INSULIN-LIKE GROWTH FACTOR SIGNALING DURING MYOGENESIS

by

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INTRODUCTION

Myogenesis

Muscle and myogenesis

In mammals, muscle constitutes a large part of the body mass. Muscle is a tissue specialized for contraction. Based on the difference in function, structure, and development, muscle tissue can be divided into four main categories – skeletal muscle, cardiac muscle, smooth muscle, and myoepithelium. This thesis will focus on the molecular mechanisms regarding skeletal muscle development.

Skeletal muscle, under the control of nervous system, is responsible for voluntary movements. Mammalian skeletal muscle is composed of large cells with elongated shape. Therefore, these muscle cells are often referred to as muscle fibers. There are two broad types of voluntary muscle fibers: slow twitch and fast twitch, which are innervated by different motor neurons (Pette, 2002). Slow twitch fibers contract for long periods of time but generate less force, while fast twitch fibers contract more rapidly and powerfully but fatigue very rapidly. The different contractile properties are imparted by the expression of specific myosin isoforms. Recent studies have revealed that fiber composition is regulated by signaling molecules and transcriptional factors, such as calcineurin, CaM kinase, Hedgehog, and peroxisome-proliferator-activated receptor-gamma co-activator (PGC) (Arany et al., 2007; Blagden et al., 1997; Du et al., 1997; Lin et al., 2002).

Each skeletal muscle fiber is a syncytium, containing many nuclei within a common cytoplasm. The multinucleated skeletal muscle cells are formed by the fusion of muscle precursor cells – myoblasts. Myogenesis is a highly ordered process (Buckingham et al., 2003). During embryonic development, the muscle precursor cells commit to myogenic linage. After a period of proliferation, the myoblasts withdraw from cell cycle, express muscle-specific genes, and eventually fuse with one another to form multinucleated myotubes. This process is known as myoblast differentiation. It is subjected to the intricate regulation of intrinsic muscle specific transcription factors, such as myogenic regulatory factors (MRFs) (Weintraub, 1993), and extrinsic growth hormones.

Mature muscle cells express muscle-specific proteins matching with specific physical activities. Of note, before myoblast differentiation, the presence of these proteins is either not detectable or exist in very low concentrations. Proteins, that are parts of the contractile apparatus, are abundantly expressed, including specific isoforms of actin, myosin, tropomyosin, and troponin. Muscle tissue consumes much of the body's energy and produces adenosine triphosphate (ATP) to power the contractile apparatus. In line with this, muscles adopt specialized metabolism by expressing creatine kinase to regenerate ATP during burst activity (van Deursen et al., 1993). Mature muscle cells also express acetylcholine receptors on the surface within specialized neural muscular junction called synapse, which renders them sensitive to the stimuli coming from the nerve terminal.

Terminal myoblast differentiation and proliferation are often considered as two mutually exclusive events. After differentiation, the multinucleated muscle cells do not divide and the nuclei stop replicating their DNA. In humans, the number of multinucleated skeletal muscle fibers is determined before birth. Postnatal muscle growth is mainly achieved

by muscle hypertrophy – the increase in the size of muscle fibers without an increase in muscle fiber number. The increase in muscle fiber size is achieved by individual myoblasts being recruited to and subsequently fusing with existing multinucleated muscle fibers.

Transcription factors in myogenesis

Myoblast differentiation is controlled by at least two families of transcription factiors – the *MyoD* family of basic helix-loop-helix proteins (also known as MRFs) and the *Myocyte enhancer factor-2* (*MEF2)* family of MADS box proteins (Fig. 1.1). MRF is a family of helix-loop-helix proteins including MyoD, Myf5, Myogenin, and Mrf4 (Weintraub, 1993). These transcription factors are normally expressed in muscle cells.

MyoD was initially identified by its ability to convert non-myogenic cells into myoblasts (Davis et al., 1987). Davis et al. screened a myocyte cDNA library and cloned *MyoD* cDNA. Expressing *MyoD* under the control of a viral promoter converted C3H10T1/2 fibroblasts into stable myoblasts (Davis et al., 1987). Later, *Myogenin* was identified as a gene homologous to *MyoD*. Transfection of *Myogenin* into C3H10T1/2 fibroblasts induced the expression of muscle-specific markers (Wright et al., 1989). The highly basic region in MyoD, immediately upstream of the helix-loop-helix motif, was identified as a critical region for muscle-specific gene activation (Davis et al., 1990). Crystal structure of MyoD basic helix-loop-helix domain – DNA complex was solved and provided insights for myogenic induction based on protein-DNA interaction (Ma et al., 1994). Specifically, two critical residues required for the myogenic activities, Ala-114 and Thr-115, are not available for protein-protein interaction but are buried at the protein-DNA interface instead (Ma et al., 1994).

Considerable efforts have been taken to delineate the roles of MRFs regulating myogenesis. The majority of these studies used transgenic mice approach. Considering the dominant role of *MyoD* in myogenesis, it is rather surprising that mice lacking *MyoD* are viable and fertile (Rudnicki et al., 1992). *Myf5* knockout mice reveal no morphological abnormalities in the skeletal muscle (Braun et al., 1992). These results suggest that *MyoD* and *Myf5* may act redundantly during skeletal muscle morphogenesis. Indeed, *Myf5* mRNA levels are elevated in postnatal *MyoD* null mice, indicating functional overlapping of *MyoD* and *Myf5* (Rudnicki et al., 1992). Combined inactivation of *MyoD* and *Myf5* results in a complete lack of skeletal muscle formation (Rudnicki et al., 1993). Together, these studies suggest that either *Myf5* or *MyoD* is required for skeletal myoblasts development and indicate that these factors play functionally redundant roles in myogenesis.

Among the four MRFs, *Mrf4* displays unique biphasic expression pattern (Bober et al., 1991; Hinterberger et al., 1991). *Mrf4* expresses transiently in the somite myotome between embryonic days 9 and 12 and is then repressed (Hinterberger et al., 1991). *Mrf4* expression increases again sharply during late fetal muscle development, and *Mrf4* continues to be expressed at a high level after birth (Bober et al., 1991). The high level of *Mrf4* expression is maintained in adulthood which, in contrast with the decreased expression of the other three muscle regulatory factors, makes *Mrf4* the predominant factor in adult muscle (Hinterberger et al., 1991). Germ-line inaction of functional *Mrf4* causes subtle reductions in the expression of certain muscle-specific genes with dramatically elevated *Myogenin* expression, suggesting potential compensatory effect from *Myogenin* (Patapoutian et al., 1995; Zhang et al., 1995). *Myogenin* knockouts have widespread muscle loss (Hasty et al., 1993; Nabeshima et al., 1993). More specifically, *Myogenin* null mutants have severely

reduced skeletal muscle fiber density in the limbs, tongue, and diaphragm (Hasty et al., 1993). In these affected regions, multinucleated muscle fibers are replaced by mononucleated myoblasts, indicating defective myoblast terminal differentiation. MyoD expression, however, remains comparable between mutant and wild-type mice. Therefore, this suggests that Myogenin is important for terminal differentiation and cell fusion, but not cell commitment to myogenic lineage (Hasty et al., 1993).

In addition to MRFs, the *MEF2* family proteins also regulate myogenesis. *MEF2* was first identified as a factor with muscle-specific DNA binding activity (Gossett et al., 1989). There are four members of *MEF2* family (*MEF2A, MEF2B, MEF2C, and MEF2D*), and they all contain characteristic MADS domain (Potthoff and Olson, 2007). Of the four members in *MEF2* family, *MEF2C* is particularly interesting based on its temporal and spatial expression pattern during myogenesis. *MEF2C* is expressed in the skeletal muscle cells of somite myotome, which precedes the expression of the other *MEF2* genes (Edmondson et al., 1994). After birth, *MEF2C* expression is restricted to skeletal muscle, brain, and spleen, while other *MEF2* genes show ubiquitous expression (Martin et al., 1993; McDermott et al., 1993).

MRF and *MEF2* families act in a cooperative way to regulate myogenesis. When introduced into nonmuscle cell types, each of the four MRFs, MyoD, myf5, Myogenin, and MRF4, can induce myogenic differentiation (Emerson, 1993; Lassar and Munsterberg, 1994; Olson, 1990; Wright, 1992). In contrast, MEF2 family members cannot induce myogenesis in transfected fibroblasts (Molkentin et al., 1995). When coexpressed with MyoD or Myogenin, they dramatically increase the extent of myogenic conversion compared with expressing either MyoD or Myogenin alone (Molkentin et al., 1995). Furthermore, it is demonstrated

that the synergistic effect requires direct interactions between MEF2 and MRF (Molkentin et al., 1995).

Signaling pathways regulating myogenesis

Myoblast differentiation is intricately regulated by a plethora of transcriptional factors and growth factors. Together, these cell autonomous and non-autonomous cues regulate cell proliferation and differentiation and therefore ensure the proper progression of myogenesis. The effect of various growth factor signaling pathways on myogenesis will be discussed here with the exception of Insulin-like growth factor (IGF) signaling, which will be further addressed in a separate segment (Fig. 1.2).

Hedgehog has been shown as an important myogenic regulator *in vivo*. It is secreted from the notochord and ventral neural tube, and has been shown to mediate the induction and expansion of muscle-specific gene expression during embryonic development (Brand-Saberi, 2005). In mouse embryos, sonic hedgehog has been proposed as a survival factor and regulates muscle patterning and development through *Myf5* activation (Borycki et al., 1999; Chiang et al., 1996; Kruger et al., 2001). Another factor secreted by neural tube is Wnt family proteins. It is demonstrated that combining sonic hedgehog with Wnt family members is sufficient to induce myogenic gene expression in the somite (Munsterberg et al., 1995).

Fibroblast growth factors (FGFs) are induced by retinoic acid (RA) and regulate myogenesis (Groves et al., 2005; Hamade et al., 2006). Inhibiting RA production reduces expression of the myogenic markers myoD and myogenin in somites, while exogenous RA induces increased expression of these genes and strongly induces premature myoD expression in the presomitic mesoderm. Hamade et al. further demonstrates that FGF8 expression in the somites is regulated by RA and RA fails to promote myogenic gene

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expression in the absence of FGF8 signaling (Hamade et al., 2006). Reciprocal and complex regulation between MRFs and FGF signaling has been documented. Inhibiting FGF receptor 4 (FGFR4) leads to down-regulation of *MyoD* and *Myf5* (Marics et al., 2002). Ectopic expression of either *MyoD* or *Myf5* can induce *FGFR4* expression (Delfini and Duprez, 2004).

In addition to the signaling pathways regulating muscle development, other pathways have evolved to control muscle growth. One well-known signaling molecule is myostatin. Myostatin belongs to the TGFβ super-family of signal proteins, and it is normally made and secreted by skeletal muscle cells. Myostatin provides negative feedback to limit muscle growth. In cattle breeding, two breeds of cattle that were bred for large muscles have both turned out to harbor mutations in *myostatin* gene. Mice with a loss-of-function mutation in the *myostatin* gene have enormous muscles with increase in both the number and the size of the muscle cells (Lee and McPherron, 1999). *In vitro* recombinant myostatin proteins inhibit cell proliferation, DNA synthesis, and protein synthesis in C2C12 myoblasts, suggesting that myostatin negatively regulates muscle mass by inhibiting muscle growth or regeneration (Taylor et al., 2001). Normally, small amount of myostatin can be detected in the circulation of adult humans. Increased myostatin is associated muscle wasting in patients with chronic diseases, such as human immunodeficiency virus (HIV) and cancer (Gonzalez-Cadavid et al., 1998; Hellerstein et al., 1990; Sellmeyer and Grunfeld, 1996). In addition, TGFβ signaling is reported to be constitutively active in aged muscle precursor cells, which potentially links to age-related muscle defects (Beggs et al., 2004).

Another signaling pathway that has been gathering general public interest is steroid hormone. Anabolic steroids increase protein synthesis in cells. As a result, cell mass

increases. In muscle tissue, anabolic steroids lead to hypertrophy and increased physical strength. These hormones are often used in medical treatment for chronic wasting (Basaria et al., 2001). Because of the performance enhancing effects, anabolic steroids may be abused and can cause adverse side effects (Cohen and Hickman, 1987; De Piccoli et al., 1991; Wilson, 1988). Other steroid hormones also regulate muscle metabolism and physiology. used in treating muscle-related diseases. For example, glucocorticoids and its derivatives have shown long-term beneficial effects in slowing the progression of muscular dystrophy (DeSilva et al., 1987; Fenichel et al., 1991; Mendell et al., 1989).

IGF

The IGF ligands

Insulin-like growth factors (IGFs), including IGF-I and IGF-II, are small polypeptide hormones with an approximate molecular weight of 7 kDa (Daughaday and Rotwein, 1989). They are members of the structurally related insulin/IGF super family. Mature IGF-I and IGF-II peptides consist of A, B, C, and D domains. The A and B domains of IGFs are homologous to the A and B domains of insulin, and the C domains of IGFs share sequence homology to the C peptide of proinsulin which is cleaved off in mature insulin during prohormone processing. IGFs contain an additional D domain, which is not found in insulin (Adamo et al., 1993; LeRoith and Roberts, 1993).

The synthesis of IGF-I, IGF-II, and insulin is regulated by distinct mechanisms (Jones and Clemmons, 1995). Unlike insulin and other peptide hormones, IGFs are not synthesized and stored within specialized cells in a given tissue, but are ubiquitously produced and

released by virtually every tissue. Insulin concentration *in vivo* is primarily gauged by blood glucose fluctuation. IGF-I synthesis is regulated by a variety of factors, including pituitary growth hormone (GH). And, IGF was initially discovered by its ability to stimulate cartilage sulfation and to replace the "sulfation factor activity" of GH (Salmon and Daughaday, 1957). The expression of *IGF-I* and *IGF-II* genes is subjected to complex regulatory mechanism at the levels of transcription and translation, including, but not limited to, multiple promoters, different transcription initiation sites, alternative splicing, and different polyadenylation signals (Hall et al., 1992; Kim et al., 1991). For instance, IGF-II expression in human liver has been shown to be regulated by four promoters with three promoters used in the fetal liver and all four promoter used from the age of 2 months after birth (Li et al., 1996). In addition, *IGF-II* gene is imprinted - only one allele is active, depending on parental origin - and this pattern of expression is maintained epigenetically in almost all tissues (Chao and D'Amore, 2008).

