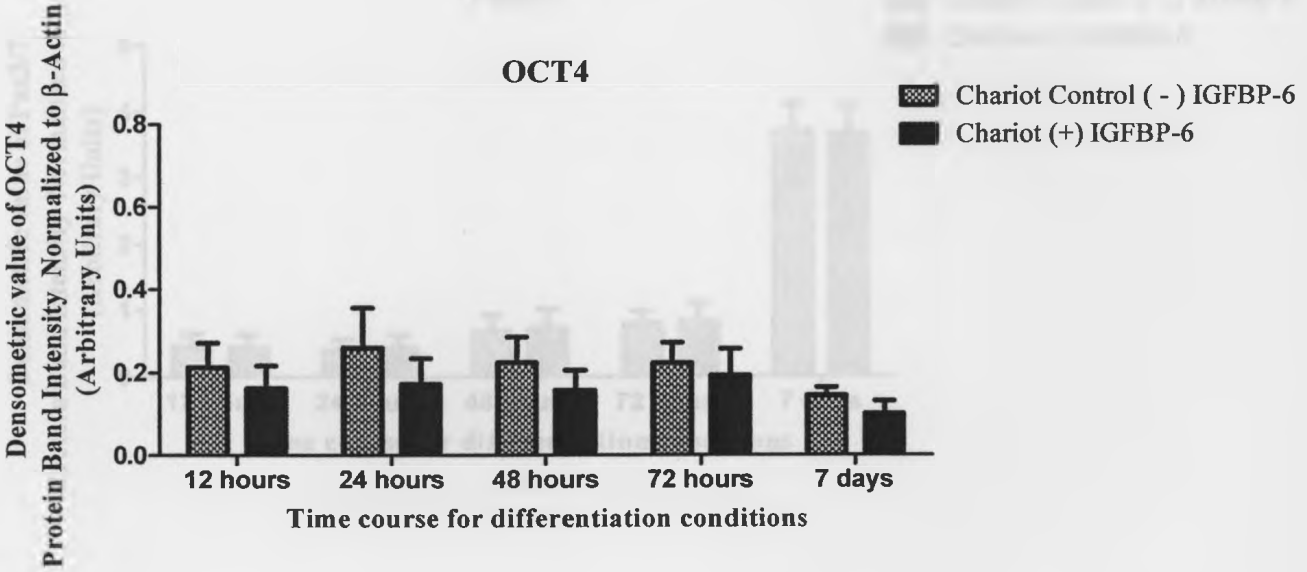
(A)


Chariot Control (–) IGFBP-6
12h 24h 48h 72h 7d
Chariot (+) IGFBP-6
12h 24h 48h 72h 7d
IGFBP-6
β- Actin
29 kDa
43 kDa

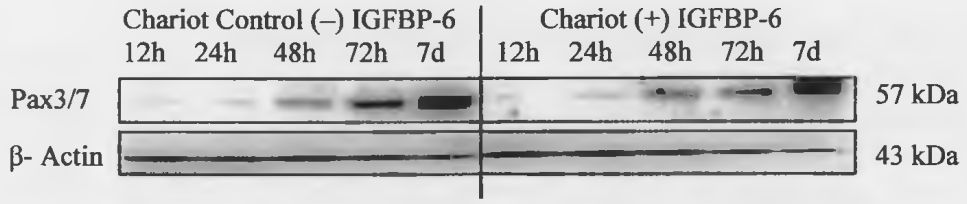

IGFBP-6
Chariot Control (-) IGFBP-6
Chariot (+) IGFBP-6
Densometric value of IGFBP-6
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0
1
2
3
4
5
12 hours
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions

(B)


Chariot Control (–) IGFBP-6
12h 24h 48h 72h 7d
Chariot (+) IGFBP-6
12h 24h 48h 72h 7d
OCT4
β- Actin
45 kDa
43 kDa


OCT4
Densometric value of OCT4
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0.8
0.6
0.4
0.2
0.0
12 hours
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions
Chariot Control (-) IGFBP-6
Chariot (+) IGFBP-6

(C)


Chariot Control (–) IGFBP-6
12h 24h 48h 72h 7d
Chariot (+) IGFBP-6
12h 24h 48h 72h 7d
Pax3/7
57 kDa
β- Actin
43 kDa

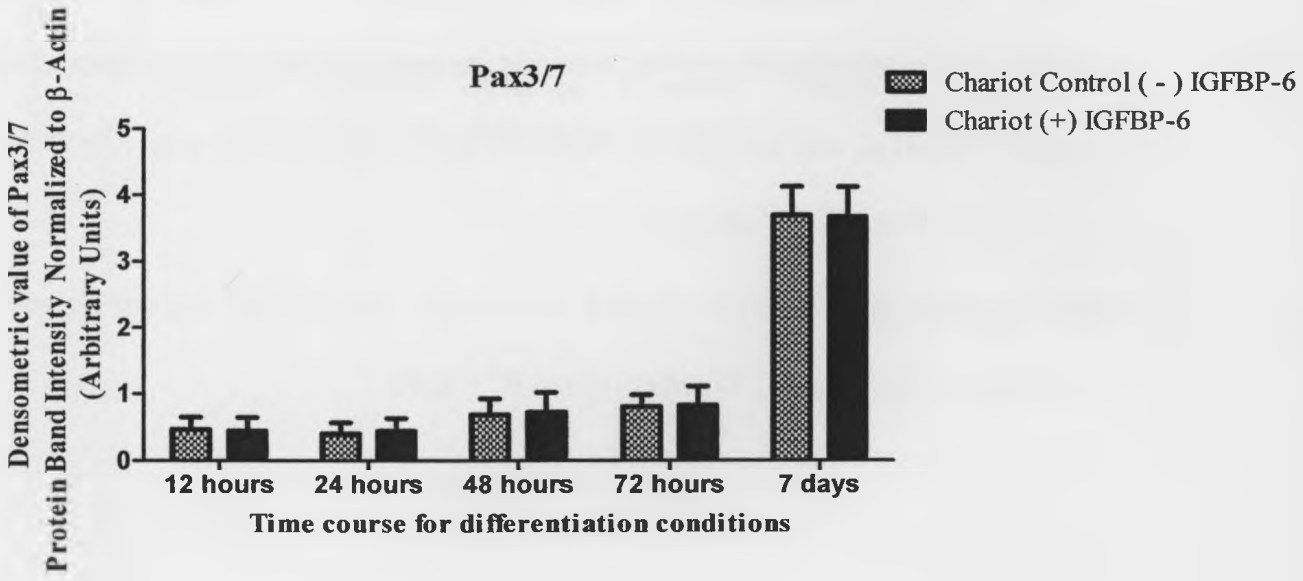

Pax3/7
Chariot Control (-) IGFBP-6
Chariot (+) IGFBP-6
Densometric value of Pax3/7
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0
1
2
3
4
5
12 hours
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions

Figure 15: ELISA for the time course experiment for increased intracellular IGFBP-6 by Chariot™ in PMSCs under differentiation conditions.

IGFBP-6 levels secreted into the media increased over time after using Chariot™ to increase the intracellular IGFBP-6 (0.75 μg/ well of recombinant human IGFBP-6 protein) compared the control (PMSCs alone) and to the Chariot™ control (Chariot™ complex only) in differentiation conditions; Chariot™ was applied only once at the beginning of the experiment. IGFBP-6 levels are presented as means and standard error of the mean (SEM) as calculated from triplicate experiments. (Two-way ANOVA followed by a t-test, *= $P<0.05$, ***=$P<0.001$, n=3).

ELISA sensitivity: Highest amount detectable was 60,000 pg/ml; lowest amount detectable was 82.3 pg/ml. Standard curve $R^2 = 0.98$

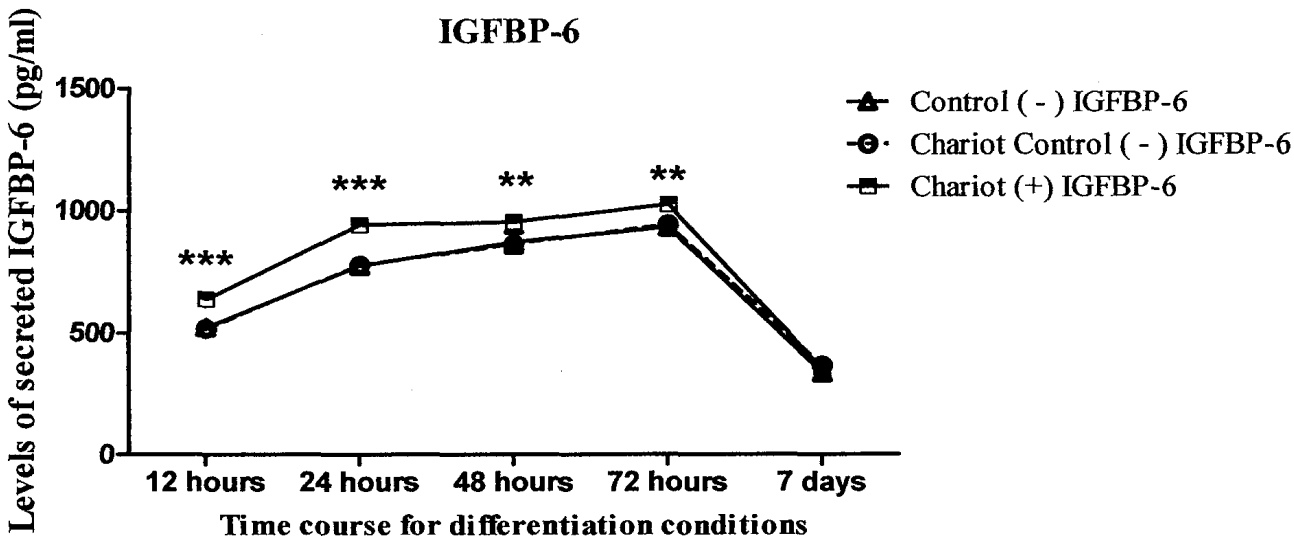

IGFBP-6
Levels of secreted IGFBP-6 (pg/ml)
1500
1000
500
0
***
***
**
**
12 hours
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions
Control (-) IGFBP-6
Chariot Control (-) IGFBP-6
Chariot (+) IGFBP-6

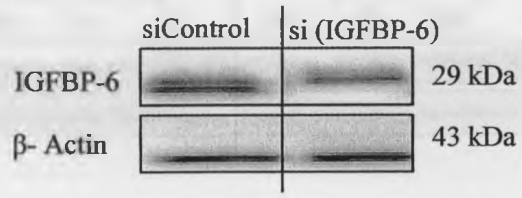
Figure 16: Time course experiment for silencing IGFBP-6 expression in PMSCs under non-differentiation conditions.

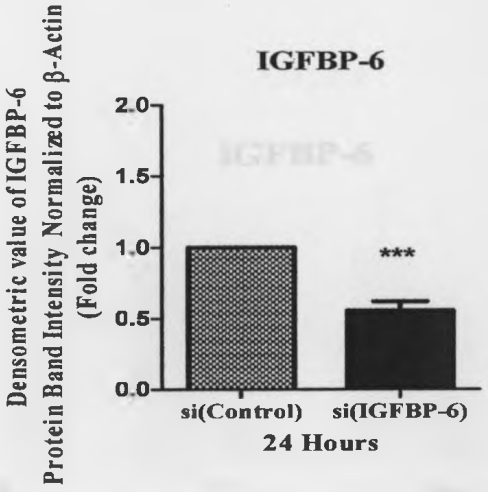
Images show Western blot analysis of lysates from cells treated with siRNAs against IGFBP-6, or scramble siRNA as a control, over time in PMSCs grown in muscle growth media; siRNA treatment was applied only once at the beginning of the experiment. The top panel shows a representative western blot of three independent experiments and the lower panel shows a diagram of the statistical analysis.

(A) After 24 hours, Silencing IGFBP-6 significantly decreases **IGFBP-6** levels by 50% compared to the scrambled siRNA. (One-way ANOVA followed by a t-test, ***=P<0.001). **(B)** Silencing IGFBP-6 is transient and **IGFBP-6** is significantly increased at day 7. **(C)** Significant decrease in **OCT4** levels at each time point with IGFBP-6 silencing. **(D)** Silencing IGFBP-6 significantly increase **Pax3/7** levels at 24 hours in non-differentiation conditions.

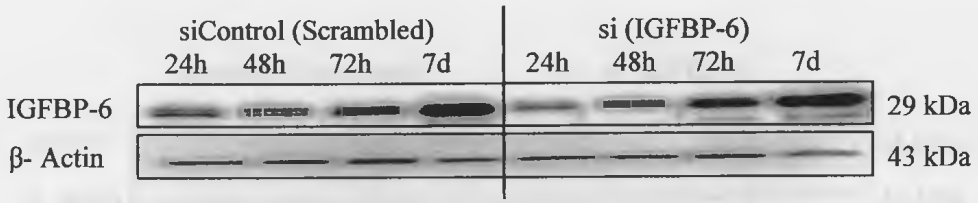
IGFBP-6 levels were quantified and normalized to β-Actin. The IGFBP-6 levels are presented as mean and standard errors of the mean (SEM) as calculated from triplicate experiments. (*) indicates comparison between the different conditions, while (#) indicates comparison within the same treatment over time. (Two-way ANOVA followed by a t-test, *= P<0.05, **=P<0.01).

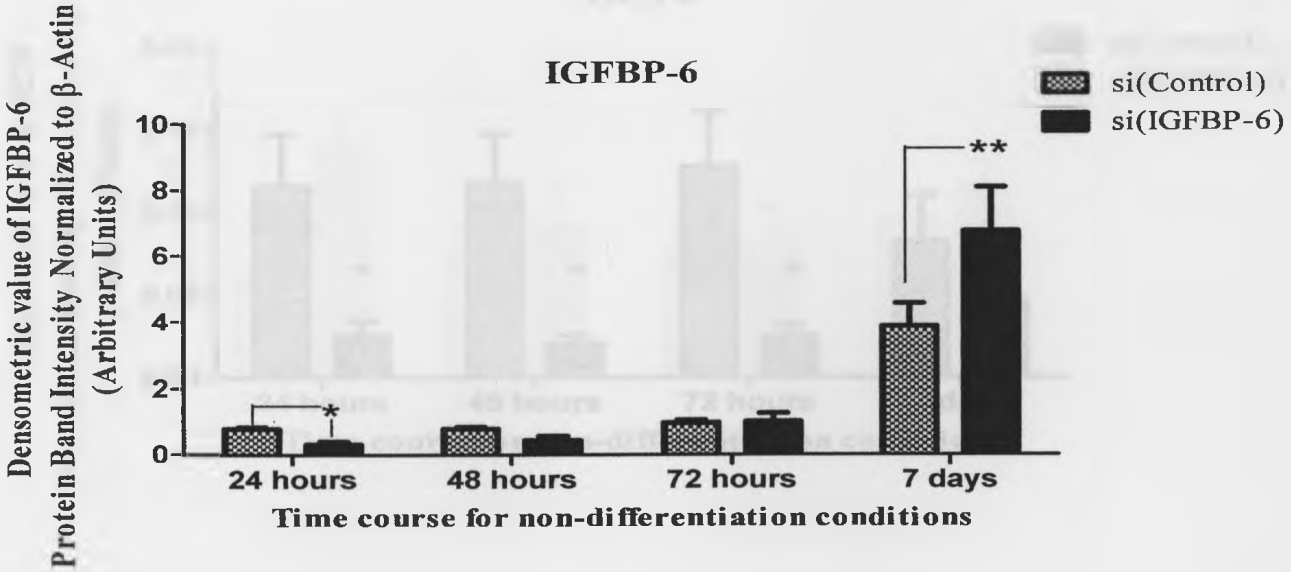
(A)


siControl
si (IGFBP-6)
IGFBP-6
29 kDa
β- Actin
43 kDa

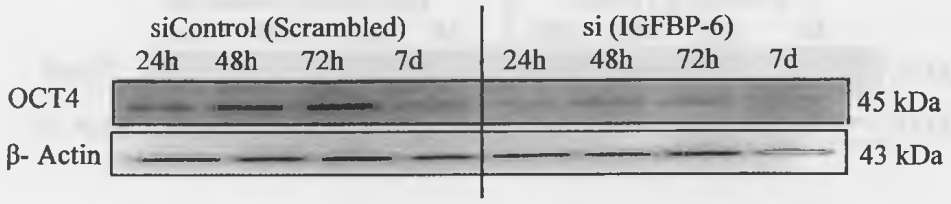

IGFBP-6
Densometric value of IGFBP-6
Protein Band Intensity Normalized to β-Actin
(Fold change)
2.0
1.5
1.0
0.5
0.0
***
si(Control)
si(IGFBP-6)
24 Hours

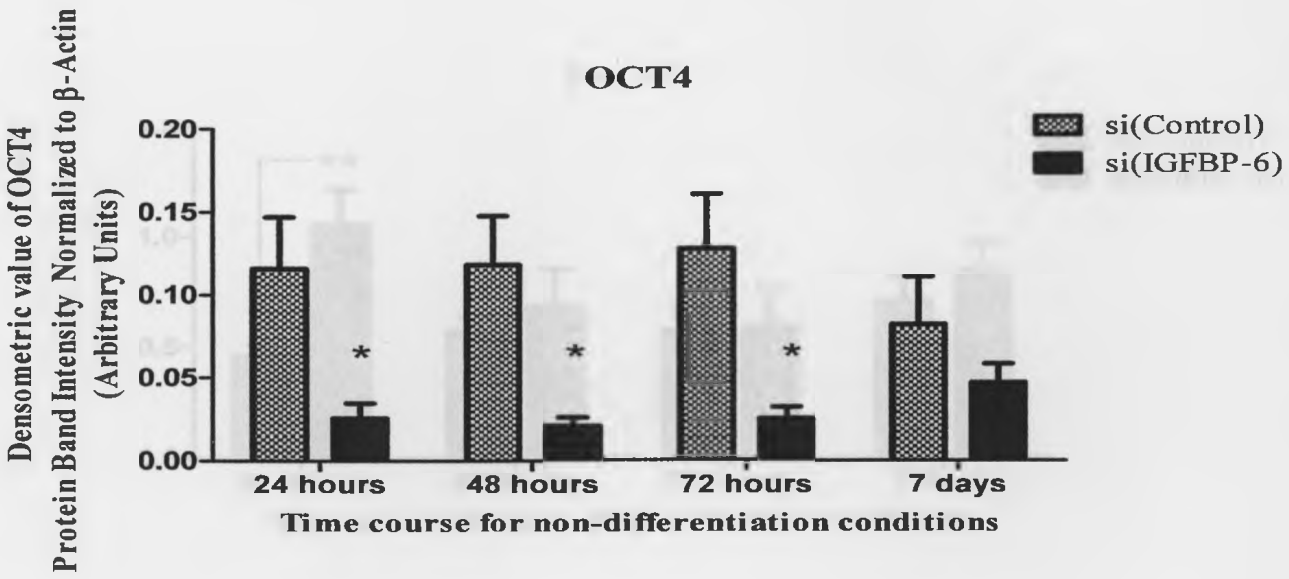
(B)


siControl (Scrambled)
24h 48h 72h 7d
si (IGFBP-6)
24h 48h 72h 7d
IGFBP-6
β- Actin
29 kDa
43 kDa

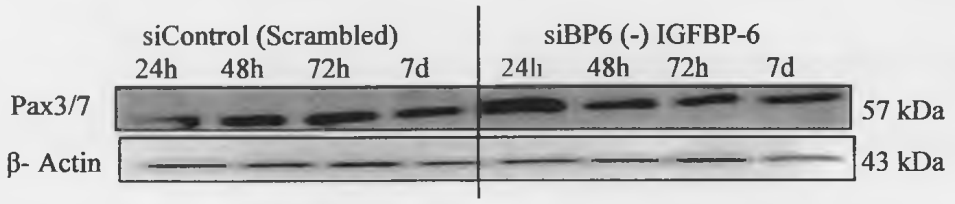

IGFBP-6
si(Control)
si(IGFBP-6)
Densometric value of IGFBP-6
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
10
8
6
4
2
0
*
**
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions

(C)


siControl (Scrambled)
24h 48h 72h 7d
si (IGFBP-6)
24h 48h 72h 7d
OCT4
45 kDa
β- Actin
43 kDa