IGFs are critical for normal growth in mammals and other vertebrates. *IGF-I* or *IGF-II* knockout mice have 60% the birth weight of normal control mice (Baker et al., 1993; Liu et al., 1993). *IGF-I* knockout mice also have increased neonatal death rate and reduced postnatal growth rate if they survive. Mice with null mutations in both *IGF-I* and *IGF-II* die invariably after birth (Baker et al., 1993; Liu et al., 1993). Compared with *IGF-I* knockout mice, *GH/IGF-I* double-knockout mice have more dramatically reduced body growth weighing only approximately 17% of normal mice (Lupu et al., 2001). Therefore, the GH-IGF pathway is the main determinant of the body growth. IGF-I infusion restores growth in GH-deficient mice (Guler et al., 1988). Administration of IGF-I to rats increases protein synthesis and body growth (Tomas et al., 1992). Overexpression of IGF-I in mice increases

the body weight by 30% (Mathews et al., 1988). Loss of imprinting (LOI) caused IGF-II overexpression is often associated with somatic overgrowth (Morison et al., 1996; Morison and Reeve, 1998). Beckwith-Wiedemann syndrome (BWS) is one example. It is characterized by fetal and neonatal overgrowth, and is often accompanied by an increased risk of childhood cancers (Morison and Reeve, 1998). Tissues that are affected by hyperplasia and malignancy in BWS are those having the highest levels of expression of IGF-II (Hedborg et al., 1994).

IGFs are also important in the development and function of the central nervous system (CNS), skeletal muscle, and reproductive organs. In humans, a homozygous partial deletion of *IGF-I* gene is associated with mental retardation and sensorineural deafness, in addition to prenatal and postnatal growth retardation (Woods et al., 1996). At the cellular level, significant decrease in auditory neuron number and increased apoptosis of cochlear neurons are observed in *IGF-I* knockout mice (Camarero et al., 2001). Characteristic underdevelopment of muscle tissue is observed in more than 95% of perinatally dead *IGF-I*-null pups (Powell-Braxton et al., 1993). IGF-I deletion in mice causes infertility (Baker et al., 1996). Male mice have drastically reduced testosterone levels, and female mice fail to ovulate and have uterus with hypoplastic endometrium (Baker et al., 1996).

IGFs are generally considered as potent survival factors and mitogens. Abnormally high levels of IGFs are found in various tumor cells (LeRoith and Roberts, 2003). Epidemiological studies have identified increased level of IGF-I level as a risk factor for development of breast, prostate, colon, and lung cancer (LeRoith and Roberts, 2003). Reduced circulating IGF-I levels are associated with Type I diabetes, and IGF-I treatment improves glucose and protein metabolism and attenuates diabetic cardiomyopathy (Carroll et

al., 2000; Norby et al., 2002). Overexpression of IGF-I in mouse pancreatic β cells

specifically leads to improvement of type I diabetes (George et al., 2002). IGF-I treatment

increases insulin sensitivity and improves glycemic control in patients with type 2 diabetes

(Moses et al., 1996). In addition, IGF-I has been shown to have beneficial effects on bone,

muscle and neuronal tissues. Overexpression of IGF-I in the osteoblasts of transgenic mice

leads to improved bone structure, including increased bone density and mineralization (Zhao

et al., 2000). IGF-I overexpression in skeletal muscle tissue caused muscle hypertrophy and

sustained regenerative capacity (Barton-Davis et al., 1998; Musaro et al., 2001). IGF-I

expression in the central nervous system positively correlates with increased brain growth,

promoted neurogenesis, process outgrowth, and synaptogenesis, and inhibited neuronal

apoptosis (D'Ercole et al., 2002). IGF-I treatment delays amyotrophic lateral sclerosis (ALS)

progression in a mouse model (Kaspar et al., 2003).

The IGF-I receptor

At the cellular level, IGFs induces a variety of cellular responses, including cell

proliferation, differentiation, migration, and survival. IGFs exert these biological actions

primarily through the type I IGF receptor (IGF-IR) (Fig.1.3). The IGF-IR has two

extracellular α subunits and two transmembrane β subunits linked by disulfide bonds. The α

subunit contains a cysteine-rich IGF binding site. The β subunit has tyrosine kinase activity,

which depends on an ATP binding and tyrosine phosphorylation. Binding to ligand will

induce conformational changes of the receptor and facilitate tyrosine autophosphorylation, an

event eventually leading to receptor activation (Hubbard, 1997; Wei et al., 1995). Due to its

functional importance, the tyrosine cluster in the β subunit has been subjected to intense

study (Cianfarani et al., 2007).

The IGF-IR exhibits high sequence identity with the insulin receptor (IR) (Abbott et al., 1992; LeRoith et al., 1995). Given the significant structural similarity between IGFs and insulin, and their respective receptors, it is not surprising that cases of cross-activation and hybrid receptors have previously been reported, though the functional importance of these interactions still remains elusive (Soos et al., 1990; Taguchi and White, 2008).

Ligand binding of the IGF-IR induces its autophosphorylation. The activated IGF-IR in turn activates multiple signal transduction cascades, including the phosphatidylinositol 3-kinase (PI3K)-Akt cascade and the Raf- mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase kinase (MEK)-extracellular signal-regulated kinase (Erk) cascade (Cianfarani et al., 2007; Coolican et al., 1997; Duan et al., 2000; Imai and Clemmons, 1999; White, 2003). IGF-IR interacts with and signals through the adaptor molecules including Insulin Receptor Substrate (IRS) and the Src homology collagen (Shc) proteins (p46/p52/p66) (Dupont and LeRoith, 2001). IRS-1 as one of the well-established primary substrates has been extensively studied (Lavan et al., 1997a; Lavan et al., 1997b; Patti et al., 1995; Sun et al., 1991). IRS-1 acts as a multisite 'docking' protein by binding to downstream signal-transducing molecules. Phosphorylation of multiple tyrosine residues results in the association of IRS-1 with the Src homology 2 (SH2) domains of other cytoplasmic signaling proteins, including PI3K and growth factor receptor-bound protein 2 (Grb2). Activated PI3K synthesizes membrane associated phosphorylated inositols, which in turn activate phosphoinositol-dependent kinases (PDKs). PDKs then activate other protein kinases including Akt/Protein Kinase B, p70S6K, and protein kinase C (PKC) (Cianfarani et al., 2007). The activated IGF-IR also recruits the guanine-nucleotide-exchange factor Sos to IRS-1 through the SH2 domain of the adaptor Grb2 (Dupont and LeRoith, 2001). This leads

to the activation of the small G-protein Ras, which activates the protein serine kinase Raf and the Erk signaling cascade.

The IGF-IR is a key component mediating IGF functions *in vivo*. Due to the ubiquitous expression pattern, nearly every tissue is adversely affected by a lack of IGF function, including skeletal muscle, bone, lung, skin, and nervous system (Allan et al., 2001; Bondy et al., 1990; Jones and Clemmons, 1995). For example, mice lacking a functional IGF-IR gene exhibited even more severe growth retardation, weighing only 45% that of their wild type littermates, and these mice died shortly after birth from respiratory failure (Baker et al., 1993; Liu et al., 1993). Conditional knockout is used to further characterize the IGF-IR function in individual tissues and has yielded detailed information with IGF function in specific tissue and development. Selectively disrupting *IGF-IR* gene in mouse osteoblasts caused a striking decrease in bone volume, connectivity, and trabecular number, which is also accompanied by a significant decrease in the rate of mineralization (Zhang et al., 2002). Deletion of the *IGF-IR* in pancreatic β cells caused defects in glucose-stimulated insulin secretion and impaired glucose tolerance (Kulkarni et al., 2002). IGF-IR inactivation in the brain impaired remyelination in response to neurotoxicant induced demyelination (Mason et al., 2003). Conditional IGF-IR inactivation in adipose tissue did not affect adipogenesis and instead resulted in increased adipose tissue mass (Kloting et al., 2008). Insulin-stimulated glucose uptake into adipocytes was unaffected by the deletion of the IGF-1R. Surprisingly, IGF-IR deletion in adipose tissue led to elevated IGF-I concentration in circulation and had a systemic effect on somatic growth (Kloting et al., 2008). IGF-IR conditional knockout in the liver decreased the capacity for regeneration and revealed IGF-IR/IRS-1/Erk signaling cascade as the intracellular pathway controlling the cell cycle progression in the regenerating

liver (Desbois-Mouthon et al., 2006). Of note, the *in vivo* function of the IGF-IR is evolutionarily conserved, as studies in zebrafish have generated similar results. Schlueter et al. showed IGF-IRs in zebrafish are required for embryo viability and proper growth, and especially the development of eye, inner ear, heart, and muscle (Schlueter et al., 2006). Further study, using antisense morpholino oligonucleotides (MO) or a dominant-negative IGF-IR fusion protein, revealed the cellular actions of this essential pathway during vertebrate embryogenesis (Schlueter et al., 2007). At the cellular level, IGF1R inhibition increased caspase activity and neuronal apoptosis. Coinjection of antiapoptotic bcl2-like mRNA attenuated the elevated apoptosis. Cell cycle analysis demonstrated cell cycle progression defects in IGF-IR-deficient embryos independent of apoptosis (Schlueter et al., 2007).

The IGF-II receptor

The IGF-II receptor (IGF-IIR) also acts as a mannose-6-phosphate (M6P) receptor and is distinct from the IGF-IR both structurally and functionally. The IGF-IIR is a monomeric protein with an extracellular domain composed almost exclusively of 15 cysteine-based repeats. IGF-IIR preferentially binds to IGF-II, and it has about 100 times less affinity for IGF-I than IGF-II. The IGF-IIR binds and targets IGF-II for lysosomal degradation without inducing a specific cellular response, as it has no intrinsic tyrosine kinase activity (Kornfeld, 1992). Mouse knockout models of the IGF-IIR suggest that it mainly functions as a sink for IGF-II to prevent overgrowth during fetal development (Wylie et al., 2003). Loss of the imprinted IGF-II/M6P receptor results in fetal overgrowth and perinatal lethality (Lau et al., 1994).

Interestingly, recent reports showed that IGF-II binding to IGF-II/M6P receptors stimulates the Erk MAPK cascade by triggering sphingosine kinase (SK)-dependent transactivation of sphingosine-1 phosphate (S1P) receptors. (El-Shewy et al., 2007; El-Shewy HM, 2008). Both IGF-I and IGF-II potently activated Erk MAPK in HEK293 cells. IGF treatment promoted translocation of SK from the cytosol to the plasma membrane and thereby caused a significant increase in S1P concentration, which is essential for IGF-stimulated Erk MAPK activation (El-Shewy et al., 2006). Endogenous IGF-IR and IGF-IIR can independently initiate Erk MAPK signaling (El-Shewy et al., 2007). Knockdown of IGF-IR expression by RNAi reduced the IGF-I response to a greater extent than the IGF-II-induced response. In contrast, IGF-IIR knockdown markedly reduced IGF-II-stimulated Erk phosphorylation, with no effect on the IGF-I-induced response. El-Shewy et al. further demonstrated that PKC mediates IGF-II activated Erk MAPK phosphorylation via SK activation (El-Shewy HM, 2008).

The IGF binding proteins

Besides IGFs and IGF receptors, another key component of the IGF signaling pathway is IGF binding proteins (IGFBPs). IGFBPs are a family of secreted proteins that specifically bind IGF-I and IGF-II with high affinities that are equal to or greater than those of the IGF-IR. IGFBPs function as carrier proteins for circulating IGFs and regulate IGF turnover, transport, and tissue distribution, thus determining physiological concentrations of IGFs (Jones and Clemmons, 1995). In addition, compared to insulin, IGF is present in higher concentrations in extracellular body fluid. However, IGF predominantly exists in complex with IGFBP. Thus, the IGF/IGFBP complexes in circulation and tissues help to prevent

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potential hypoglycemic effect generated by cross-binding of IGFs to the insulin receptor (Rajaram et al., 1997).

There are six members of IGFBP family, including IGFBP-1 to -6. They have been isolated and characterized from human and a variety of vertebrate species (Duan, 2002; Duan and Xu, 2005; Firth and Baxter, 2002). All six IGFBPs share a common domain structure arrangement (Fig. 1.4). They all have a highly conserved N-terminal domain (N domain) and C-terminal domain (C domain), and a variable central domain (L domain). The N-domain and C-domain contain multiple conserved cystine residues, which form intra-domain disulfide bonds within the N domain and C domain, thereby defining their overall globular structure (Chelius et al., 2001; Forbes et al., 1998; Neumann and Bach, 1999). Although the crystal structure of full length IGFBP has not been resolved, the L domain is often predicted as a flexible linker region connecting both N and C domain, based on the fact that it contains no intra-domain disulfide bonds (Firth and Baxter, 2002).

The N-domain contains the major high affinity IGF-binding site, but the C-domain also contributes to the ligand binding to some degree (Brinkman et al., 1991; Clemmons, 2001; Hobba et al., 1998; Zeslawski et al., 2001). The molecular details of IGF-IGFBP interaction have been described using data generated from structural analysis. Kalus et al. have produced two N-terminal fragments of IGFBP-5 in Escherichia coli – the first encoding the N-terminal domain of the protein (residues 1-104) and the second, named as mini-IGFBP-5, comprising residues Ala40 to Ile92 (Kalus et al., 1998). The complex formed by IGF and mini-IGFBP-5 shows a prototype interaction for all N-terminal domains of the IGFBP family. The solution structure of mini-IGFBP-5 was determined by nuclear magnetic resonance (NMR) spectroscopy. It displays a rigid, globular structure that consists of a

centrally located three-stranded anti-parallel beta-sheet, and further stabilized by two inside packed disulfide bridges. Several amino acid residues located in a hydrophobic patch on the surface of IGFBP-5 are determined as critical residues mediating its interaction with ligand (Kalus et al., 1998). Consistent with the results of structural analysis, mutation of five amino acid residues (Lys68Asn, Pro69Gln, Leu70Gln, Leu73Gln, and Leu74Gln) in the N-domain of IGFBP-5 results in a 1000-fold reduction in its affinity for IGF-I (Imai et al., 2000). IGFBP-5 with these five amino acid residues mutated is thereafter referred to as the ligand binding domain (LBD) mutant. However, the smaller N-terminal domain of the protein (residues 1-104) and mini-IGFBP, compared with the full-length IGFBP-5, binds to IGF less efficiently, indicating the contribution of C domain to IGF binding (Kalus et al., 1998). Indeed, mutations of two amino acid residues (G203 and Q209) in the IGFBP-5 C-domain reduced its binding affinity by seven and six fold, respectively (Bramani et al., 1999). This is further confirmed by X-ray crystallography using IGF-I, the N-terminal domain of IGFBP-4 (NBP-4, residues 3–82) and the C-terminal domain of IGFBP-4 (CBP-4, residues 151–232). Although CBP-4 does not bind individually to either IGF-I or NBP-4, in the ternary complex, CBP-4 contacts both (Siwanowicz et al., 2005). Therefore, both the N-domain and the C-domain of IGFBP-5 are important for its IGF-binding capacity, with the N-domain being its predominant IGF-binding site.

IGF binds to IGFBP and IGF-IR at different regions. Zeslawski et al. reported that a solvent-exposed hydrophobic patch is located on the IGF-I pole opposite to the mini-IGFBP-5 binding region and marks the IGF-I receptor binding site (Zeslawski et al., 2001). Binding of IGFBP to IGF excludes its accessibility to IGF-IR. X-ray structure of the ternary complex of IGF-I, the N-terminal fragment, and the C-terminal fragment of IGFBP-4 or IGFBP-1

demonstrated that the C-terminal region of IGFBP blocks the IGF-IR binding region of IGF-I (Sitar et al., 2006; Siwanowicz et al., 2005).

The C-domain often mediates the interactions between IGFBPs with other proteins. For instance, both IGFBP-3 and IGFBP-5 bind to the acid-labile subunit (ALS) through their C-domains (Firth and Baxter, 2002; Guler et al., 1987). The ternary complex (IGF-IGFBP-ALS) not only serves as a reservoir for IGF release, but also greatly increases the half-life of IGFs and contributes to transport and protection of IGF. Circulating IGF system is severely disrupted in ALS knockout mice (Ueki et al., 2000). Specifically, circulating IGF-I showed a 65% reduction in ALS knockout mice (Yakar et al., 2002). IGF-IGFBP-3-ALS is the predominant ternary complex in circulation (Jones and Clemmons, 1995). IGFBP-5 level in circulation is less than 10% of total IGFBPs (Rajaram et al., 1997). This is consistent with the fact that IGFBP-5 has low expression in the liver but high expression in peripheral tissues (Schneider et al., 2000).

Of the three domains, the central L-domain is the least conserved and often contains sites for post-translational regulation, including glycosylation, phosphorylation, and proteolysis (Clemmons, 2001; Firth and Baxter, 2002). The post-translational modification is important for IGFBP function in terms of regulating IGF availability, which will be further illustrated in details in the following section.

Despite the significant sequence homology among the six IGFBPs, each IGFBP exhibits distinct structural and biochemical properties based on glycosylation, ALS binding sites, integrin binding sites, nuclear localization signals, phosphorylation, and proteolysis (Duan, 2002; Duan and Xu, 2005). For example, human IGFBP-1 and IGFBP-2 have an arginine-glycine-glutamate (RGD) sequence in their C-domain. The RGD sequence mediates

the binding of these IGFBPs to integrins. Indeed, IGFBP-1 has been shown to bind specifically to the α5β1 integrins and stimulate cell motility (Jones et al., 1993c). Intriguingly, both IGFBP-3 and IGFBP-5 have a heparin binding motif (HBM) in their C-domain. HBMs have been found in proteins that capable of binding to glycosaminoglycan (GAG) side chains of many cell surface and extracellular matix (ECM) proteins. Biochemical studies have confirmed that IGFBP-3 and IGFBP-5 are associated with the cell surface and extracellular matrix (Jones et al., 1993b).

The function of IGFBPs

Cellular actions of IGFBPs

IGFBP regulates the endocrine functions of IGF by increasing the halflife as well as facilitating the transportation and tissue distribution of of IGF ligand. For example, the ternary complex formed between IGF, IGFBP-3 and ALS is the major existing form of IGF in circulation (Jones and Clemmons, 1995). IGFBP-1 is predominantly expressed in liver. Its expression is subjected to nutrient and hypoxic stress regulation (Jones and Clemmons, 1995; Kajimura et al., 2005b; Seferovic et al., 2008).

In addition to the endocrine function, IGFBPs also modulate IGF autocrine and paracrine activity in local tissues. The expression of most IGFBPs, including IGFBP-2 to -6, is detected in many peripheral tissues. In culture, most mammalian cells express more than one IGFBPs (Jones and Clemmons, 1995). IGFBPs bind to IGFs with high affinity and are subjected to a variety of post-translational regulations. Therefore, they have been postulated as local regulators for paracrine and autocrine IGF actions. The six IGFBPs exhibit diverse

actions in modulating IGF actions, including cell proliferation, differentiation, survival, and migration (Firth and Baxter, 2002; Jones and Clemmons, 1995). IGFBP can both inhibit and potentiate IGF actions, depending on specific cellular context and experimental conditions(Firth and Baxter, 2002). In vascular smooth muscle cell (VSMC), when added together with IGF-I, exogenous IGFBP-4 exerts inhibitory effect on IGF-I-induced DNA synthesis, while IGFBP-5, on the other hand, potentiates the effect of IGF-I (Duan and Clemmons, 1998). Hsieh et al. examined the effects of individual IGFBPs in VSMC. IGFBP-2 and IGFBP-4 both inhibit IGF-I-stimulated DNA synthesis and cell migration. IGFBP-5 has an inhibitory effect on IGF-I-stimulated DNA synthesis, but it strongly potentiates IGF-I-induced cell migration (Hsieh et al., 2003).

Some IGFBPs have been shown to have intrinsic biological activities that are IGF-independent. It was reported that the binding of IGFBP-1 to integrins via its RGD motif stimulates cell migration (Jones et al., 1993c). Intriguingly, both IGFBP-3 and IGFBP-5 have a nuclear localization sequence (NLS) in their C-domain. Several studies have demonstrated the nuclear localization of IGFBP-3 and IGFBP-5 (Li et al., 1997; Schedlich et al., 2000; Schedlich et al., 1998; Wraight et al., 1998; Xu et al., 2004; Zhao et al., 2006). Exogenously added radioactive IGFBP-5 is translocated into the cell nucleus (Xu et al., 2004). Fusion protein composed of a peptide corresponding to the NLS of IGFBP-5 and enhanced green fluorescence protein (EGFP) is targeted to the nucleus (Schedlich et al., 2000). Nuclear localization of endogenous IGFBP-5 was detected in the mouse embryonic skeletal cells (Zhao et al., 2006). Altogether, these studies suggest that IGFBP may be present in the nucleus. However, the nuclear function of these IGFBPs remains elusive.

In vivo functions of IGFBPs

Gain-of-function approach has often been used to elucidate the *in vivo* actions of IGFBPs using the mouse genetic model (Schneider et al., 2000; Silha and Murphy, 2002). Ubiquitous expression of IGFBP-1, -2, -3 and -5 in transgenic animals leads to growth inhibition (Salih et al., 2004; Silha and Murphy, 2002). Special caution needs to be guarded when interpreting the various phenotypes exhibited by these transgenic animals, because the transgene expression may not correlate with normal physiological conditions.

In order to address the physiological function of endogenous IGFBPs, loss-of-function approaches have been employed in mouse and zebrafish models. IGFBP-1 knockout mice do not exhibit obvious overall growth phenotype under normal conditions, but their livers are more sensitive to apoptotic stimuli and show impaired liver regeneration after hepatectomy (Leu et al., 2003a; Leu et al., 2003b). This phenotype is consistent with the fact that IGFBP-1 is a minor IGF carrier in circulation under normal conditions. Its circulating level is very low under normal, fed condition, but is up-regulated by low nutrient levels and stress (Tazuke et al., 1998). Pintar and colleagues have reported that while IGFBP-2-null mice have normal total body weight, these animals have altered internal organ size, including smaller spleen and bigger liver (Wood et al., 2000). Gender-specific changes in skeletal structure are observed in IGFBP-2 knockout mice (DeMambro et al., 2008). IGFBP-2 deficient male mice have reduced cortical bone area and decreased trabecular bone volume fraction, which correlates with lower numbers of osteoblasts and osteoclasts and reduced serum osteocalcin (DeMambro et al., 2008). IGFBP-5 knockout mice have subtle defects of mammary gland involution, with the whole-body growth, selected organ weights, and body composition are essentially normal (Ning et al., 2007). It is reasonable to speculate that

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functional compensation by other members of the IGFBP family may have prevented the manifestation of more dramatic phenotypes in single IGFBP knockout mice. Indeed, elevated levels of IGFBP-1, IGFBP-3, IGFBP-5, and IGFBP-4 were detected in the IGFBP-2-null mice (DeMambro et al., 2008; Wood et al., 2000). Mice lacking either IGFBP-3, -4, or -5 had no apparent deficiencies in growth or metabolism. Mice homozygous for targeted defects in IGFBP-3, -4, and -5 showed significantly diminished postnatal growth and enhanced glucose metabolism (Ning et al., 2006). Triple knockout mice also demonstrated significantly smaller quadriceps muscle. Moreover, the complexity of the regulatory roles of IGFBPs in growth has been shown by a recent study focusing on IGFBP-4 (Ning et al., 2008). Although the *in vitro* studies have shown IGFBP-4 to be exclusively inhibitory, mice lacking IGFBP-4 leads to a prenatal growth deficit correlating with the timing when IGF-II growth deficit first arises, which strongly suggests that IGFBP-4 is required for optimal IGF-II-promoted growth during fetal development. However, mice encoding a mutant IGFBP-4 protease (pregnancy-associated plasma protein-A, PAPP-A), which facilitates IGF-II release from an inactive IGF-II/IGFBP-4 complex *in vitro*, are even smaller than IGFBP-4 mutant mice. And, the more modest growth deficit is completely restored in double *IGFBP-4/PAPP-A*-knockout mice. Taken together, these results elegantly not only demonstrate that IGFBP functions as a local reservoir to optimize IGF actions during normal embryogenesis, but also establish that proper post-translational modulation of IGFBP (e.g. proteolysis) is important for embryogenesis.

More recently, zebrafish has evolved to be a promising model organism to study the embryonic functions IGF signaling *in vivo*. It has advantages over mouse model. Their *ex utero* embryogenesis and transparent tissues facilitate non-invasive *in vivo* cell imaging and

experimental manipulation during development (Eisen, 1996). Zebrafish possess all the essential components of IGF signaling pathway, including *IGF1*, *IGF2*, *IGF1R*, and several *IGFBP*s genes (Ayaso et al., 2002; Eivers et al., 2004; Wood et al., 2005a). The expression of several *IGFBP*s was found to be tissue and stage specific (Kajimura et al., 2005b; Kamei et al., 2008; Li et al., 2005; Maures et al., 2002; Maures and Duan, 2002; Wood et al., 2005b). Studies in zebrafish have revealed functions of IGFBP during embryogenesis, which have yet been revealed in mouse studies. Morpholino (MO) targeting zebrafish IGFBP2 resulted in several cardiovascular-specific defects, including reduced blood cell number and circulation, cardiac dysfunction, and angiogenic defects associated with sites of *IGFBP2* expression (Wood et al., 2005b). *IGFBP3* deficiency resulted in delayed pharyngeal skeleton morphogenesis and reduced pharyngeal cartilage differentiation, as well as reduced inner ear size and defects in sensory hair cell formation (Li et al., 2005). In mammals, hypoxia induces IGFBP-1 expression and causes growth retardation. In zebrafish, ubiquitous overexpression of *IGFBP1* in zebrafish embryos significantly reduces growth and delays development; however, under hypoxic conditions, *IGFBP1* knockdown alleviates the hypoxia-induced growth and developmental defects (Kajimura et al., 2005a; Maures and Duan, 2002). Further study by Kamei et al. has revealed that zebrafish IGFBP-1 as co-orthologs of mammalian IGFBP-1 – *IGFBP-a* and *IGFBP-1b* (Kamei et al., 2008). Interestingly, these two genes display distinct temporal expression pattern in response to hypoxia during embryogenesis and exhibit different biochemical properties (Kamei et al., 2008). Taken together, these studies demonstrate that IGFBPs in zebrafish function as important mediators of the endocrine, paracrine and autocrine actions of IGFs during embryonic development, which could be evolutionarily conserved.

Post-translational regulation of IGFBPs

Post-translational modifications play important roles in regulating IGFBP actions, including proteolysis, phosphorylation, and glycosylation. Post-translational modification of IGFBPs in turn affects their stability, binding capacity to IGF ligands, and association with cell surface, which therefore is subjected to intense investigation.

Three potential N-glycosylation sites (Asn-X-Ser/Thr) have been located in the L-domain of the human IGFBP-3 sequence (Asn89, Asn109, and Asn172). Single and combinational mutants of these sites are generated. Mutations of these glycosylation sites, however, affect neither ligand binding nor ALS binding. It is proposed that glycosylation in IGFBP-3 may regulate the turnover rate and proteolysis of IGFBP-3 (Firth and Baxter, 1995). IGFBP-6 is O-glycosylated. The glycosylation sites of IGFBP-6 have been identified as Thr126, Ser144, Thr145, Thr146, and Ser152 (Neumann et al., 1998). Glycosylated IGFBP-6 exhibited greater resistance to proteolysis by chymotrypsin and trypsin than nonglycosylated IGFBP-6 (Neumann et al., 1998).

IGFBP-1 and -3 have been shown to be phosphorylated on serine residues (Coverley and Baxter, 1997; Hoeck and Mukku, 1994; Jones et al., 1991). IGFBP-1 phosphorylation has been shown to be physiologically important. For example, Jones et al. showed that *in vitro* cultured HepG2 cells secrete predominantly the phosphorylated IGFBP-1, while *in vivo* IGFBP-1 from human amniotic fluid and human fetal serum contains a large proportion of nonphosphorylated IGFBP-1.This suggests that IGFBP-1 is secreted as a phosphorylated protein and is subsequently dephosphorylated *in vivo*. Moreover, phosphorylated IGFBP-1 has a 6-fold higher binding affinity for IGF-I than the dephosphorylated form (Jones et al., 1991). The phosphorylation sites of IGFBP-1 are subsequently mapped to Ser101, Ser119,

and Ser169. Mutation of Ser101 leads to reduced affinity for IGF-I (Jones et al., 1993a).

Interestingly, hypoxia and leucine deprivation not only increase IGFBP-1 protein expression, but also increase the phosphorylated form of IGFBP-1 in HepG2 cells, which functionally increases IGFBP-1 binding to IGFs (Seferovic et al., 2008).

Proteolytic cleavage directly controls IGFBP abundance and releases IGFs from the IGFBP-IGF complexes (Bunn and Fowlkes, 2003). Detection of IGFBP fragments in circulation and other biological fluids, as well as various conditioned media from *in vitro* cultured cells, supports that physiological importance of IGFBP proteolysis as a means of regulating IGFBP availability. Some IGFBPs bind to components of the extracellular matrix (IGFBP-2, -3, and -5) or the cell membrane (IGFBP-1, -2, -3, and -5), thus providing a potential mechanism to concentrate IGF activity within discrete regions (Clemmons, 1998). Most of the proteolytic sites are identified in the L-domain of IGFBPs and the proteolytic fragments have greatly reduced or no affinity for IGFs (Firth and Baxter, 2002). Some of the proteases responsible for IGFBP proteolysis have been identified, including plasmin, thrombin, complement protein 1s (C1s), metalloproteinases (MMPs), pregnancy-associated plasma protein A (PAPP-A), and PAPP-A2 (Bunn and Fowlkes, 2003). Complement components C1r and C1s secreted by human fibroblasts have been purified and identified as IGFBP-5 specific proteases, as no protease cleavage activity is detected against IGFBP-1 (Busby et al., 2000).The functional importance of IGFBP proteolysis has been demonstrated by recent *in vivo* studies (Nichols et al., 2007; Ning et al., 2008). IGFBP proteolysis may be directly involved in disease pathogenesis. For example, infusing protease-resistant form of IGFBP-4 significantly inhibited IGF-I actions and cell proliferation, which led to reduced neointimal expansion in a porcine model of atherosclerosis (Nichols et al., 2007).