OCT4
Densometric value of OCT4
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0.20
0.15
0.10
0.05
0.00
*
*
*
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions
si(Control)
si(IGFBP-6)

(D)


siControl (Scrambled)
siBP6 (-) IGFBP-6
24h
48h
72h
7d
24h
48h
72h
7d
Pax3/7
57 kDa
β- Actin
43 kDa

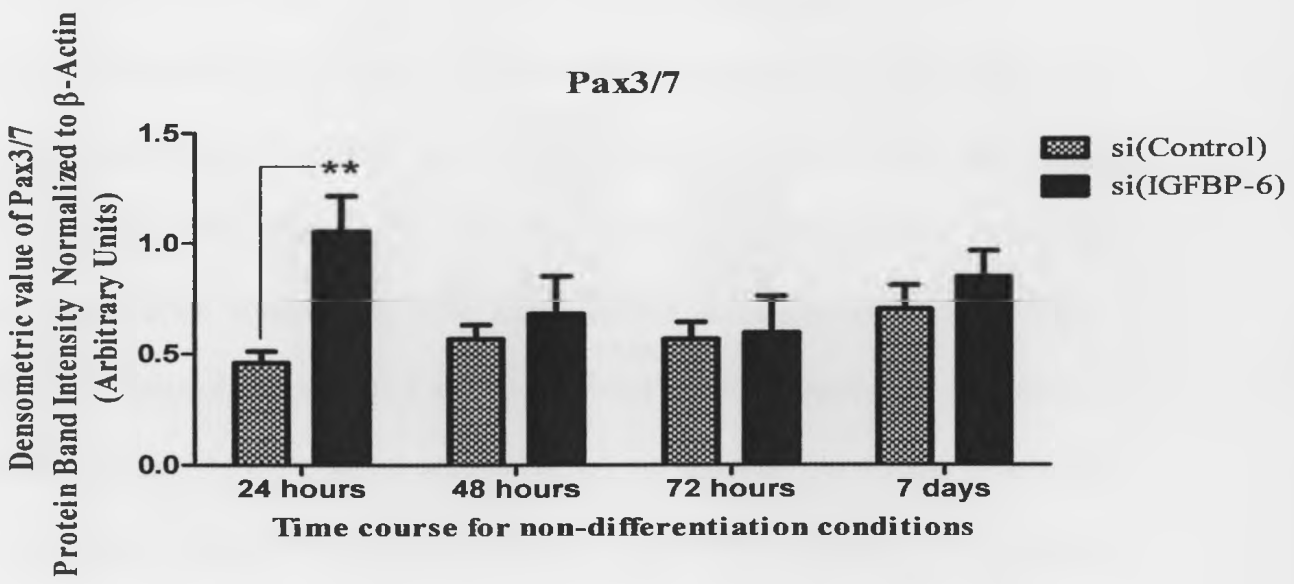
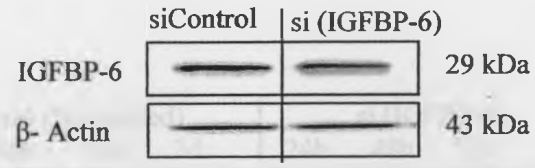
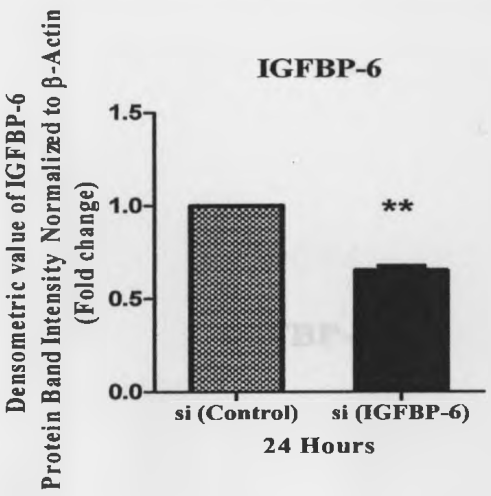

Pax3/7
Densometric value of Pax3/7
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
1.5
1.0
0.5
0.0
**
si(Control)
si(IGFBP-6)
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions

Figure 17: Time course experiment for silencing of IGFBP-6 expression in PMSCs under differentiation conditions.

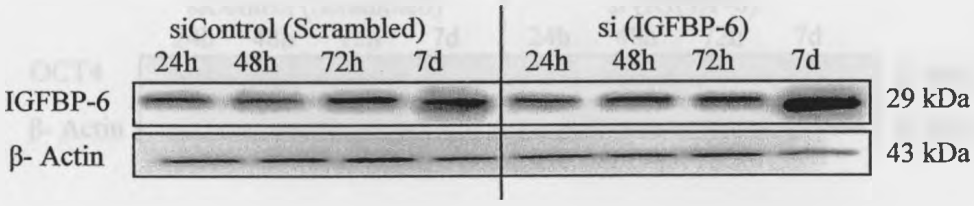
Images show Western blot analysis of lysates from cells treated with siRNA to IGFBP-6, or scramble siRNA as a control over time in PMSCs grown in muscle differentiation media; siRNA treatment was applied only once at the beginning of the experiment. The top panel shows a representative western blot of three independent experiments and the lower panel shows a diagram of the statistical analysis. **(A)** Silencing IGFBP-6 with siRNAs significantly decreased **IGFBP-6** levels by 40% at 24 hours compared to controls (scrambled siRNA) (One-way ANOVA followed by a t-test, **=$P<0.01$). **(B)** Silencing IGFBP-6 was transient and **IGFBP-6** levels were significantly increased at day 7. **(C)** Concurrent with reduction in IGFBP-6, there was a significant decrease in **OCT4** levels, **(D)** with a significant increase in **Pax3/7** levels at each time point in differentiation conditions. By western blot, IGFBP-6 levels were quantified and normalized to β-actin. IGFBP-6 levels are presented as means and standard error of the mean (SEM). (Two-way ANOVA followed by t-test, *=$P<0.01$***=$P<0.001$).

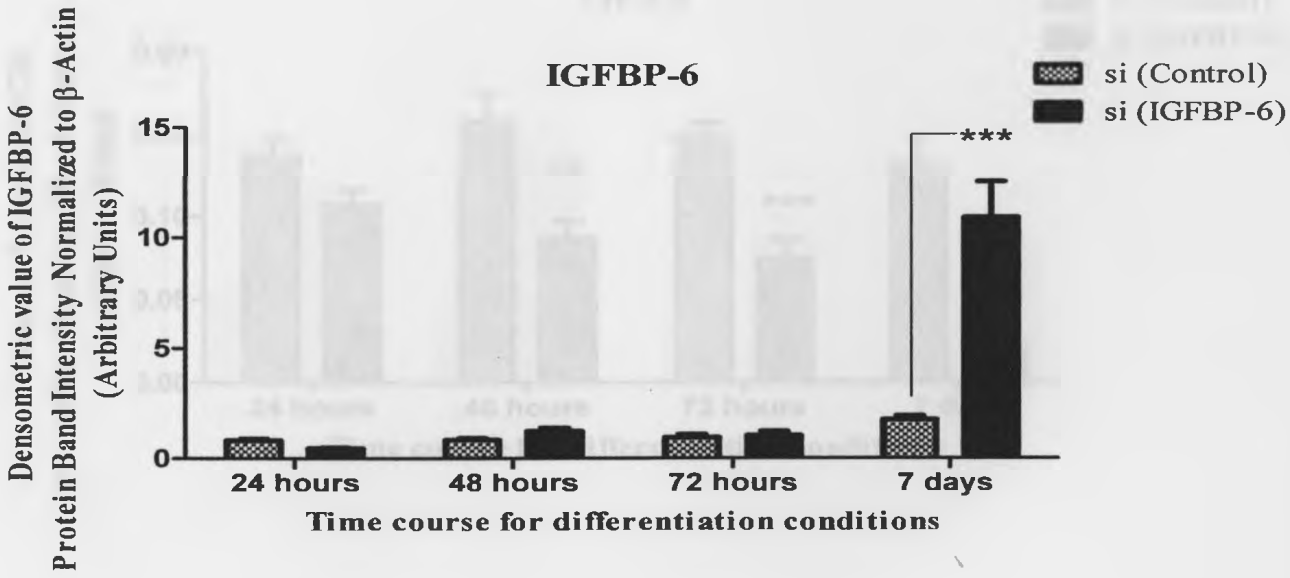
(A)


siControl
si (IGFBP-6)
IGFBP-6
29 kDa
β- Actin
43 kDa

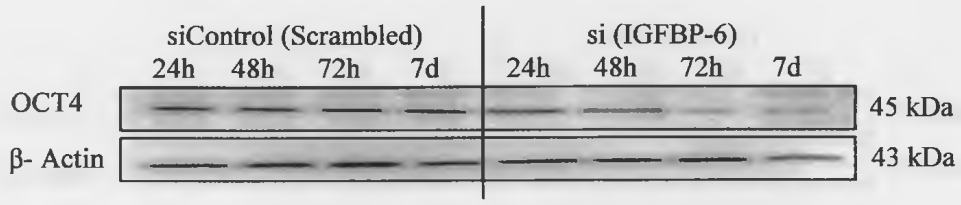

IGFBP-6
Densometric value of IGFBP-6
Protein Band Intensity Normalized to β-Actin
(Fold change)
1.5
1.0
0.5
0.0
**
si (Control)
si (IGFBP-6)
24 Hours

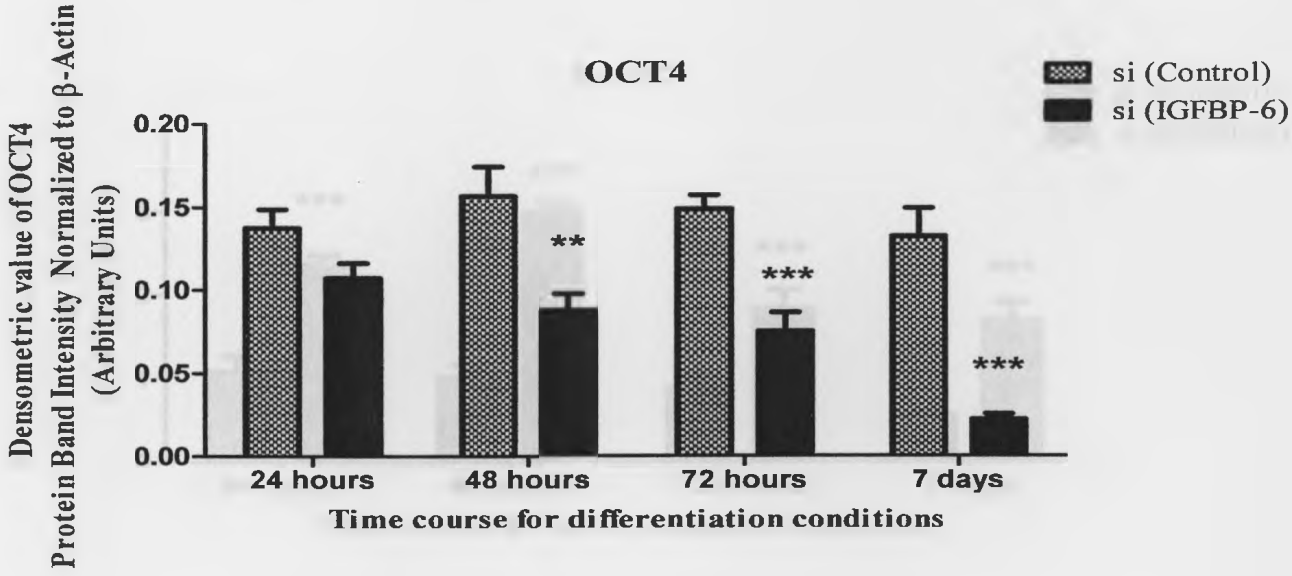
(B)


siControl (Scrambled)
si (IGFBP-6)
24h 48h 72h 7d
24h 48h 72h 7d
IGFBP-6
β- Actin
29 kDa
43 kDa

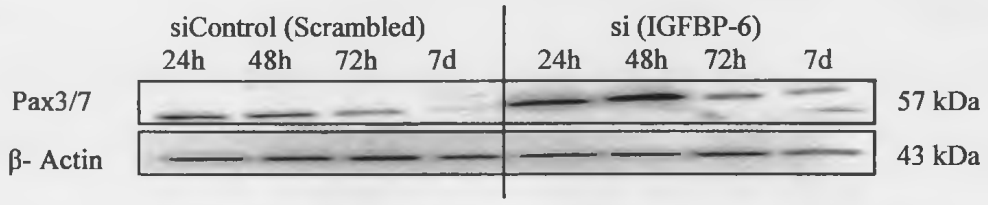

IGFBP-6
si (Control)
si (IGFBP-6)
***
Densometric value of IGFBP-6
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
15
10
5
0
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions

(C)


siControl (Scrambled)
si (IGFBP-6)
24h 48h 72h 7d 24h 48h 72h 7d
OCT4
45 kDa
β- Actin
43 kDa


OCT4
si (Control)
si (IGFBP-6)
Densometric value of OCT4
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0.20
0.15
0.10
0.05
0.00
**
***
***
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions

(D)


siControl (Scrambled)
24h 48h 72h 7d
si (IGFBP-6)
24h 48h 72h 7d
Pax3/7
57 kDa
β- Actin
43 kDa

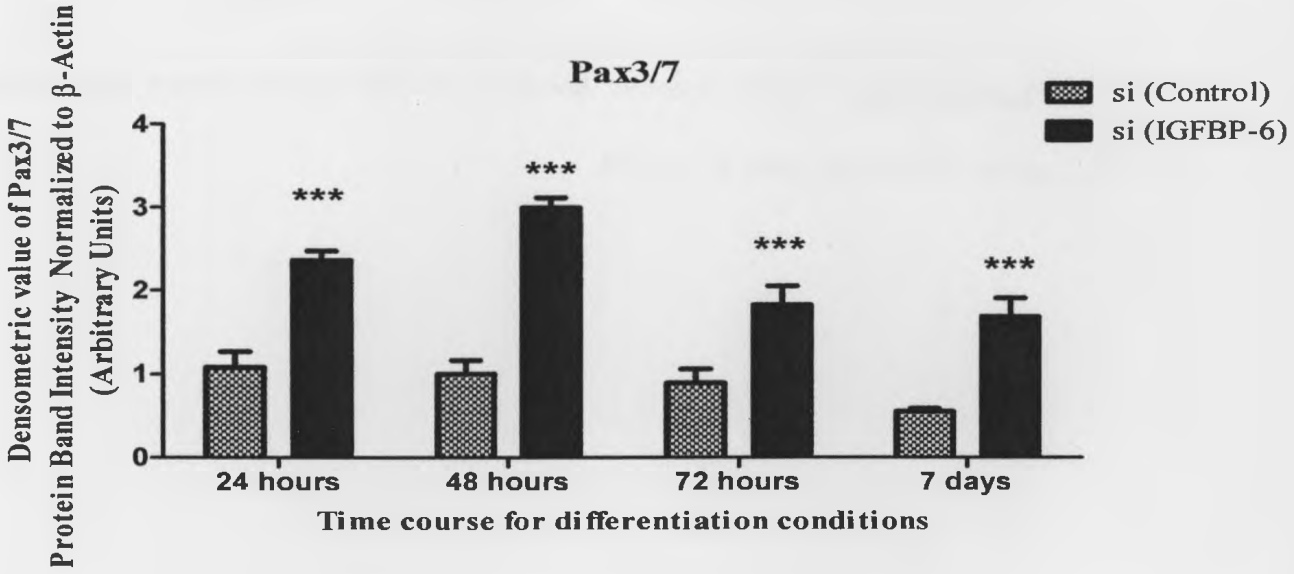

Pax3/7
si (Control)
si (IGFBP-6)
Densometric value of Pax3/7
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0
1
2
3
4
***
***
***
***
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions

Figure 18: ELISA for the time course experiment for silencing IGFBP-6 expression in PMSCs under non-differentiation conditions.

IGFBP-6 secreted into the media decreased significantly at each time point after treatment with siRNA against IGFBP-6 compared to the scrambled siRNA. siRNA treatment was applied only once at the beginning of the experiment. IGFBP-6 levels are presented as mean and standard error of the mean (SEM) as calculated from triplicate experiments. (Two-way ANOVA followed by a t-test, $P<0.001$, $n=3$).

ELISA sensitivity: Highest amount detectable 60,000 pg/ml; lowest amount detectable 82.3 pg/ml. Standard curve $R^2 = 0.99$

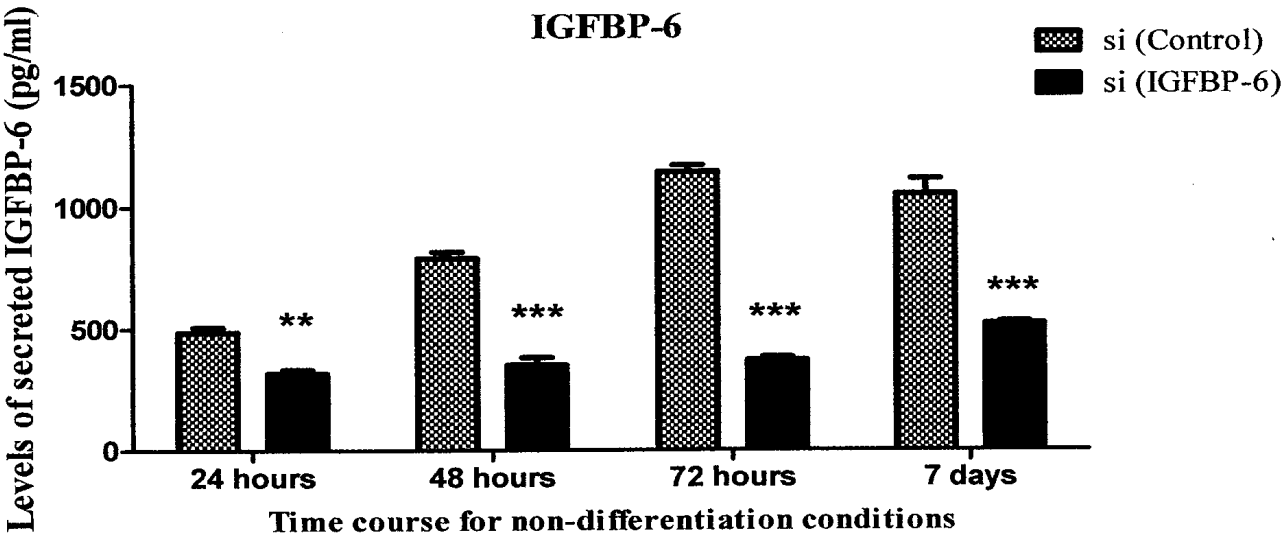
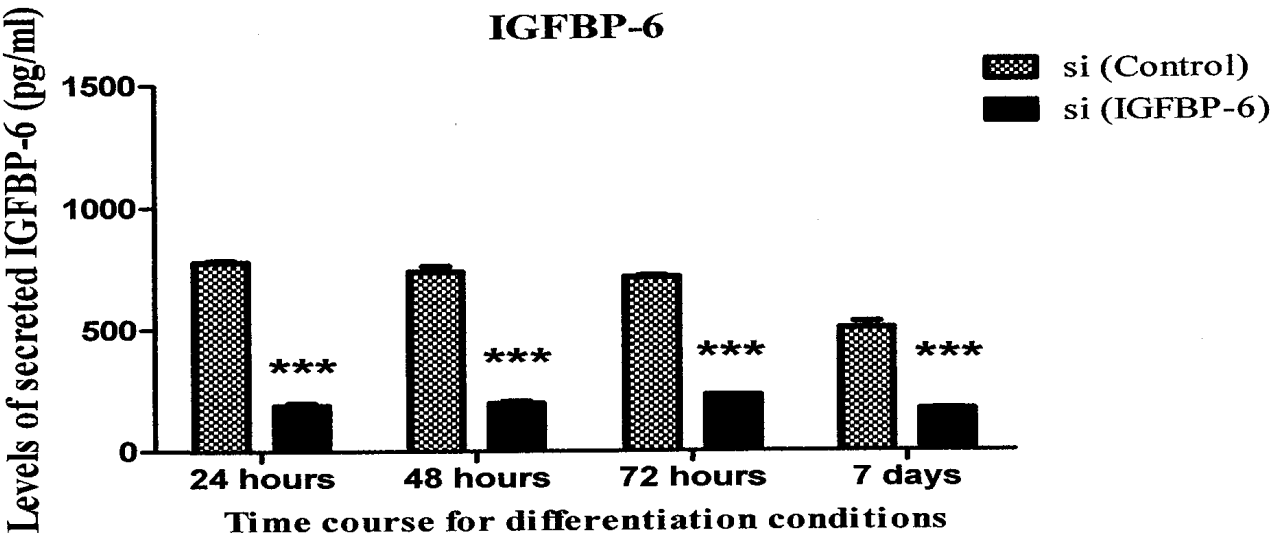

IGFBP-6
si (Control)
si (IGFBP-6)
Levels of secreted IGFBP-6 (pg/ml)
1500
1000
500
0
**
***
***
***
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions

Figure 19: Time course of changes in IGFBP-6 secreted into the media following silencing of IGFBP-6 expression in PMSCs under differentiation conditions.