IGF and muscle

IGF and muscle biology

IGF signaling is an important regulator for muscle physiology. In vivo manipulation of IGF systems is often associated with muscle mass/growth. This has been summarized in the previous topics about *in vivo* studies of IGF ligands, IGF receptors and IGFBPs, which will not be reiterated here.

IGFs play critical roles in skeletal muscle differentiation and growth. Mice deficient in IGF signaling show muscle hypoplasia and die short after birth due to impaired muscle mass to properly inflate the lungs (Liu et al., 1993; Powell-Braxton et al., 1993). Conversely, mice with IGF-I overexpression in muscle have larger muscle fibers and enhanced muscle strength during aging (Barton et al., 2002; Barton-Davis et al., 1998; Coleman et al., 1995; Kaspar et al., 2003). Consistent with the *in vivo* studies, in cultured muscle cells, IGF-II levels increase dramatically during differentiation (Florini et al., 1993; Florini et al., 1991). IGF-II overexpression accelerates myoblast differentiation (Stewart et al., 1996).

Adult skeletal muscle exhibit high plasticity compared with other tissues. Skeletal muscle hypertrophy refers to its ability to increase its mass as an adaptation to mechanical loading generated by and imposed on muscle. Exercises, especially resistance training, can cause muscle hypertrophy (Phillips, 2007). Remarkably, skeletal muscle tissue is able to regenerate after damage. Both the hypertrophy and regeneration processes are mediated by satellite cells. Satellite cells were initially identified by electron microscopy based on their anatomical localization and distinct morphology (Mauro, 1961). Satellite cells are defined as

muscle-specific adult stem cells residing in specific niche (Kuang et al., 2008). Owing to their availability and remarkable capacity to regenerate, satellite cells and their descendent myoblasts have been considered as powerful candidates for cell-based therapies to treat diseases, including muscular dystrophies and other neuromuscular diseases (Kuang and Rudnicki, 2008). There is a growing interest in the specific importance of IGF-I-mediated actions on skeletal muscle satellite cells (Philippou et al., 2007).

Studies established IGF as a critical factor for adult muscle regeneration and hypertrophy. Viral mediated IGF-I overexpression in differentiated muscle fibers prevents aging-related muscle changes in old adult mice with significant increase in strength (Barton-Davis et al., 1998). Rosenthal and colleagues generated transgenic mice with a locally acting isoform of IGF-I expressed in skeletal muscle. Remarkably, these mice do not exhibit age-related muscle atrophy and retained the proliferative response to muscle injury characteristic of younger animals (Musaro et al., 2001). Moreover, this transgenic model exhibits enhanced repair of skeletal muscle damage. Increased recruitment of proliferating bone marrow cells to injured muscles was observed with elevated bone marrow stem cell production and subsequent recruitment to the injury site (Musaro et al., 2004). Interestingly, locally produced IGF-I is increased following acute muscle damage or chronic aerobic exercise, with no changes in circulating IGF-I (Bamman et al., 2001; Hambrecht et al., 2005). This suggests that local autocrine and/or paracrine IGF-I rather than liver-derived IGF-I is important for skeletal muscle regeneration and adaptation.

Despite the advancement made by these studies, the molecular mechanism of IGF actions during myogenesis is still not completely understood. IGF plays dual roles during myogenesis. It not only stimulates myoblast proliferation but also promotes myogenic

differentiation, two mutually exclusive cellular events (Florini et al., 1996). It is still unclear how the specificity of cellular responses to IGF stimulation is established. A thorough understanding the molecular mechanism of IGF actions on myogenesis will eventually help to realize the maximal therapeutical potential of muscle stem cells.

IGFBP-5 and myogenesis

Myogenesis is a highly ordered process, which occurs during embryogenesis (Buckingham et al., 2003). Myogenic process can be recapitulated in cultured myoblasts. It includes a series of cellular events – myoblast proliferation, cell cycle withdraw, differentiation, and cell fusion. It is subjected to the intricate regulation of intrinsic muscle specific transcription factors and extrinsic growth hormones, including IGF. The *in vitro* myoblast differentiation system presents a unique paradigm to assess the function of IGF system in a physiologically relevant environment. IGF is established as positive regulator for myogenesis. However, the precise function of IGFBP-5 in this process is under debate.

IGFBP-5 is the highly conserved across species (Beattie et al., 2006). IGFBP-5 is highly expressed in peripheral tissues, such as muscle and bone, and functions as a local regulator for IGF actions (Conover, 2008; Florini et al., 1996). Transgenic mice are generated in order to address the tissue specific function of IGFBP-5 *in vivo*. Overexpression of IGFBP-5 in murine bone tissues under the osteocalcin promoter resulted in a transient decrease in trabecular bone volume and osteoblastic function (Devlin et al., 2002). In transgenic mice expressing IGFBP-5 in the mammary gland, both mammary cell number and milk synthesis were decreased prematurely with increased apoptosis, probably due to the inhibited survival effect of IGF-I (Tonner et al., 2002).

IGFBP-5 is subjected to post-translational modulation. IGFBP-5 phosphorylation and glycosylation have been reported (Coverley and Baxter, 1997; Firth and Baxter, 2002). IGFBP-5 secreted by osteosarcoma cells migrates in electrophoresis as a triplet and is sensitive to O-glycanase treatment, indicating it is O-glycosylated (Conover and Kiefer, 1993). Thr152 is identified as O-glycosylation site in IGFBP-5 fragments from human serum (Standker et al., 1998). 12 potential phosphorylation sites have been predicted in IGFBP-5 with 6 sites clustered in the L-domain (Schneider et al., 2002). A recent study use mass spectrometry to characterize the in vivo phosphorylation and glycosylation of human IGFBP-5 (Graham et al., 2007). Phosphorylation and O-glycosylation both affected IGFBP-5 binding to heparin but not IGF binding or ternary complex formation with the acid-labile subunit (Graham et al., 2007). The most studied post-translational modification of IGFBP-5 is proteolysis. IGFBP-5 fragments are frequently found in biological fluid and is conditioned media of cell cultures (Schneider et al., 2000). In most cases, the cleavage site is located to the L-domain, generating two fragments of 20-23 kDa and 14-17 kDa. The complement components C1r and C1s are identified as IGFBP-5 proteases (Busby et al., 2000; Moralez et al., 2003). Proteolytic cleavage of IGFBP-5 results in release of IGF and enhancement of IGF action.

IGFBP-5 is the major IGFBP secreted by skeletal muscles. IGFBP-5 is expressed in the myotomal compartments during early development in rodent and zebrafish (Green et al., 1994; Wood et al., 2005a). IGFBP-5 expression is induced during cultured myoblast differentiation (James et al., 1993). IGFBP-5 protein is secreted within 12 h of the onset of differentiation in these cells and that it is the only IGFBP produced in several fusing skeletal muscle cell lines (James et al., 1993). In cultured myoblast, IGFBP-5 expression levels

increased dramatically during myogenic differentiation (Bayol et al., 2000; Rotwein et al., 1995).

These studies of IGFBP-5 expression pattern indicate that IGFBP-5 may modulate IGF-II actions during muscle differentiation. However, studies *in vitro* and *in vivo* have yielded conflicting results depending on the experimental approaches used and specific conditions of different assays. For example, IGFBP-5 overexpression in cultured myoblasts under the control of a constitutive promoter caused inhibitory effects on myoblast differentiation process (Cobb et al., 2004; James et al., 1996; Mukherjee et al., 2007). Interestingly, when purified exogenous IGFBP-5 was added to cultured myoblasts together with IGF-I at a certain molecular ratio, it stimulated IGF-I-induced cell differentiation (Ewton et al., 1998). Transgenic mice with ubiquitously overexpressed IGFBP-5 from early development exhibited compromised muscle development (Salih et al., 2004). IGFBP-5 knockout mice have no reported abnormalities on muscle development, which could result from the potential compensatory effects from other IGFBPs expressed in the single knockout mice (Ning et al., 2007). Indeed, IGFBP-3, -4, and -5 triple knockout mice showed smaller body size and significantly reduced quadricep muscles (Ning et al., 2006).

Overexpression of IGFBP-5 often causes inhibitory effects, which can be rationally attributed to their competition for the ligand with the cell surface receptors. This notion is supported by the fact that IGFBP-5 binds to IGFs with affinities higher than that of IGF-I receptor. And, the IGF-I/IGFBP-5 complex is unable to interact with IGF-IR (Kalus et al., 1998; Zeslawski et al., 2001). Special caution is needed when interpreting the overespression data. The inhibitory effects of IGFBP-5 are often observed when IGFBP-5 was added in molar excess or overexpressed. Moreover, given the fact that endogenous IGFBP-5 is already

expressed at high levels from the early onset of myoblast differentiation, the physiological relevance of the overexpression study needs to be established.

Whether manipulating IGFBP-5 by overexpression is physiologically relevant needs to be determined based particular cellular context. It is possible that while at low concentrations IGFBP-5 potentiates IGF actions, it inhibits IGF actions at high concentrations (Moralez et al., 2003). It was reported that IGFBP-5 at a higher concentration inhibits IGF-induced human fibroblast proliferation, while it potentiates IGF actions in the same cell type at a lower concentration (Imai et al., 2000; Jones et al., 1993b). Several mechanisms have been proposed to explain how IGFBP-5 potentiates the effects of IGFs. Studies by Clemmons and colleagues have shown that binding of IGFBP-5 to ECM or glycosaminoglycans at the cell surface reduces its binding affinity for IGFs (Arai et al., 1996; Arai et al., 1994; Parker et al., 1998). It has been proposed that binding of the IGF/IGFBP-5 complex to cell surface components reduces the affinity of IGFBP-5 for IGF-I, thereby releases IGF-I to its receptors. As a result, it potentiates the IGF actions. Binding of IGFBP-5 to the ECM proteins is independent from its ability to bind to IGFs. Therefore, it was postulated that the potentiation effect of IGFBP-5 originates from increased bioavailability of IGF-I to the IGF-I receptor after sequestation and concentration of the IGF-I/IGFBP-5 to the cell surface.

Project summary

In this thesis, I intend to address how IGF signaling specificity is established. To this purpose, I use *in vitro* cultured C2C12 myoblast differentiation model. C2C12 myoblasts can

be induced to differentiate rapidly *in vitro*, forming myotubes and producing characteristic muscle proteins (Yaffe and Saxel, 1977). The well-orchestrated myogenic process comprises both cell proliferation and differentiation. Previous studies have established IGF-II as a critical autocrine factor in myoblast differentiation (Florini et al., 1993; Florini et al., 1991; Stewart et al., 1996). IGF system is expressed in this model with IGF-II being the major isoform of IGFs and IGFBP-5 being the dominant IGFBP (Ren et al., 2008a). Therefore, the in vitro myoblast differentiation model presents a unique platform to study how cells respond to IGF with distinct outcomes.

In Chapter II of my thesis, I showed that local IGF binding proteins play intricate roles in regulating IGF function. Realizing the caveats associated with overexpression study, loss-of-function approach was used in this study. In the C2C12 myoblast differentiation model, induction of IGFBP-5 expression precedes that of IGF-II. Knockdown IGFBP-5 by siRNA impairs myogenesis and suppresses IGF-II gene expression. IGF-II exerts autocrine regulation via a positive regulatory mechanism through PI3K-Akt signaling pathway. Adding IGF-II or constitutively activating Akt rescues that IGFBP-5 knockdown-caused defects. However, an IGF analogue that binds to the IGF-IR but not IGFBP has only a limited effect. When added with low concentrations of IGF-II, IGFBP-5 restores IGF-II expression and myogenic differentiation, whereas an IGF-binding-deficient IGFBP-5 mutant has no effect. Taken together, these results demonstrated that local IGFBP-5 promotes IGF-II actions in myoblast differentiation by binding to and switching on the IGF-II auto-regulation loop.

In Chapter III of my thesis, I investigated how IGF signaling specificity is established in response to particular microenvironment conditions, for example hypoxia (lack of oxygen). I demonstrate that IGF promotes myoblast differentiation under normoxia but