IGFBP-6 levels secreted into the media decreased significantly after treatment with siRNA against IGFBP-6 compared to controls (scrambled siRNA); siRNA treatment was applied only once at the beginning of the experiment. IGFBP-6 levels are presented as means and standard error of the mean (SEM) as calculated from triplicate experiments. (Two-way ANOVA followed by a t-test, $P<0.001$, $n=3$).

ELISA sensitivity: Highest amount detectable was 60,000 pg/ml; lowest amount detectable was 82.3 pg/ml. Standard curve $R^2 = 0.98$


IGFBP-6
si (Control)
si (IGFBP-6)
Levels of secreted IGFBP-6 (pg/ml)
1500
1000
500
0
***
***
***
***
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions

4.3 Extracellular Actions of IGFBP-6 on Developing Muscle Cells

IGFBP-6 is present in the mesenchymal stem cell microenvironment, and extracellular IGFBP-6 is expected to regulate the bioavailability of IGFs (especially IGF-II). Since IGF-II has been recently identified as one of the main regulators of the embryonic stem cell proliferation, the excess of IGFBP-6 is expected to deplete the system of IGF-II, and therefore, down-regulate stem cell proliferation (95, 96, 97, 98, 135).

To test the effects of extracellular IGFB-6 on developing muscle cells, placental mesenchymal stem cells were grown in 6-well plates under either muscle growth media for non-differentiation or muscle differentiation media for differentiation conditions; purified human IGFBP-6 recombinant protein (750 ng/ml) was added to the media only once at the beginning of the experiment and a time course experiment was done. Cells were lysed after 24, 48, 72 hours and 7 days and western blots were used to determine protein levels for IGFBP-6, OCT4, and Pax3/7.

Compared to the negative control, the extracellular IGFBP-6 in either muscle growth media or in muscle differentiation media had no significant effect on PMSC expression of IGFBP-6 at each time point under both non-differentiation (Figure 20A) and differentiation conditions (Figure 21A); moreover, under both conditions IGFBP-6 levels increased over time regardless of the treatment condition.

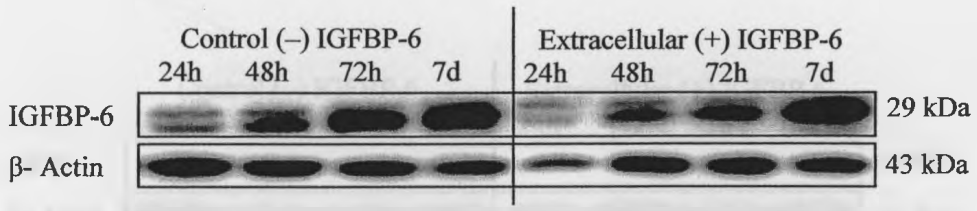
OCT4 levels at each time point were not changed under both non-differentiation (Figure 20B) and differentiation conditions (Figure 21B). On the other hand, there was a significant increase in Pax3/7 at day 7 with extracellular IGFBP-6 under non-differentiation conditions (Figure 20C); while there was no significant change in Pax3/7

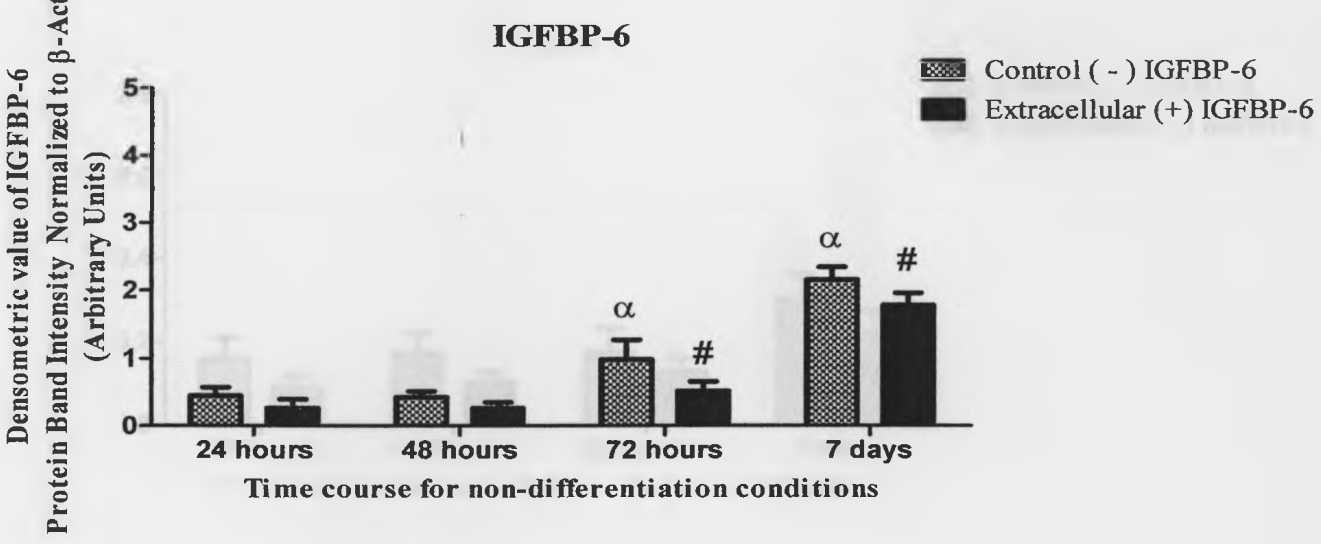
levels over time under differentiation conditions (Figure 21C). However, under both conditions, IGFBP-6 levels increased over time regardless of treatment condition. This indicates that extracellular IGFBP-6 has no significant effects on Pax3/7 levels once the cells are committed to the muscle lineage.

Figure 20: Changes in IGFBP-6 levels when extracellular IGFBP-6 was increased in PMSCs under non-differentiation conditions.

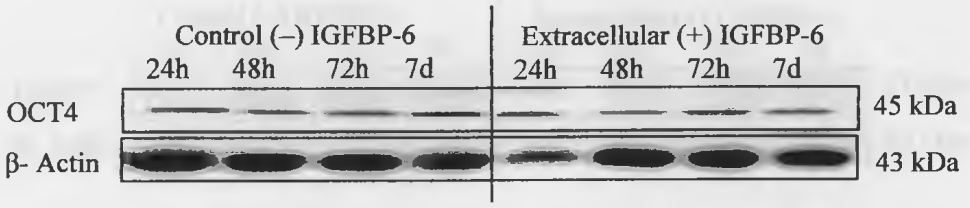
Western blots were used to examine cell lysate over time in PMSCs grown in muscle growth media after IGFBP-6 was added to the media (0.75 μg/well of recombinant human IGFBP-6 protein); the top panel shows a representative western blot of three independent experiments and the lower panel shows a diagram of the statistical analysis. Extracellular IGFBP-6 had **no significant** effects on **(A) IGFBP-6** levels, as the levels were increased over time following both treatments, and **(B) OCT4** levels; but **(C) Pax3/7** levels were significantly increased at day 7 in non-differentiation conditions compared to control (-) IGFBP-6 (PMSCs in muscle growth media only). IGFBP-6 levels were quantified and normalized to β-Actin. IGFBP-6 levels are presented as means and standard error of the mean (SEM). (*) indicates comparison between the different treatments, while (#) indicates comparison within the extracellular treatment over time, and (α) is to compare within the control over time. (Two-way ANOVA followed by a t-test, $P<0.001$, $n=3$).

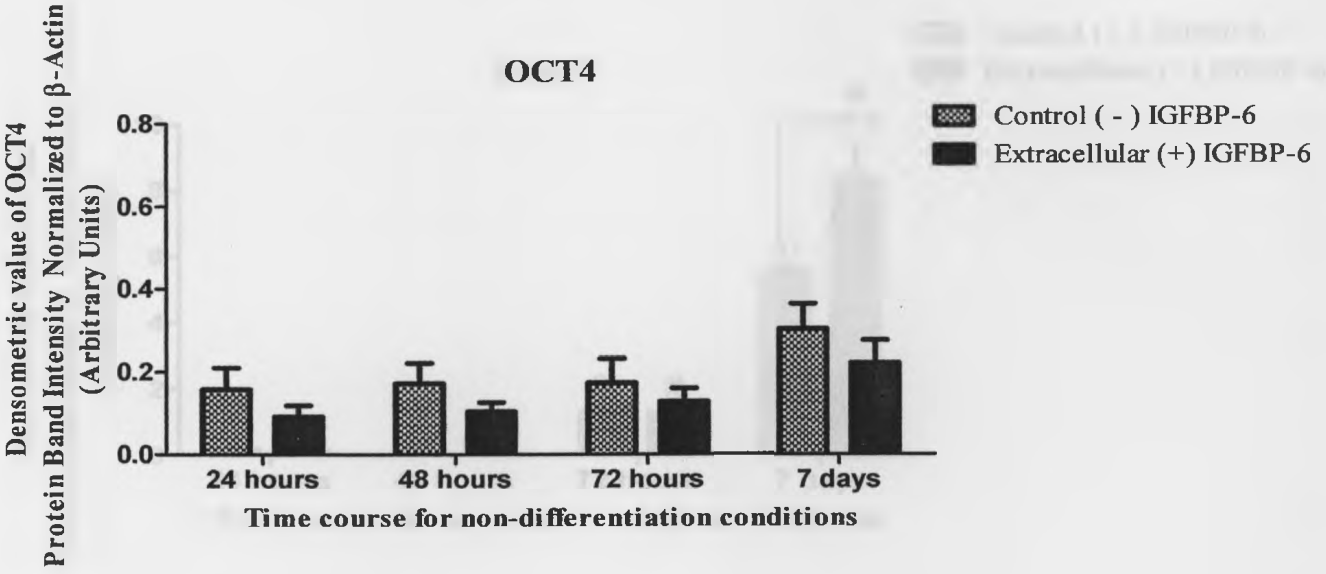
(A)