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stimulates proliferation under hypoxia. HIF-1 facilitates cellular adaptation to hypoxia by reprogramming cell metabolism. IGF sustains cell proliferation under hypoxia by further enhancing HIF-1-mediated anaerobic glycolysis. Hypoxia differentially regulates IGF downstream signaling events. It inhibits myogenic Akt-mTOR and p38 pathways by suppressing Akt signaling and inducing negative regulators for mTOR. In contrast, mitogenic Erk1/2 MAPK, as a major IGF downstream signaling cascade, is enhanced under hypoxia leading to increased cell proliferation and decreased differentiation. Abrogating p38 activity directly contributes to increased proliferation. These findings demonstrate a dynamic cellular response to IGF signaling, where a functional switch is originated from the integrated growth factor signal and oxygen availability in their microenvironments.
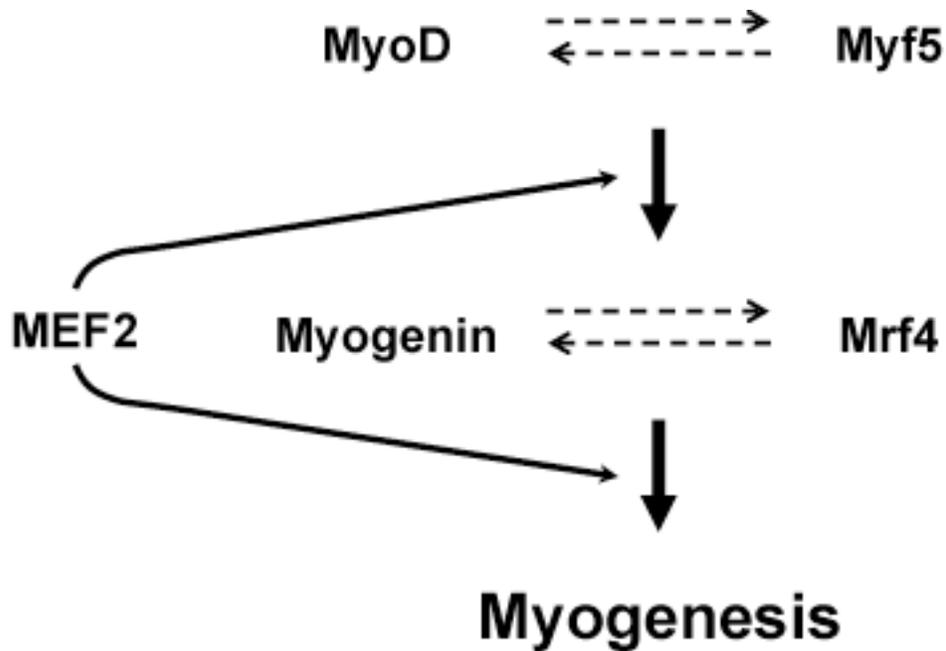
Fig. 1.1 Major transcription factor network in myogenesis. MRF family includes MyoD, Myf5, Myogenin, and Mrf4. MyoD and Myf5 act upstream of Myogenin and Mrf4. Dashed arrows represent potential compensation between two transcription factors when one is absent. MRF and MEF2 families act in a cooperative way to regulate myogenesis. MEF2 family members synergize with MRF family members and increase the myogenic potential.
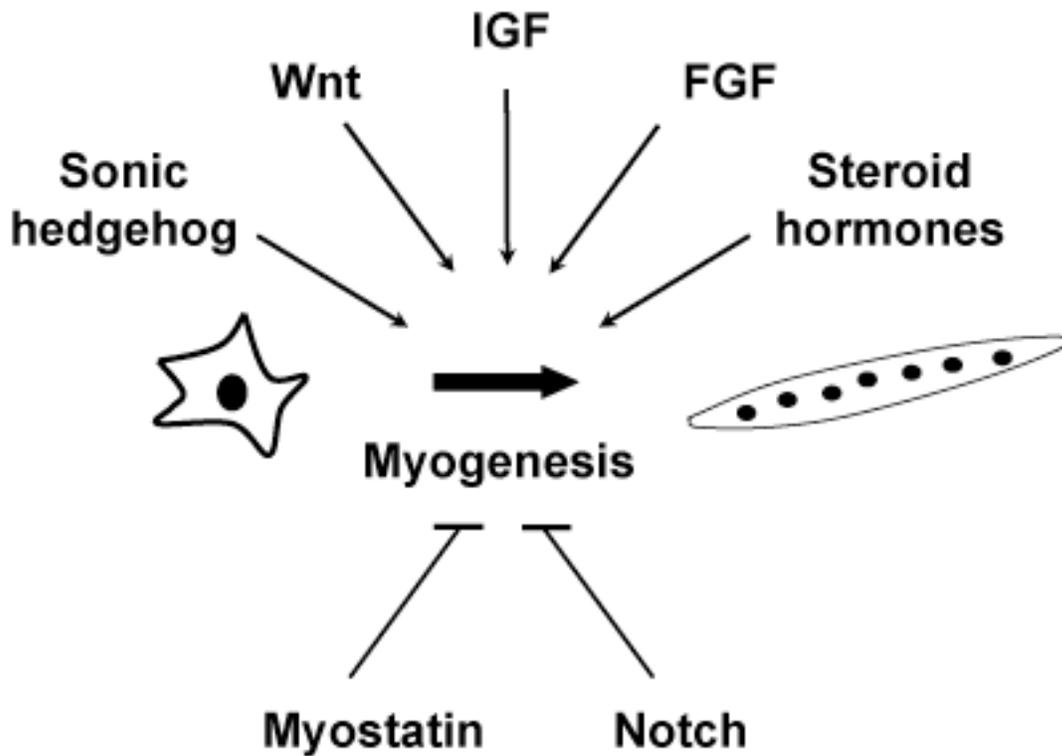
Fig. 1.2 Illustration of major signaling pathways regulating myogenesis. Myogenesis is a well-orchestrated process, balanced by both positive and negative regulators. Sonic hedgehog and Wnt have been shown as factors secreted from notochord that positively regulate myogenesis. Multiple growth factors, including IGF and FGF, regulate myogenic process. Anabolic steroid hormones are strong inducers for muscle hypertrophy. Myostatin and Notch are demonstrated as negative regulators by *in vitro* and *in vivo* studies.
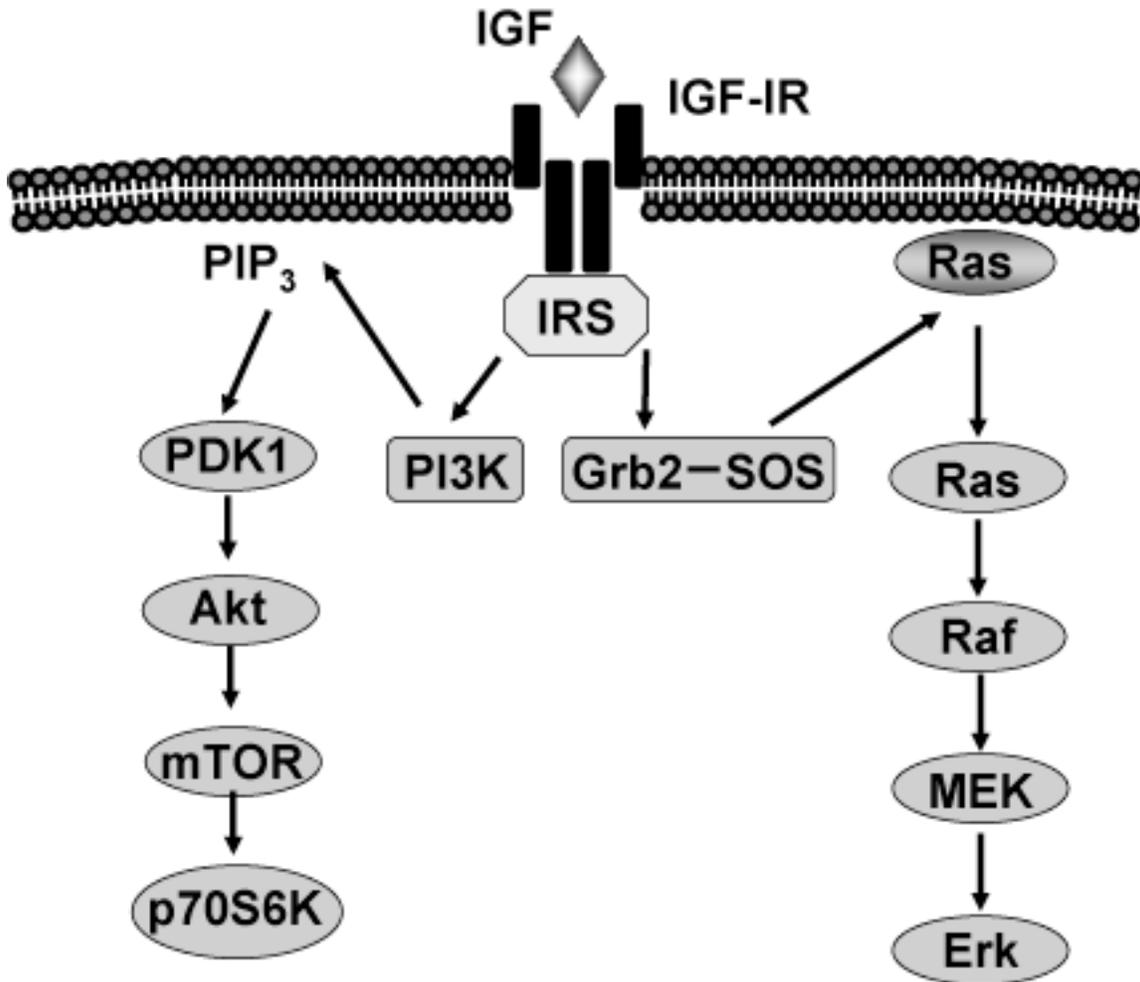
Fig. 1.3 IGF-IR and major downstream effectors in IGF signaling pathway. IGF binds to

IGF-IR and activates the receptor tyrosine kinase. IRS associates with activated receptor via

the phosphorylated tyrosine motif. IRS functions as a critical scaffold adaptor that connects

the downstream PI3K-Akt and Erk MAPK pathways.
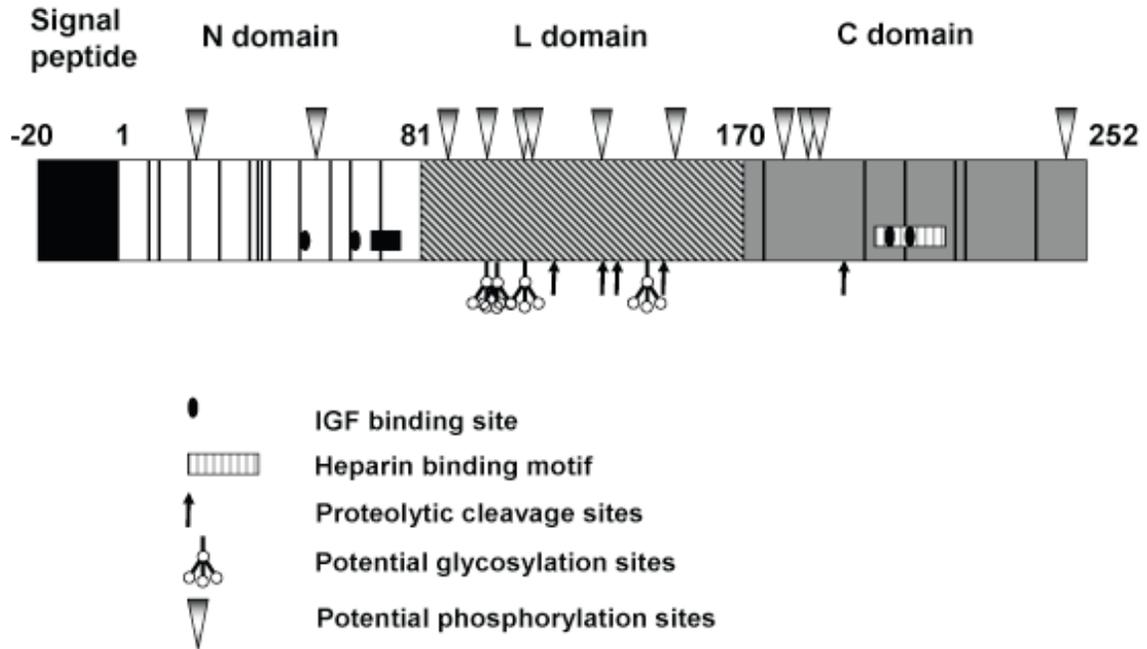
Fig. 1.4 Schematic representation of IGFBP-5 domain arrangement. Vertical lines represent

Cysteine residues. IGF binding sites and heparin binding motif are depicted using symbols.

Potential phosphorylation, glycosylation, and proteolytic cleavage sites are marked. Figure is

adapted from (Schneider et al., 2002).

CHAPTER II

IGFBP-5 REGULATES MUSCLE CELL DIFFERENTIATION BY BINDING TO IGF-II AND SWITCHING ON THE IGF-II AUTO-REGULATION LOOP

Abstract

IGF-II stimulates both mitogenesis and myogenesis through its binding and activation of the IGF-I receptor (IGF-1R). How this growth factor pathway promotes these two opposite cellular responses is not well understood. We tested the hypothesis that local IGF binding protein-5 (IGFBP-5) plays a crucial role in promoting the myogenic action of IGF-II. IGFBP-5 is induced prior to the elevation of IGF-II expression during myogenesis. Knockdown of IGFBP-5 impairs myogenesis and suppresses IGF-II gene expression. IGF-II up-regulates its own gene expression via the PI3K-Akt signaling pathway. Adding IGF-II or forced expression of constitutively active Akt rescues the IGFBP-5 knockdown-caused defects. An IGF analog that binds to the IGF-IR but not IGFBP had limited effect. When added with low concentrations of IGF-II, IGFBP-5 restores IGF-II expression and myogenic differentiation, whereas an IGF-binding deficient IGFBP-5 mutant had no effect. These findings suggest that IGFBP-5 promotes muscle cell differentiation by binding to and switching on the IGF-II auto-regulation loop.

[1]

Introduction

Myogenesis is a highly ordered and balanced process, which includes myoblast proliferation, cell cycle withdrawal, differentiation, and cell fusion (Buckingham et al., 2003). This process is regulated by a variety of hormones and growth factors. Insulin-like growth factors (IGFs), including IGF-I and IGF-II, play critical roles in skeletal muscle differentiation and growth, as well as adult muscle regeneration and hypertrophy (Musaro et al., 2004; Musaro et al., 2001). Mice deficient in IGF ligand or IGF-I receptor exhibit muscle hypoplasia and die shortly after birth due to impaired muscle mass to inflate their lungs (Liu et al., 1993; Powell-Braxton et al., 1993). Transgenic mice with overexpression of IGF-1 in muscle have larger muscle fibers and enhanced muscle strength during aging (Barton et al., 2002; Barton-Davis et al., 1998; Coleman et al., 1995; Kaspar et al., 2003). In cultured muscle cells, IGF-II levels increase dramatically during myogenesis. IGF-II antisense oligonucleotides can abolish differentiation (Florini et al., 1993; Florini et al., 1991), and IGF-II overexpression accelerates myoblast differentiation (Stewart et al., 1996).

While most growth factors stimulate myoblast proliferation and inhibit myogenic differentiation, IGFs are unique in that they not only stimulate myoblast proliferation but also promote myogenic differentiation, two mutually exclusive processes (Florini et al., 1996; Rosenthal and Cheng, 1995). These actions are mediated through the IGF-I receptor (IGF-IR), a transmembrane tyrosine kinase, and activation of the IGF-IR initiates downstream signaling cascades including the phosphatidylinositol 3-kinase (PI3K) -Akt pathway. Earlier studies have shown that there is a temporal separation between these two responses to IGFs: IGF treatment causes a proliferative response in the first 24-36 h, and this is followed by subsequent increase in myogenic differentiation. The increase in differentiation is not

secondary to increased cell number after IGF treatment (Florini et al., 1996) . Rosenthal and

Cheng (1995) have reported a biphasic effect of IGF-I on pRb, a regulator of cell cycle

progression that is present in the hyperphosphorylated state during cell proliferation and

hypophosphorylated state during differentiation. They found that IGF treatment resulted in

persistent Rb hyperphosphorylation for over 24 h before hypophosphorylation became the

dominant form. Several laboratories have shown that activation of PI3K, Akt, and FoxO

promotes myogenic differentiation and survival (Coolican et al., 1997; Engert et al., 1996;

Jiang et al., 1999; Kaliman et al., 1996; Lawlor et al., 2000; Lawlor and Rotwein, 2000a;

Lawlor and Rotwein, 2000b; Rommel et al., 2001; Rommel et al., 1999; Wilson and

Rotwein, 2007; Wilson et al., 2004). Rotwein and colleagues have reported that in cultured

muscle cells secreted IGF-II stimulates the IGF-IR, PI3K, and Akt to induce the expression

of the cyclin-dependent kinase inhibitor p21 and Myogenin, and through this mechanism,

maintains myoblast viability during early myogenesis (Lawlor et al., 2000; Lawlor and

Rotwein, 2000a; Lawlor and Rotwein, 2000b; Wilson and Rotwein, 2007; Wilson et al.,

2004). Despite these advances in various intracellular signaling mechanisms underlying IGF

actions in myogenesis, it remains puzzling how the activation of the same IGF-IR by the

same ligand (IGF-II) can elicit opposite biological responses.

We now understand that most, if not all IGFs in the extracellular environment are

bound to IGF binding proteins (IGFBPs). IGFBPs are a family of secreted proteins that

specifically bind IGFs with affinities that are equal to or greater than those of the IGF-IR. Six

distinct IGFBPs, designated as IGFBP-1 to -6 have been isolated and characterized in

humans and a variety of other vertebrate species (Clemmons, 2001; Duan, 2002; Firth and

Baxter, 2002). IGFBP-5, the most conserved IGFBP, is the major protein secreted by skeletal

muscles. IGFBP-5 is expressed in the myotomal compartments during early development in rodent and zebrafish (Green et al., 1994; Wood et al., 2005a) and its expression is induced during muscle differentiation (James et al., 1993). In cultured myoblasts, IGFBP-5 expression levels increased dramatically during myogenic differentiation (Bayol et al., 2000; Rotwein et al., 1995). These findings suggest that IGFBP-5 may play a role in modulating the actions of IGF-II on muscle differentiation. The precise role(s) of IGFBP-5 in myogenesis, however, remains under debate. Knockout of the IGFBP-5 in mice had a minimal effect on muscle size and whole body size, but IGFBP-3, -4, and -5 triple knockout mice showed smaller body size and reduced quadriceps muscles (Ning et al., 2006), indicating the potential compensatory effects from other IGFBPs in the single knockout mice. When overexpressed in vivo in transgenic mice (Salih et al., 2004) and in vitro in cultured murine muscle cells under the control of a constitutive promoter (Cobb et al., 2004; James et al., 1996; Mukherjee et al., 2007), IGFBP-5 inhibits muscle differentiation. When exogenous IGFBP-5 was added to cultured rat myoblasts together with IGF-I at an appropriate ratio, it stimulated IGF-I induced differentiation (Ewton et al., 1998). While these gain-of-function studies, either adding large amounts of purified IGFBP-5 to or stably and constitutively overexpressing of IGFBP-5 in cultured myoblasts, are useful in demonstrating the biological capabilities of IGFBP-5, they do not necessarily provide insight into the physiological function(s) of the endogenous protein. Furthermore, interpretation of these data is not always straightforward because the IGFBP-5 expression is dramatically induced during myogenesis.

The objective of this study is to investigate the physiological role(s) of endogenous IGFBP-5 in regulating IGF-II actions in skeletal muscle differentiation. Our results suggest the induction of IGFBP-5 precedes that of IGF-II in cultured C2C12 myoblast cells and in

primary skeletal muscle cells. Knockdown of IGFBP-5 impairs myogenic differentiation and reduces the expression of Myogenin and myosin heavy chain (MHC). Interestingly, knockdown of IGFBP-5 suppresses IGF-II expression and reduces IGF-IR-mediated signaling activity. Further mechanistic analyses suggest that IGFBP-5 promotes muscle cell differentiation by binding to IGF-II and enhances the IGF-II autocrine regulatory loop.

Results

Induction of IGFBP-5 precedes the induction of IGF-II during myogenesis

C2C12 myoblasts undergo terminal differentiation after switching to horse serum containing differentiation medium (DM). As shown in Fig. 2.1A, at 24 h after the induction of differentiation, the expression of IGFBP-5 mRNA showed a ~7 fold increase over the 0h control ($p<0.05$). There was a further increase (~5 fold) at 48 h. Thereafter, the IGFBP-5 mRNA levels decreased considerably, but remained higher than 0h. In comparison, while the absolute IGF-II mRNA level (copy number/μl) remained extremely low, the relative levels of IGF-II mRNA increased dramatically at 48h (51 fold over 24h, $p <0.05$). There was a further ~4 fold increase at 72h and an additional ~2 fold increase at 96h. RT-PCR analysis indicated that there are no significant changes in the mRNA levels of IGF-IR, IGFBP-2, and IGFBP-4 (data not shown, also see Fig. 2.6B). These results suggest that while both IGF-II and IGFBP-5 expression are induced during myogenic differentiation in the C2C12 model, the induction of IGFBP-5 expression precedes that of IGF-II.

Induction of IGFBP-5 expression is required for myogenic differentiation

To determine the role of endogenous IGFBP-5, pSUPER-BP5, a plasmid that generates siRNA specifically targeting IGFBP-5 mRNA, was transfected to cultured C2C12 cells. The effect in silencing the IGFBP-5 gene was examined by directly measuring changes in IGFBP-5 mRNA, as well as changes in IGFBP-5 protein levels. Introducing pSUPER-BP5 into these cells resulted in a marked reduction of IGFBP-5 mRNA levels, whereas the empty pSUPER plasmid had no such effect (Fig 2A). Likewise, ligand blot analysis of the conditioned media revealed a similar decrease in IGFBP-5 protein levels (Fig. 2B). The specificity of IGFBP-5 siRNA was confirmed by the unchanged levels of IGFBP-4 mRNA, a related protein, and GAPDH mRNA, a housekeeping gene. Quantification of three independent experiments showed that IGFBP-5 siRNA caused a 72.8% decrease (p <0.05) in IGFBP-5 mRNA levels and a 71.2% decrease ($p < 0.05$) in protein levels.

When switched to DM, the empty control vector transfected myoblasts formed morphologically distinctive multinucleated myotubes, while pSUPER-BP5 transfected myoblasts remained as individual mononucleated cells (Fig. 2.2C). We also examined the expression of Myosin heavy chain (MHC), a marker for terminal myogenic differentiation, by immunostaining and Western blot. Both assays showed a dramatic decrease in MHC expression in the pSUPER-BP5 transfected cells (Fig. 2.2D and E). Quantification of Western blot data from four independent experiments showed that the MHC levels were decreased by 84.4% (p< 0.05). To rule out the possibility of any non-specific interferon responses associated with siRNA, a pSUPER construct expressing siRNA with a sequence unrelated to IGFBP-5 was engineered and introduced to C2C12 cells. This construct did not affect differentiation or MHC expression (Fig. 2.2C-E). Taken together, these results indicated that knockdown of IGFBP-5 impairs myogenic differentiation in C2C12 cells.

Knockdown of IGFBP-5 suppresses myogenin expression but expression of Myogenin cannot "rescue" the myogenic defects caused by IGFBP-5 knockdown

The muscle-specific myogenic regulatory factors (MRFs), including MyoD, Myf5, MRF4, and Myogenin regulate myoblast specification and differentiation through their ability to activate muscle specific structural genes, such as MHC (Perry and Rudnick, 2000). We next examined whether the expression of some of these critical myogenic transcription factors is altered by IGFBP-5 knockdown. There was little difference of MyoD levels between the IGFBP-5 knocked down cells and control cells through the time course of differentiation (Fig. 2.3A). Myogenin was not detectable in undifferentiated myoblast cells or within the first 24 h after DM was added (Fig. 2.3B). At 48 h, Myogenin was easily detectable and its levels increased dramatically at 72 and 96 h in the control group. At 48 and 72 h, Myogenin was not detected in pSUPER-BP5 transfected cells (Fig. 2.3B). Its levels were markedly lower even at 96 h. Northern blot analysis revealed similar decreases in myogenin mRNA levels (Fig. 2.3C), indicating that knockdown of IGFBP-5 suppresses myogenin gene expression.

To determine whether myogenin mediates the myogenic action of IGFBP-5, we co-transfected C2C12 myoblasts with pSUPER-BP5 and a Myogenin expression plasmid. Western blot analysis verified the successful expression of the Myc-tagged Myogenin transgene, while the endogenous Myogenin was suppressed by IGFBP-5 siRNA (Fig. 2.4A). As shown in Fig. 2.4B, cells co-transfected with Myogenin and pSUPER-BP5 were indistinguishable from those transfected with pSUPER-BP5 alone, i.e., they had minimal MHC expression and did not form myotubes when grown in DM. To confirm that the

myogenin transgene is functional in this particular experimental setting, the transcriptional activity of Myc-Myogenin was examined. Myogenin forms heterodimers with other ubiquitous basic helix-loop-helix transcription factors and binds to the *cis*-control elements termed E-boxes often found in the promoters of muscle-specific genes (Buckingham, 2001; Sabourin and Rudnicki, 2000). 4RTK is a reporter construct that contains the firefly luciferase gene driven by a minimal thymidine kinase (TK) promoter linked with four copies of the E-box derived from the mouse muscle creatine kinase promoter. As shown in Fig. 2.4C, when co-transfected with 4RTK, the Myc-Myogenin caused a 6.1 fold increase in luciferase activity, suggesting that it was functional. Furthermore, when transfected to C2C12 cells, Myogenin significantly increased differentiation at 24 and 36 h after the induction of differentiation (Fig. 2.4D), suggesting that overexpression of Myogenin is capable of promoting differentiation. Taken together, these data indicate that while knockdown of IGFBP-5 reduces myogenin gene expression, myogenin alone is insufficient to promote myogenic differentiation in the absence of IGFBP-5.

Knockdown of IGFBP-5 suppresses IGF-II gene expression and exogenous IGF-II "rescues" the myogenic defects caused by IGFBP-5 knockdown

It has been previously reported that autocrine IGF-II is a critical regulator of myogenesis and IGF-II exerts its actions primarily through PI3-kinase/Akt pathway in differentiating C2 myoblasts (Wilson et al., 2004). We therefore examined the impact of IGFBP-5 knockdown on IGF-II expression. No IGF-II mRNA was detected by RT-PCR in the control undifferentiated C2C12 myoblast cells (Fig. 2.5A). 24 h after the addition of DM, the IGF-II mRNA levels were easily appreciated and showed further increases at 48 h and

thereafter. IGF-II mRNA expression was undetectable at 24 h and were markedly lower at 48 h in the pSUPER-BP5 transfected cells. This effect appears to be specific to the IGF-II gene, as knockdown of IGFBP-5 did not reduce the mRNA levels of other members of the IGF signaling pathway expressed in these cells, including IGF-I, IGF-IR, IGFBP-2 and -4 (Fig. 2.5A). The effect on IGF-II gene expression was further examined by qRT-PCR. IGF-II mRNA levels were significantly lower in IGFBP-5 knocked down cells at 24, 48, and 72 h after the induction of differentiation (Fig. 2.5B). There was a clear trend of reduction at 96 h, although the difference was not statistically different due to the greater variations in the control group at this time point. Knockdown of IGFBP-5 markedly decreased phospho-Akt levels, whereas the levels of total Akt remained comparable to those of the control. When quantified and expressed as the ratio of phospho-Akt/total Akt, the levels in the IGFBP-5 deficient group showed a 94% reduction (Fig. 2.5C), indicating that knockdown of IGFBP5 leads to a reduction in IGF-1R mediated signaling activity.

Since knockdown of IGFBP-5 suppresses IGF-II expression and reduces the IGF signaling activity, we asked whether IGFBP-5 affects myogenesis via IGF-II. To test this idea, DM supplemented with exogenous IGF-II was added to the IGFBP-5 knocked down and control cells. As shown in Fig. 2.6A, while pSUPER-BP-5 transfected cells remained as mononucleated myoblasts, addition of exogenous IGF-II not only increased myotube formation in the control group, it also reversed the myogenic defects caused by IGFBP-5 knockdown. IGF-I, which signals through the same IGF-IR, had a similar effect. Western blot analysis indicated that knockdown of IGFBP-5 suppressed MHC and Myogenin expression and addition of IGF-II restored their expression (Fig. 2.6B). This action of exogenous IGF-II is likely mediated by the IGF-IR and Akt signaling pathway because

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knockdown of IGFBP-5 reduced phospho-Akt levels and IGF-II increased phospho-Akt levels in differentiating cells (Fig. 2.6C).

If IGFBP-5 indeed promotes myogenesis through the IGF-IR-PI3K-Akt signaling pathway, then activation of Akt should also "rescue" IGFBP-5 knockdown-induced differentiation defects. To test this idea, myrAkt, a constitutively active form of Akt, was introduced to the cells. As shown in Fig. 2.6D, C2C12 cells co-transfected with pSUPER-BP5 and a myrAkt expressing plasmid clearly formed myotubes when growing in DM, whereas cells transfected with pSUPER-BP5 did not. Cells transfected with the myrAkt plasmid alone showed enhanced differentiation. Similarly, expression of myrAkt restored pSUPER-BP5 caused reduction in Myogenin and MHC expression (Fig. 2.6E). Taken together, these findings suggest that knockdown of IGFBP-5 suppresses IGF-II expression, which in turn leads to reduced signaling intensity of the IGF-IR-PI3K-Akt signaling pathway.

IGF-II up-regulates its own gene expression via the PI3K-Akt signaling pathway

Although it has been shown that autocrine IGF-II production increases dramatically during muscle differentiation, the regulatory mechanisms of IGF-II gene expression is not well understood. Given that fact that IGF-II gene expression is continuously and dramatically elevated during myogenesis (see Fig. 2.1), we speculated that IGF-II might up-regulate its own gene expression through a positive auto-regulatory loop. Two independent approaches, adding exogenous IGF-II and overexpressing myrAkt, were taken to address this issue. As shown in Fig. 2.7A, addition of IGF-II to wild-type C2C12 cells resulted in a significant increase in IGF-II mRNA levels. Likewise, expression of myrAkt significantly increased

IGF-II mRNA levels (Fig. 2.7C). These results suggest that IGF-II up-regulates its own gene expression through the PI3K and Akt signaling pathway.

In addition to IGF-II, Myogenin expression was also increased by exogenous IGF-II or myrAkt expression (Fig. 2.7B and D). These data are consistent with previous reports (Florini et al., 1993; Xu and Wu, 2000). However, expression of Myogenin did not restore the reduced IGF-II expression caused by IGFBP-5 knockdown cells (Fig. 2.7E). While the increased Myogenin plays a role in myogenesis, it does not appear to play any direct role in the expression of the IGF-II gene during myogenesis.