Control (–) IGFBP-6
24h 48h 72h 7d
Extracellular (+) IGFBP-6
24h 48h 72h 7d
IGFBP-6
β- Actin
29 kDa
43 kDa

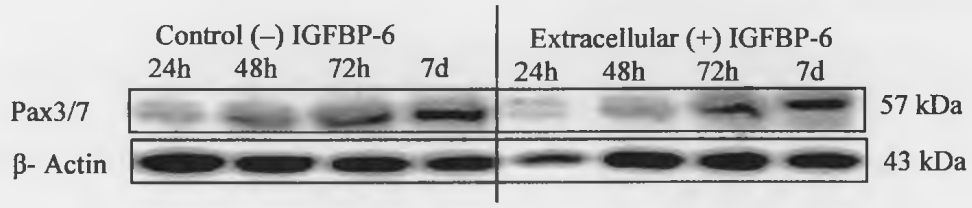

IGFBP-6
Control (-) IGFBP-6
Extracellular (+) IGFBP-6
Densometric value of IGFBP-6
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0
1
2
3
4
5
α
#
α
#
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions

(B)


Control (–) IGFBP-6
24h 48h 72h 7d
Extracellular (+) IGFBP-6
24h 48h 72h 7d
OCT4
45 kDa
β- Actin
43 kDa


OCT4
Densometric value of OCT4
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0.8
0.6
0.4
0.2
0.0
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions
Control (-) IGFBP-6
Extracellular (+) IGFBP-6

(C)


Control (–) IGFBP-6
24h 48h 72h 7d
Extracellular (+) IGFBP-6
24h 48h 72h 7d
Pax3/7
57 kDa
β- Actin
43 kDa

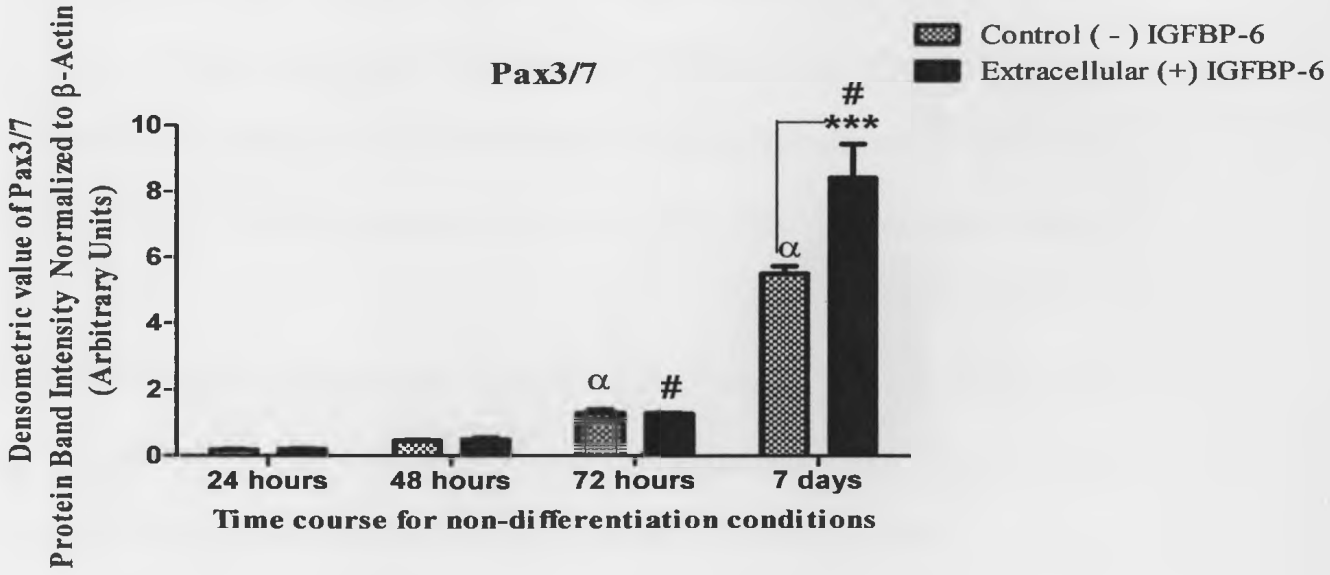
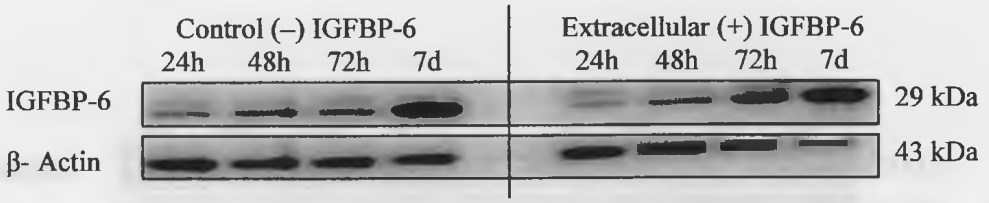

Pax3/7
Control (-) IGFBP-6
Extracellular (+) IGFBP-6
Densometric value of Pax3/7
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
10
8
6
4
2
0
α
#
***
24 hours
48 hours
72 hours
7 days
Time course for non-differentiation conditions

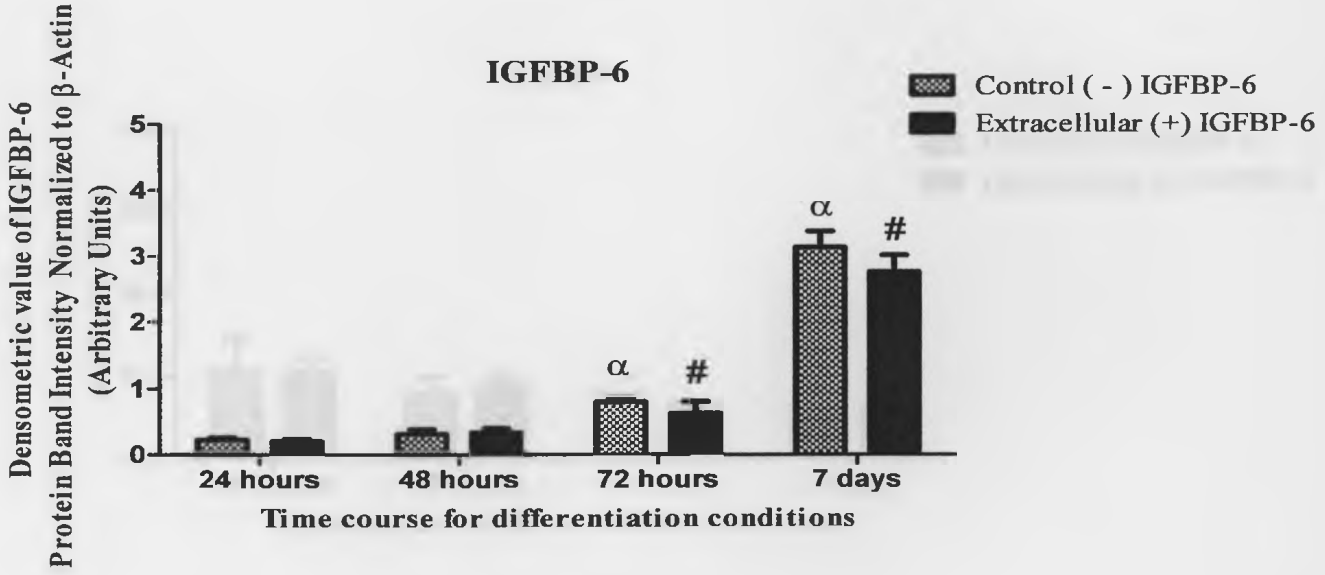
Figure 21: Changes with time after addition of extracellular IGFBP-6 in PMSCs under differentiation conditions.

Quantification of IGFBP-6, OCT4 and Pax3/7 in lysates from PMSCs grown in muscle differentiation media and treated with the addition of extracellular IGFBP-6 at the beginning of the experiment (0.75 μg/well of recombinant human IGFBP-6 protein); the top panel shows a representative western blot from three independent experiments and the lower panel shows quantification and statistical analysis. Extracellular IGFBP-6 had **no significant** effects on **(A) IGFBP-6** levels, **(B) OCT4** levels, and **(C) Pax3/7** levels over time under differentiation conditions compared to control (-) IGFBP-6 (PMSCs in muscle differentiation media only) with an overall increase in IGFBP-6 and Pax3/7 over time with in the same treatment.

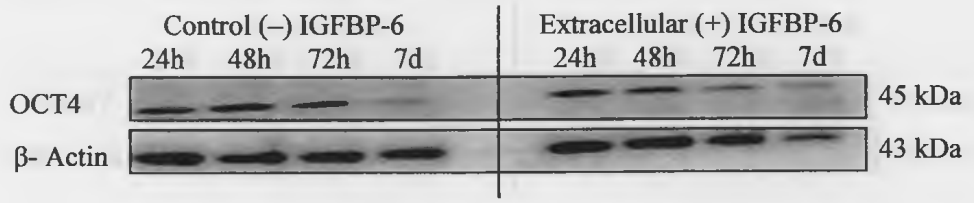
By western blot, levels were quantified and normalized to β-Actin. Error bars represent the standard error of the mean (SEM). (*) indicates comparison between the different treatments, while (#) indicates comparison within the extracellular treatment over time, and (α) indicates comparison within the control over time. (Two-way ANOVA, $P<0.001$, $n=3$).

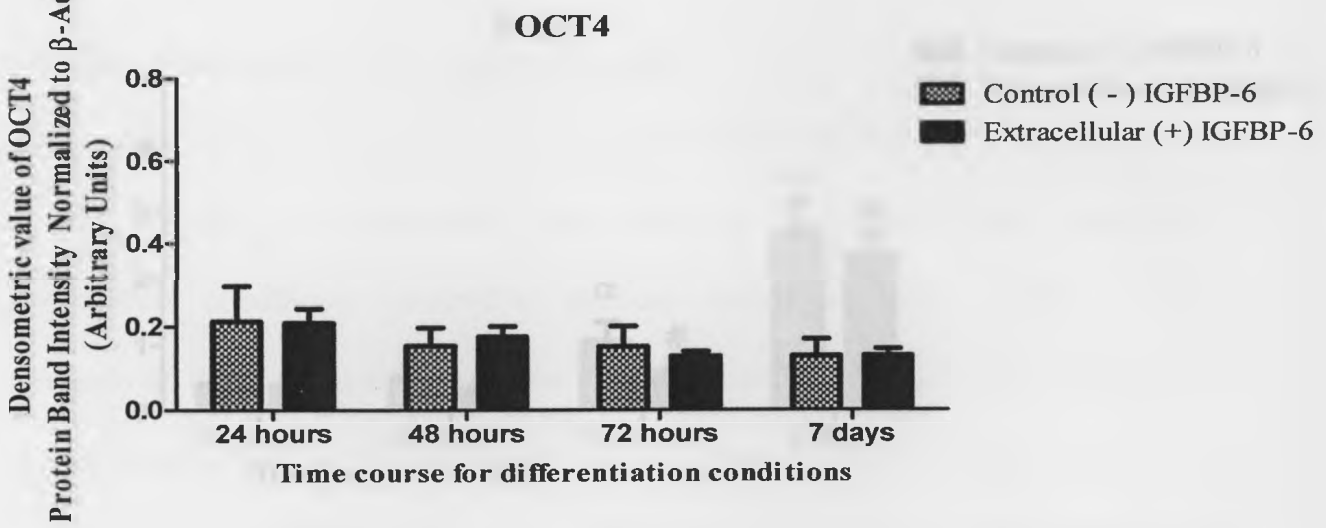
(A)


Control (–) IGFBP-6
24h 48h 72h 7d
Extracellular (+) IGFBP-6
24h 48h 72h 7d
IGFBP-6
β- Actin
29 kDa
43 kDa

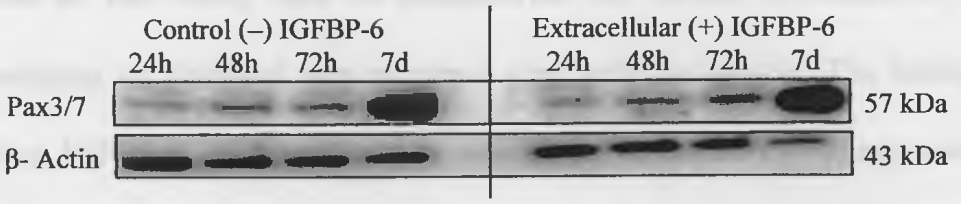

IGFBP-6
Control (-) IGFBP-6
Extracellular (+) IGFBP-6
Densometric value of IGFBP-6
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0
1
2
3
4
5
α
#
α
#
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions

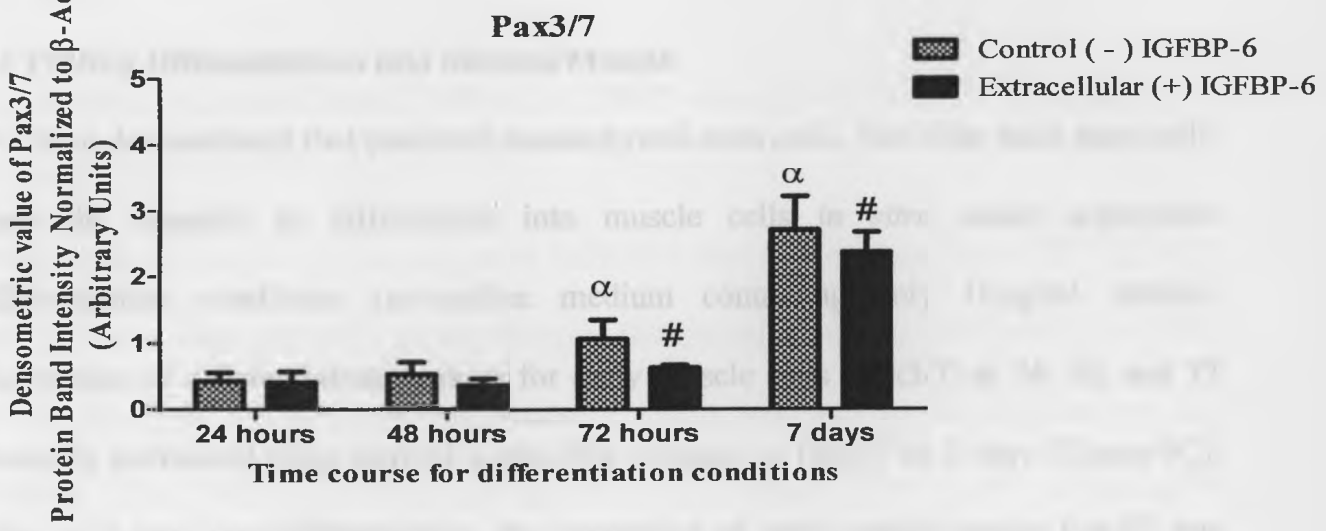
(B)


Control (–) IGFBP-6
24h 48h 72h 7d
Extracellular (+) IGFBP-6
24h 48h 72h 7d
OCT4
β- Actin
45 kDa
43 kDa


OCT4
Densometric value of OCT4
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0.8
0.6
0.4
0.2
0.0
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions
Control (-) IGFBP-6
Extracellular (+) IGFBP-6

(C)


Control (–) IGFBP-6
24h 48h 72h 7d
Extracellular (+) IGFBP-6
24h 48h 72h 7d
Pax3/7
57 kDa
β- Actin
43 kDa


Pax3/7
Control (-) IGFBP-6
Extracellular (+) IGFBP-6
Densometric value of Pax3/7
Protein Band Intensity Normalized to β-Actin
(Arbitrary Units)
0
1
2
3
4
5
α
#
α
#
24 hours
48 hours
72 hours
7 days
Time course for differentiation conditions

5.0 DISCUSSION

The aim of this study was to characterize the effects of IGFBP-6 on the early differentiation of placental mesenchymal stem cells to muscle. The biologic effects of increasing IGFBP-6, as determined by expression of markers of pluripotency (OCT4) or commitment (Pax3/7), were more apparent under the non-differentiation conditions before PMSCs commit to the muscle lineage, while silencing IGFBP-6 had a significant biologic effect under both non-differentiation and differentiation conditions.

5.1 PMSCs Differentiation into Skeletal Muscle

We have demonstrated that placental mesenchymal stem cells, like other adult stem cells, have the capacity to differentiate into muscle cells *in vitro* under appropriate differentiation conditions (serum-free medium containing only 10μg/ml insulin). Expression of differentiation markers for early muscle cells (Pax3/7) at 24, 48, and 72 hours by immunoblotting showed a two-fold increase in Pax3/7 at 3 days (Figure 9C). Also, at 3 days post-differentiation, the expression of early muscle marker Pax3/7 was shown using confocal microscopy (Figure 9A), as well as the presence of the multi-nucleation process (Figure 9B). Moreover, at 14 days post-differentiation, PMSCs expressed late muscle differentiation markers Myogenin (Figure 9D) and MyoD (Figure 9E), as well as the formation of multi-nucleated fibers (Figure 10C). This data supports what is known in the stem cell field, as it has been reported that under the appropriate niche conditions, adult human mesenchymal stem cells have the capacity for myogenic differentiation (43).

IGFBP-6 was reported to be expressed at high levels in developing muscle cells (134) and the findings from our studies support that PMSCs express the protein in significant levels

during differentiation. When incubated in the differentiation microenvironment, IGFBP-6 levels doubled at 3-days as demonstrated by immunoblotting (Figure 9C) and confocal microscopy (Figure 8C).

In contrast, the pluripotency marker, OCT4, remained unchanged during differentiation (Figure 11B). These findings indicate that IGFBP-6 is an integral protein during muscle differentiation and that more studies are required to address why OCT4 levels are not changing.

5.2 Increase in Intracellular IGFBP-6 in PMSCs using the Chariot™ Transport System

Previous studies in our laboratory have indicated that the biologic role of IGFBP-6 in muscle cells (using RD cells) is dependent on the intracellular location of IGFBP-6 either in the cytoplasm or in the nucleus, and the intracellular effects of IGFBP-6 may be different from its effect in the extracellular compartment. We used the Chariot™ transport system for our studies because it is non-cytotoxic, serum independent (136), and has been tested in mammalian cells (137, 138), and therefore, it allows us to increase intracellular IGFBP-6 in differentiating muscle cells and compare and contrast with the extracellular effects of the protein during the same process. Our studies using an indicator protein, β-galactosidase (Figure 8), demonstrated to us that the system could be used to transport IGFBP-6 in a similar manner into PMSCs.

Our studies showed that PMSCs responded differently under non-differentiation conditions compared to differentiation conditions. Under non-differentiation conditions (PMSCs grown in muscle growth media), IGFBP-6 levels significantly increased at 12

hours using Chariot™ compared to the Chariot™ control (Chariot™ complex only). IGFBP-6 levels were stable after 12 hours until the significant increase at 7days due to the increase in intracellular of IGFBP-6 (Figure 12A).

On the other hand, under differentiation conditions (PMSCs grown in muscle differentiation media), there was no significant change in IGFBP-6 levels at any of the time points (Figure 14A) indicating that IGFBP-6 intracellular increase by Chariot™ was more effective in introducing the IGFBP-6 protein into the cell under non-differentiation conditions before PMSCs commit to the muscle lineage.