Endogenous IGFBP-5 is not localized in the nucleus and forced nuclear expression of the IGFBP-5 transactivation domain does not increase IGF-II expression

Recent studies have shown that IGFBP-5 is not only secreted, but can also enter the nucleus in certain cell type and that IGFBP-5 contains a functional transactivation domain in its conserved N-domain (Firth and Baxter, 2002; Xu et al., 2004; Zhao et al., 2006). We wondered whether IGFBP-5 could exert a direct effect on IGF-II transcription in the nucleus. To test this idea, immunocytochemical experiments were carried out to determine whether endogenous IGFBP-5 is present in the nucleus in differentiating C2C12 cells. Confocal microscopy analysis showed that endogenous IGFBP-5 signal was detected in the Golgi apparatus, in the shape of a crescent moon outside of the nucleus (Fig. 2.8A, panels a-d). This is consistent with the fact that IGFBP-5 is secreted from these cells. To show that the IGFBP-5 antibody could detect nuclear IGFBP-5 if present, we generated a plasmid that expresses a non-secreted form of IGFBP-5 (with the signal peptide deleted) with an EGFP tag at its N-terminus, and introduced it into cells by transient transfection. The non-secreted

EGFP-mIGFBP-5 was clearly localized in the nucleus as indicated by the overlap of GFP signal and IGFBP-5 immunoreactivity with DAPI signal (Fig. 2.8A, panels e – h). These results indicate endogenous IGFBP-5 is not localized in the nucleus in myoblasts. Next, we transfected C2C12 cells with an IGFBP-5 construct that contains its functional transactivation domain (Xu et al., 2004) and examined its impact on the IGF-II gene expression. As shown in Fig. 2.8B, no significant increase was detected in IGF-II mRNA expression levels in the control or IGFBP-5 transfected cells, although the IGFBP-5 fusion protein was highly expressed (Fig. 2.8C). In comparison, myrAkt overexpression significantly increased IGF-II mRNA levels (Fig. 2.8B). Taken together, these results suggest that endogenous IGFBP-5 is not localized in the nucleus and forced nuclear expression of the IGFBP-5 transactivation domain does not increase IGF-II expression in differentiating myoblast cells

IGFBP-5 promotes myogenic differentiation by switching on the IGF-II auto-regulation loop

How does IGFBP-5 act to increase IGF-II gene expression and to promote myogenesis then? IGFBP-5 has been shown to be located on the cell surface and/or extracellular matrix in fibroblasts and muscle cells (Clemmons et al., 2002; Firth and Baxter, 2002). Since IGF-II stimulates its own gene expression through a positive auto-regulatory loop, we hypothesized that cell-surface associated IGFBP-5 up-regulates IGF-II gene expression and promotes myogenesis by binding to IGF-II and enhances its interaction with the IGF-IR, thereafter amplifying IGF signaling activity during myogenesis. To test this hypothesis, immunocytochemical staining was performed. As shown in Fig. 2.9A, IGFBP-5

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signal was clearly observed on the surface in cells that were fixed in a buffer containing no detergent and therefore with the integrity of the plasma membrane intact (panel a). When the plasma membrane was permealized, however, no such signal was observed, even though intracellular IGFBP-5 was clearly visible (Fig. 2.9A, panel b). We further demonstrated that intact IGFBP-5 is present on the surface of differentiating myoblasts and is capable of IGF binding by treating C2C12 cells with a high salt buffer to release its cell surface bound IGFBP-5. As shown in Fig. 2.9B, no cell surface associated IGFBP-5 was found in myoblasts prior to the induction of differentiation. In contrast, a large amount of intact IGFBP-5 was detected on these cells 24 h after the induction of differentiation.

Next, we compared the myogenic activities of IGF-II or Des(1-6)IGF-II. Des(1-6)IGF-II binds to the IGF-1R but does not bind to IGFBPs. As shown in Fig. 2.9C, compared to IGF-II, Des(1-6)IGF-II was significantly less potent in stimulating differentiation. Since IGFBP-5 is the predominant IGFBP secreted by these cells, the reduced activity of Des(1-6)IGF-II is consistent with the notion that endogenous IGFBP-5 binds to IGF-II and promotes IGF-II actions. To test this further and to determine whether the potentiation effect of IGFBP-5 is indeed due to its binding to IGF-II, IGF-II and IGFBP-5 were added individually or in combination to cells transfected with pSUPER or pSUPER-BP5. pSUPER-BP5 transfected cells did not form myotubes and had significantly reduced MHC levels (Fig. 2.9D). Addition of a relatively high concentration (150 ng/ml) of IGF-II partially "rescued" these myogenic defects caused by IGFBP-5 knockdown. At a low concentration (50 ng/ml), however, IGF-II by itself had little effect. When the low concentration of IGF-II (50 ng/ml) was added in combination with an equal molar concentration of IGFBP-5 (210 ng/ml) to pSUPER-BP5 transfected cells, these cells showed comparable degree of myotube formation

and MHC expression to those of the high IGF-II (150 ng/ml) group and the control pSUPER group. IGFBP-5 (210 ng/ml) alone had no rescuing effect (Fig. 2.9D). These results suggest that IGFBP-5 does not act directly to affect myogenic differentiation. Rather it works primarily by potentiating or amplifying IGF-II actions.

Next, LBD-IGFBP-5, an IGFBP-5 mutant with amino acid substations in the ligand binding domain and has 800-fold lower affinity to IGFs (Imai et al., 2000), was used to examine the mechanistic basis of IGFBP-5 action. As shown in Fig. 2.9D, when added together with IGF-II (50 ng/ml) to pSUPER-BP5 transfected cells, IGFBP-5-LBD did not increase myotube formation or MHC expression, suggesting that the action of IGFBP-5 in promoting myogenic differentiation is dependent on its ability to bind to IGF-II.

The IGFBP-5 and IGF-II genes are similarly induced in primary myoblasts and IGFBP-5 knockdown inhibits primary cell differentiation

To establish the physiological relevance of the observations made with C2C12 cells, we examined the IGF-II and IGFBP-5 mRNA expression patterns in primary skeletal myoblasts prepared from neonatal mice. As shown in Fig. 2.10A, 24 h after the initiation of differentiation, the expression of IGFBP-5 mRNA showed a 2.9 fold increase over the 12h control ($p < 0.05$). Thereafter, the IGFBP-5 mRNA levels returned to the basal levels. In comparison, the IGF-II mRNA level remained unchanged at this early stage of differentiation. The IGF-II mRNA levels increased significantly at 48h (4.6 fold over 12h, $p < 0.05$) and remained significantly high at 48h (3.6 fold, $p < 0.05$). Therefore, the overall patterns of IGF-II and IGFBP-5 induction and their temporal relationship are similar in these primary cells, although the magnitude of inductions is lower. To examine the role of IGFBP-

5, primary skeletal muscle cells were transfected with pSUPER or pSUPER-BP5. When switched to DM, the vector transfected myoblasts formed morphologically distinctive multinucleated myotubes and expressed MHC (Fig. 2.10B). In comparison, most pSUPER-BP5 transfected myoblasts had fewer MHC-positive cells. The differentiation index in the pSUPER-BP5 transfected group is 37.5% (Fig. 2.10C), which is significantly lower than that of the control group (58.4%, $p<0.05$). These findings suggest that the induction of IGFBP-5 occurs earlier than that of IGF-II and that IGFBP-5 promotes myogenic differentiation in primary skeletal muscle cells.

Discussion

In this study, we have explored the role(s) of endogenous IGFBP-5 in modulating the action of autocrine IGF-II in promoting myogenic differentiation. We show that knockdown of IGFBP-5 impairs myogenic differentiation. Addition of IGF-II "rescues" IGFBP-5 knockdown-induced myogenic defects, while Des(1-6)IGF-II, an IGF-II analog with greatly reduced binding affinity for IGFBP, is significantly less effective. These data strongly argue that the endogenously secreted IGFBP-5 is a differentiation-promoting factor in skeletal muscle cells. This conclusion makes physiological sense in light of the highly induced IGFBP-5 expression prior to an appreciable increase in IGF-II production during early myogenesis. It is also in agreement with several previous studies. It has been reported that NFB4 cells, a mutant muscle cell line derived from C2C12 cells that are deficient in IGFBP-5 and IGF-II, do not undergo differentiation under the conditions in which C2C12 cells differentiate (Sarbassov et al., 1995). When added in an appropriate ratio to IGF-I, IGFBP-5 stimulates IGF-induced myoblast differentiation in rat muscle cells (Ewton et al., 1998).

Recent loss-of-function studies in other cell types also suggest that IGFBP-5 promote bone, neuronal and epithelial cell differentiation (Kiepe et al., 2006; Lochrie et al., 2006; Tanno et al., 2005; Yin et al., 2004). More importantly, the IGFBP-5 and IGF-II genes exhibit similar expression patterns in primary skeletal muscle cells and knockdown of IGFBP-5 inhibits myogenic differentiation in primary cells. These findings suggest that IGFBP-5 promotes muscle cell differentiation and this finding is of physiological relevance.

Our finding, however, contradicts the view that IGFBP-5 is a negative factor of myogenesis. Several previous studies in C2 myoblasts have shown that stably overexpressing IGFBP-5 under a constitutive promoter impairs myogenic differentiation, presumably by binding to and sequestering IGF-II from its binding to the IGF-IR (James et al., 1996; Mukherjee et al., 2007). The different findings on the precise role IGFBP-5 in myogenesis could be due to different experimental approaches used. In the present study, we explored the role of endogenous IGFBP-5 through a loss-of-function approach followed by "rescuing" using proteins, whereas previous studies primarily relied on overexpression approaches. The different results may also be due to different cell lines used. Previous studies were conducted experiments with C2 cells (Cobb et al., 2004; James et al., 1996; Mukherjee et al., 2007). Instead, we used the C2C12 cell line, which is subcloned from the parent C2 cell line by selection for the ability to differentiate rapidly and produce extensive contracting myotubes (Silberstein et al., 1986). Moreover, we obtained similar results in primary skeletal muscle cells, strongly supporting the physiological relevance of our findings.

IGFBP-5, Myogenin, and IGF-II are all induced during differentiation (Bayol et al., 2000; Rotwein et al., 1995). The expression of IGFBP-5 correlates well with the expression of Myogenin (Bergstrom and Tapscott, 2001). Myogenin, IGF-II, and IGFBP-5 were

undetectable in the mutant NFB4 cells, which cannot go through differentiation (Sarbassov et al., 1995). In cultured muscle cells, endogenously secreted IGF-II stimulates the IGF-IR, PI3K, and Akt to induce the expression of Myogenin (Wilson et al., 2004). The functional relationship between IGFBP-5, Myogenin, and IGF-II is not well understood. In this study, we found that IGFBP-5 knockdown caused marked reduction in both Myogenin and IGF-II expression, but had little effect on MyoD expression. Forced expression of functional Myogenin, however, failed to rescue the differentiation defects caused by IGFBP-5 knockdown. Forced expression of Myogenin did not cause significant changes in either IGF-II or IGFBP-5 mRNA levels. This suggests that while IGF-II and IGFBP-5 regulates Myogenin expression, Myogenin itself is not sufficient to induce myogenic differentiation in the absence of IGFBP-5 and/or IGF-II. Other factors must also be involved in the effect of IGFBP-5 on myogenesis. Future studies are needed to identify these factors.

One of the intriguing findings made in this study is that IGFBP-5 knockdown suppresses IGF-II expression. IGFBP-5 knockdown had little effect on the IGF-I and IGF-IR mRNA levels. Knockdown of IGFBP-5 did not affect the levels of other IGFBPs synthesized by C2C12 either. The specificity of this siRNA construct has been verified using a siRNA resistant form of IGFBP-5 in U2OS cells (Yin et al., 2004). In addition, the suppressed IGF-II expression was reversed by adding IGFBP-5 together with low concentrations of IGF-II or by a high concentration of IGF-II. Expression of myrAkt also restores IGF-II expression levels. We also ruled out non-specific interferon responses using another pSUPER construct expressing siRNA with a sequence unrelated to IGFBP-5.

IGFBP-5 is not only secreted but can also enter the nucleus in certain cell type (Firth and Baxter, 2002). We have recently reported that IGFBP-5 contains a functional

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transactivation domain in its conserved N-domain and this activity is IGF-binding independent (Xu et al., 2004; Zhao et al., 2006). Furthermore, full-length IGFBP-5 has transcriptional repressing activity (Zhao et al., 2006). Our finding that IGFBP-5 knockdown suppresses IGF-II mRNA expression in differentiating muscle cells raised the possibility that IGFBP-5 may exert direct an effect on IGF-II gene transcription in the nucleus. Two approaches were taken to address this intriguing hypothesis. First, immunocytochemical staining experiments showed that endogenous IGFBP-5 protein was detected in the Golgi apparatus and on the cell surface but not in the nuclei in myoblasts. Second, expression of the IGFBP-5 transactivation domain did not cause any significant change in IGF-II mRNA expression levels. These results argue against a direct action of IGFBP-5 on the IGF-II gene transcription in this cell type.

It is of interest to find that IGF-II up-regulates its own gene expression in differentiating C2C12 myoblasts. Although previous studies have indicated that nutrients influence IGF-II gene expression in C2C12 cells through the PI3K-Akt-mTOR pathway (Erbay et al., 2003), to our knowledge, this is the first demonstration that IGF-II stimulates its own gene expression in skeletal muscle cells. Furthermore, forced expression of a constitutive Akt strongly increases IGF-II expression in these cells, suggesting that IGF-II auto-regulates itself through activating the IGF-IR-PI3K-Akt signaling pathway in C2C12 cells. This and other findings led us o propose a new model, depicted in Fig. 2.10D, on how IGFBP-5 acts to promote myogenic differentiation. According to this model, the induction of IGFBP-5 in early stages of myogenic differentiation plays a crucial role in promoting the myogenic action of autocrine IGF-II. IGFBP-5 acts by binding to IGF-II and promoting its interaction with IGF-IR. This interaction may contribute to reaching a certain threshold that

turns on the IGF-II positive auto-regulatory loop, thereby resulting in increased IGF-II gene expression. The increase in IGF-II production, in turn, results in an accelerated elevation in the IGF-IR-PI3K-Akt signaling activity, leading to increases in Myogenin, MHC expression, and myotube formation. This model is supported by several lines of evidence. First, IGFBP-5 is induced in early stages of myogenesis, prior to the elevation of IGF-II expression, in the C2C12 myoblast model and in primary cells. Second, knockdown of IGFBP-5 impairs myogenic differentiation. Third, knockdown of IGFBP-5 suppresses IGF-II gene expression and addition of exogenous IGF-II "rescues" IGFBP-5 knockdown-induced myogenic defects. Furthermore, we show that IGF-II up-regulates its own gene expression via the PI3K-Akt signaling pathway. Addition of native IGFBP-5 but not a ligand binding IGFBP-5 mutant, together with a low concentration of IGF-II, restores IGF-II expression and myogenic differentiation.

Although it is known that IGFBP-5 has IGF-independent actions (Clemmons et al., 2002; Firth and Baxter, 2002), the myogenic promotion action of IGFBP-5 is clearly IGF-dependent because: 1) immunocytochemistry revealed that endogenous IGFBP-5 is not localized to the nuclei of C2C12 cells; 2) adding high concentrations of exogenous IGF-I or IGF-II reversed IGFBP-5 knockdown caused myogenic defects; 3) Des(1-6)IGF-II, an IGF-II analog with greatly reduced binding affinity for IGFBPs has lower activity in regulating differentiation; and 4) adding native IGFBP-5, but not the LBD mutant IGFBP-5, rescued the myogenic defects in the presence of low concentrations of IGF-II. It has been shown that IGFBP-5 is not only present in the extracellular fluids, but is also localized on the cell surface and/or extracellular matrix (Clemmons, 2001). In this study, we have provided two lines of independent evidence indicating that abundant

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amounts of intact IGFBP-5 is indeed located on the surface of differentiating myoblasts and is capable of IGF binding. It is possible that cell-surface associated IGFBP-5 provides a means of attracting IGF-II to the close proximity of the IGF-1R receptor, thereby enhancing IGF-II signaling activity and initiating the IGF-II positive auto-regulation loop.

In summary, we have uncovered a novel mechanism by which the induction of IGFBP-5 promotes the myogenic action of autocrine IGF-II. We provide evidence suggesting that IGFBP-5 is located on the cell surface and binds to autocrine IGF-II and potentiates its interaction with IGF-IR, leading to the enhanced activation of the IGF-IR-PI3K-Akt signaling activity and a further increase in IGF-II gene expression. An accelerated activation of this auto-regulatory loop stimulates muscle cell differentiation after reaching a certain threshold. We have extended these findings to primary cultures, suggesting that this mechanism is of physiological relevance.