The delivery of IGFBP-6 using Chariot™ transport system had no significant effects on pluripotency of PMSCs as indicated by OCT4 levels under both non-differentiation (Figure 12B) and differentiation conditions (Figure 14B). This is a novel finding due to the fact that no studies have been performed before using Chariot™ to increase the intracellular IGFBP-6 in PMSCs and its effects on OCT4 levels. Therefore, further investigation is required.

5.3 IGFBP-6 on Muscle Differentiation

Increasing IGFBP-6 intracellularly using the Chariot™ System, we observed an increase in early muscle marker Pax3/7 at 7days under non-differentiation conditions (Figure 12C), and this increase correlated with IGFBP-6 levels (Figure 12A). In contrast, under differentiation conditions, there were no significant changes in Pax3/7 levels (Figure 14C) correlating with IGFBP-6 levels (Figure 14A). These findings suggest that intracellular IGFBP-6 is more effective in promoting muscle differentiation under non-differentiation conditions before PMSCs commit to the muscle lineage. These data support previous

reports which showed that the expression of IGFBP-6 is associated with differentiation (82).

There was a significant increase in IGFBP-6 levels secreted to the media after treatment with Chariot™ to increase intracellular IGFBP-6 with a significant decrease at day 7 under both non-differentiation conditions (Figure 13) as well as differentiation conditions (Figure 15). It is expected that introduction of IGFBP-6 into the cells is associated with an increase in the secretion of the protein into the media. However, the increase in IGFBP-6 secretion by PMSCs was longer than expected in the cells following introduction of the protein with Chariot treatment, suggesting that the endogenous synthesis of IGFBP-6 may have been stimulated by the increase in intracellular IGFBP-6. The mechanism is still unknown, and requires further studies.

5.4 Silencing IGFBP-6 under Non-differentiation and Differentiation Conditions

IGFBP-6 has a significantly greater affinity (20-100 fold), for IGF-II than IGF-I (83, 84). Since IGF-II is present in developing placental cells in greater levels than IGF-I, the reduction of IGFBP-6 in PMSCs would increase the proliferation effects of IGFs on the cells as it expected that there will be a greater availability of IGF-II.

Using siRNA against IGFBP-6, there was a significant decrease in IGFBP-6 levels, a 50% decrease in IGFBP-6 levels under non-differentiation conditions (Figure 16A) and a 40% decrease under differentiation conditions (Figure 17A). The siRNA treatment was, however, short term under both non-differentiation (Figure 16B) and differentiation conditions (Figure 17B).

One interesting finding was that the pluripotency marker, OCT4, decreased significantly with IGFBP-6 silencing (Figure 16C) and (Figure 17C). The mechanism by which this effect likely occurs is unknown and needs further investigation. Since IGFBP-6 has both intracellular as well as extracellular effects, whether the response occurs dependent or independent of IGFs (particularly IGF-II) has to be delineated.

The corollary finding of a significant increase in early muscle marker Pax3/7 at 24 hours with silencing IGFBP-6 under non-differentiation conditions (Figure 16D), indicates that reduced expression of IGFBP-6 promotes PMSCs towards the muscle differentiation process. A significant increase in Pax3/7 levels at each time point with IGFBP-6 silencing under differentiation conditions further supports this conclusion (Figure 17D). The mechanism by which reduced IGFBP-6 expression leads to enhanced muscle differentiation is unknown. The increase in Pax3/7 could be due to the presence of a greater availability of IGF-II which is recruited to the differentiation process or could be due to the IGFBP-6 independent actions. Future studies will delineate the underlying mechanism.

5.5 Increased IGFBP-6 in PMSC Microenvironment

Since there was a significant decrease in IGFBP-6 levels secreted to the media after silencing IGFBP-6 expression under both non-differentiation (Figure 18) and differentiation conditions (Figure 19), it is also possible that the biologic effect may also be due to an increase in extracellular action of IGF-II. However, the increase in IGFBP-6 by adding recombinant IGFBP-6 extracellularly had no effect.

Normally, increased IGFBP-6 in the mesenchymal stem cell microenvironment is expected to reduce the bioavailability of IGFs (especially IGF-II because of its high affinity for the peptide). Studies in various cell lines have shown mostly an inhibitory action of IGFBP-6 predominantly via IGF-II-dependent action. Human recombinant IGFBP-6 has been shown to inhibit IGF-II induced myogenesis through an IGF-II-dependent mechanism in L6A1 myoblasts (135). Neuroblastoma cells undergo a decrease both in cell proliferation and tumorigenic potency as a result of increased exogenous IGFBP-6 (95, 96). In osteoblast cells, IGFBP-6 has also been shown to bind IGF-II and modulate its effects on cell growth by reducing the bioavailability of IGF-II for its receptor in the bone microenvironment (97). In human bronchial epithelial cells, increased IGFBP-6 induces a proliferative arrest, with no evidence of apoptosis, suggesting an inhibition of IGF-II's ability to induce mitosis (98).

In contrast, our results show no significant effect of increased IGFBP-6 in the PMSC microenvironment (addition of 0.75 μg/ well of purified human IGFBP-6 recombinant protein to the media) under non-differentiation conditions on either its own expression (Figure 20A) or OCT4 (Figure 20B). Similar effects were observed under differentiation conditions (Figure 21A, 21B).

The effect of increased IGFBP-6 in the cellular microenvironment on PMSC differentiation into muscle was determined by western blot analysis for early muscle marker Pax3/7. It showed a significant increase at day 7 under non-differentiation conditions (Figure 20C), indicating that IGFBP-6 induces muscle differentiation even in non-favourable conditions. This effect was not observed in differentiation conditions, suggesting that once the PMSCs are committed into the muscle lineage, IGFBP-6 may not

have further effect. The mechanism of action, whether IGF-dependent or –independent, remains to be delineated.

6.0 SUMMARY AND CONCLUSIONS

To date, no previous studies have been performed relating PMSCs and IGFBP-6. Therefore, the data presented in this thesis gives us an insight into the effects of IGFBP-6 on PMSCs. The data in this thesis demonstrate that placental mesenchymal stem cells have the ability to differentiate into muscle cells *in vitro*, as identified by immunocytochemistry and immunoblotting for early muscle marker Pax3/7 at 3days post-differentiation, and the expression of late muscle markers Myogenin and MyoD at 14-days post-differentiation using confocal imaging. PMSCs expressed IGFBP-6 as demonstrated by immunocytochemistry and immunoblotting of muscle cells. These findings are in agreement with previous reports of PMSCs ability to differentiate into skeletal muscle and that IGFBP-6 may play a role in this process (44, 45, 134).

The Chariot™ method was used successfully to increase intracellular IGFBP-6. Under non-differentiation conditions, there was an increase in Pax3/7 at day7 with no changes in OCT4 levels. In contrast, under differentiation conditions, there were no significant changes in IGFBP-6, Pax3/7, or OCT4. Overall, there was a correlation between IGFBP-6 levels and Pax3/7. Therefore, the effect of intracellular IGFBP-6 is more prominent under non-differentiation conditions before PMSCs commit to the muscle lineage.

Conversely, the effect of decreasing IGFBP-6 expression using siRNA under both non-differentiation and differentiation conditions is transient or short term with return of IGFBP-6 to control levels by day7.

Increasing extracellular IGFBP-6 did not cause changes to some of the parameters analyzed, i.e. expression of IGFBP-6 or OCT4, unlike increasing the protein intracellularly, which is different from previous reports. This could be due to the cells used (PMSCs) or different parameters tested. Further studies are required to determine if extracellular IGFBP-6 has any biologic actions on PMSCs.

Interestingly, there was a significant increase in IGFBP-6 levels secreted to the media after increasing intracellular IGFBP-6 using Chariot^{TM} and a significant decrease after decreasing IGFBP-6 expression using siRNA.

We demonstrated that PMSCs are able to differentiate into skeletal muscle cells under appropriate conditions, and that this process may be regulated by intracellular IGFBP-6. Further studies are required to delineate the mechanisms of action of IGFBP-6 and IGF-II on this biologic effect.

7.0 LIMITATIONS

- As demonstrated in the results, PMSCs underwent differentiation even when cultured in muscle growth media (non-differentiation media). These results needs to be addressed more in order to fully understand why PMSCs undergo myogenesis under non-differentiation conditions.
- PMSCs produce their own IGFBP-6 and it is the most abundant binding protein; therefore, there might be an interaction between the native IGFBP-6 synthesized by the cells and the IGFBP-6 protein added exogenously.

- The Chariot™ system increased IGFBP-6 only transiently and is not very stable once introduced into PMSCs. Hence, a more effective method is needed to further study the effects of increasing the intracellular IGFBP-6 over a period of time.
- The recombinant human IGFBP-6 used in this study is produced in E.Coli and therefore has not undergone any post-translational processing; glycosylated IGFBP-6 shows greater resistance to proteolysis by chymotrypsin and trypsin than non-glycosylated IGFBP-6 (91), which raises questions about whether the effects observed using a recombinant IGFBP-6 represent the effects of native IGFBP-6.

8.0 FUTURE STUDIES

This study provides an important foundation for future studies on the role of IGFBP-6 on differentiation of PMSCs into muscle. Based on the data and observations in this study, further studies can be planned as follows:

- To study the signal transduction mechanisms of IGFBP-6 and IGF-II actions on muscle differentiation of PMSCs (e.g., the mitogen-activated protein kinase and the PI3K/Akt pathways) in the presence or absence of specific ERK1/2 and Akt inhibitors.
- To determine if IGFBP-6 effects on PMSCs are due to IGF-dependent or -independent functions, we will over-express a mutant form of IGFBP-6 that does not bind IGF-II and compare with a wild type form of IGFBP-6.
- To determine the interaction between IGF-II and IGFBP-6 on skeletal muscle differentiation of PMSCs.
- To determine the level of action of IGFBP-6 on pluripotency marker (OCT4) and muscle differentiation markers by determining the mRNA levels of OCT4.

- To identify further siRNAs to improve the silencing of IGFBP-6 to greater than 40-50% and to determine the biologic effects of this knockdown.
- To determine if other pluripotency markers such as NANOG and SOX2 are affected by intracellular or extracellular IGFBP-6.
- To investigate the impact of IGFBP-6 and IGF-II on other biologic effects such as proliferation and cell death (apoptosis) in addition to the differentiation events, as they are well recognized effects of the IGF system.

9.0 SIGNIFICANCE

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

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