Material and Methods

Materials and animals - Monoclonal MHC antibody (MF20) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). Antibodies against MyoD (M-318) and Myogenin (M-225) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies to Akt and phospho-Akt (Ser473) were from Cell Signaling Technology (Danvers, MA). Monoclonal anti-tubulin antibody (T 6793) was from Sigma (St. Louis, MO). The secondary antibody conjugates were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Recombinant human IGF-I, IGF-II, Des(1-6)IGF-II, and IGFBP-5 were purchased from GroPep (Adelaide, Australia). Mutant IGFBP-5 with reduced

affinity for IGF was kindly provided by Dr. David Clemmons, University of North Carolina at Chapel Hill (Imai et al., 2000). Trypsin, fetal bovine serum (FBS), horse serum (HS), Dulbeco's Modified Eagle's Medium (DMEM), OPTI-minimum essential medium, and penicillin-streptomycin were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). The BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL). Reagents for enhanced chemifluorescence were obtained from Amersham Biosciences (Piscataway, NJ). Dual-luciferase reporter assay kit was from Promega (Madison, WI). TriPure Isolation Reagent was from Roche Molecular Biochemicals (Nutlet, NJ). Oligonucleotide primers for PCR were purchased from Invitrogen. iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). All other chemicals were reagent grade and were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise noted. Mice (strains CD1) were obtained from Charles River Laboratories, Inc. (Wilmington, MA).

Plasmid construction- pSUPER Vector was kindly provided by Dr. Reuven Agami, Netherlands Cancer Institute. The construction and verification of pSUPER-BP-5, which generates IGFBP-5 siRNA, was described previously (Yin et al., 2004). Mouse IGF-II cDNA and partial mouse IGFBP-5 cDNA were amplified by RT-PCR and cloned into the pGEM-T easy vector (Promega). Myogenin plasmids (pCS2MTMyogenin and pCS2EGFP) and the Myogenin reporter construct 4RTK and control TK reporter construct (Tang et al., 2004) were generously provided by Dr. Daniel Goldman, University of Michigan. MyoD cDNA was a kind gift from Dr. Lassar Andrew, Harvard Medical School. The pCS2+myr-Akt construct, which expresses a constitutively active, membrane-localized full-length mouse Akt1 (Kohn et al., 1998), was provided by Dr. Anne Vojtek, University of Michigan. The pcDNA3.1(+)EGFP-IGFBP5, which expresses a non-secreted form of IGFBP-5 with EGFP

tagged at its N-terminus, was generated by inserting mature IGFBP-5 (with signal peptide deleted) into the pcDNA3.1(+) vector. All of the plasmids were verified by DNA sequencing.

Cell culture and transfection- Mouse C2C12 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS in a humidified air atmosphere containing 5% CO_2. For transfection, 6×10^4 cells were seeded in 6-well plates (Falcon, Corning, NY). 2 µg of pSUPER-BP5 or pSUPER plasmid DNA was transfected into cells as previously reported (Yin et al., 2004). Thirty hours after transfection, the cells were washed and incubated with fresh serum-free medium (SFM) for 48 h. At the end of incubation, the conditioned media and total RNA were prepared for further analysis. 30 h after transfection, the cells were washed with SFM and then transferred to differentiation medium (DM) consisting of DMEM plus 0.5-2% horse serum. For rescue experiments, 30 h after transfection, the cells were switched to DM plus IGF-I, IGF-II, and/or IGFBP-5 with concentration designated in each experiment. Primary mouse skeletal myoblasts were isolated from 2-5 day old mice, grown, and differentiated following previously reported method (Tang and Goldman, 2006). Briefly, the forelimbs and hindlimbs were removed from neonatal mice. Muscle mass was minced into a coarse slurry and enzymatically dissociated at 37°C for 35 – 45 min with frequent trituration. Isolated muscle cells were grown in DMEM supplemented with 10% FBS and 10% HS on collagen-coated plates at a density of 2 - 4 x 10^5 cells per 35-mm culture dish. Fugene6 (Roche) was used for transfection of these primary cells. Differentiation was induced by switching to DMEM medium containing 5% or 1% HS. All experiments were conducted in accordance with guidelines approved by the University Committee on the Use and Care of Animals, University of Michigan.

Northern blot- Total RNA was extracted using TriPure Reagent following the manufacturer's instructions. RNA samples were size-fractionated in 1.2% agarose gels, blotted, and fixed onto Hybond N membranes (Amersham Biosciences). Hybridization was performed using ^{32}P-dCTP-labeled (ICN, Irvine, CA) IGFBP-5 or IGFBP-4 cDNA probes as reported previously (Duan et al., 1996). Labeled GAPDH cDNA was used as a control. The band densities were quantified using Quantity One quantification software (Bio-Rad).

Reverse transcription (RT)-PCR and quantitative real-time RT-PCR (qRT-PCR)- After treated with DNase, RNA samples were subjected to reverse-transcription using SuperScript II reverse transcriptase (Invitrogen). Gene specific primers for IGF-I, IGF-I receptor, IGFBP-2, IGFBP-4, and S17 were described previously (Boutinaud et al., 2004; Wilson et al., 2003). IGF-II primers for RT-PCR are 5'-GGCTTCTACTTCAGCAGGC-3' (sense), 5'-GGTGGTAACACGATCAGGG-3' (antisense). The linear range of product amplification was established in pilot studies for each primer pair.

qRT-PCR was carried out using an iCycler iQ Multicolor real-time PCR detection system (Bio-Rad). Primer sequences for qRT-PCR were reported by others (Boutinaud et al., 2004). 4 µl of the cDNA product (1:20 dilution) was used as a PCR template. Plasmid cDNA for cyclophilin (Hasel and Sutcliffe, 1990), a housekeeping gene, was provided by Dr. Gregor Sutcliffe (Scripps Research Institute). Serial dilutions of the plasmids ranging from 10^8 to 10 molecules/2 µl were used for standard curve. The number of molecules of particular gene transcript was calculated based on the standard curve and normalized to the cyclophilin mRNA level. The specificity of the PCR was verified by denaturing curve analysis and direct sequencing of the products.

Immunohistochemistry- Cells cultured on 6-well dishes were washed twice with 1x

PBS before fixation in 4% paraformaldehyde for 10 min. Cells were permeablized for 5 min

in 1xPBS containing 0.2% Triton X-100 (PBST), washed twice with PBST, and incubated

with primary antibodies at 4°C overnight. The cells were next washed three times with PBST

before incubation with Cy3 conjugated secondary antibodies for 2 h at room temperature. For

experiment that required intact membrane integrity, staining was performed in 1x PBS

without detergent. Confocal images were obtained using a Leica SP5 Confocal microscope.

Other immunofluorescence or phase contrast micrographs were obtained using a Nikon

Eclipse E600 microscope. Differentiation index (%), defined as the percent of MHC positive

nuclei over total nuclei number, was determined.

Western and ligand Blot Analysis - Equal amounts of protein were separated by SDS-

PAGE and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA) and

subjected to Western blot following published procedures (Duan et al., 1996). The antibodies

were used at concentrations recommended by the commercial sources. Ligand blot analysis

was performed using digoxigenin-labeled IGF-I following published procedure (Yin et al.,

2004). To determine cell surface bound IGFBP-5, cultured cells were washed in 1X PBS

twice and then incubated briefly in a high salt buffer (2N NaCl).

Luciferase reporter assay- The transcription activity of Myogenin was determined

using the dual luciferase reporter assay kit (Promega). Briefly, myoblasts were co-transfected

with pCS2MTmyogenin or pCS2 plasmid, 4RTK or control minimal TK reporter, and an

internal control vector encoding *Renilla* luciferase. Differentiation was induced one day after

transfection. Two days after inducing differentiation, cells were washed, and cell lysates

were used to measure the firefly and *Renilla* luciferase activities. The result was expressed as

fold change over the empty control vector and 4RTK transfected group. Transfection efficiency was normalized by *Renilla* luciferase activity.

Statistical analysis- Differences among groups were analyzed by Student's *t* test or One-Way Analysis of Variance followed by Fisher's protected least significance difference test, using Prizm (GraphPad Software, Inc., San Diego, CA). Significance was accepted at $p<0.05$.

Acknowledgements
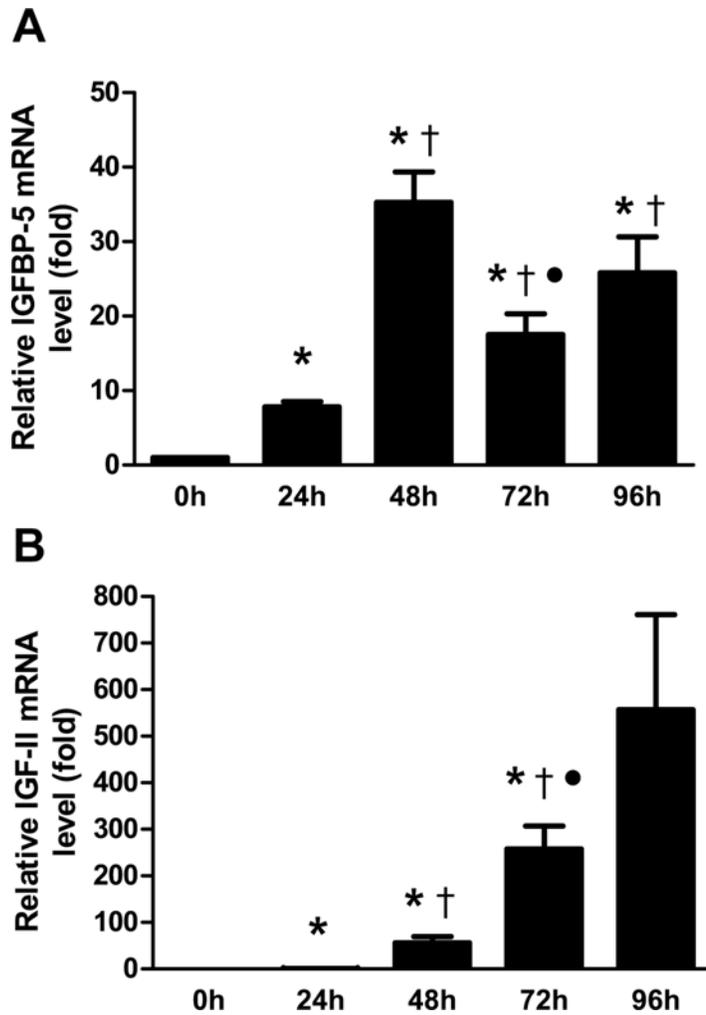
Figure 2.1. The induction of IGFBP-5 precedes that of IGF-II during myogenesis. Cultured

C2C12 myoblasts were induced to differentiate by switching to differentiation medium (DM)

containing 2% horse serum. Total RNA was extracted at different time points after the

addition of DM. IGFBP-5 mRNA (A) and IGF-II mRNA (B) levels were measured by qRT-

PCR. Values are expressed as relative levels to that of 24h for IGF-II (IGF-II mRNA levels

were under the detection limit at 0h) or to that of the 0h group for IGFBP-5 after normalized

to cyclophilin levels. Data shown are Means $\pm$ SE of three independent experiments. *

$p<0.05$, compared with the 0 h group; † $p<0.05$, compared with the 24h group; and ●

$p<0.05$, compared with the 48h group.
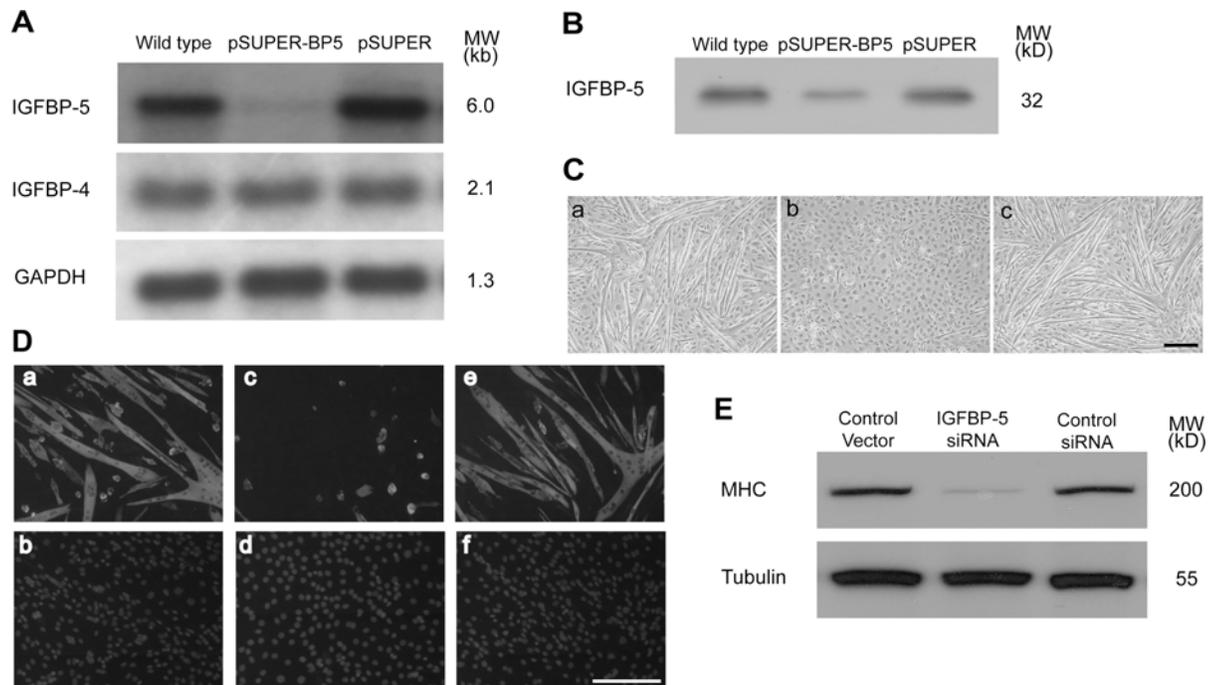
Figure 2.2. Knockdown of IGFBP-5 impairs myogenic differentiation. A) C2C12 cells were transfected with pSUPER or pSUPER-BP5. Three days later, total RNA was isolated and subjected to Northern blot analysis using the cDNA probes indicated. B) Conditioned media obtained from wild type, pSUPER-BP5 transfected, and pSUPER transfected cells were subjected to ligand blot analysis. C) Knockdown of IGFBP-5 inhibits myotube formation. C2C12 cells were transfected with the empty pSUPER vector (a), pSUPER-BP5 (b), or control pSUPER vector expressing an unrelated sequence (c). 30h after transfection, cells were induced to differentiate for 4 days. Images were representative from three reproducible experiments. Bar = 200μm. D) Knockdown of IGFBP-5 reduces MHC expression. Cells described in C) were analyzed by immunostaining for MHC (a, c, e) and counterstained with DAPI (b, d, f). Bar= 200μm. E) Western immunoblot analysis of cells described in C).
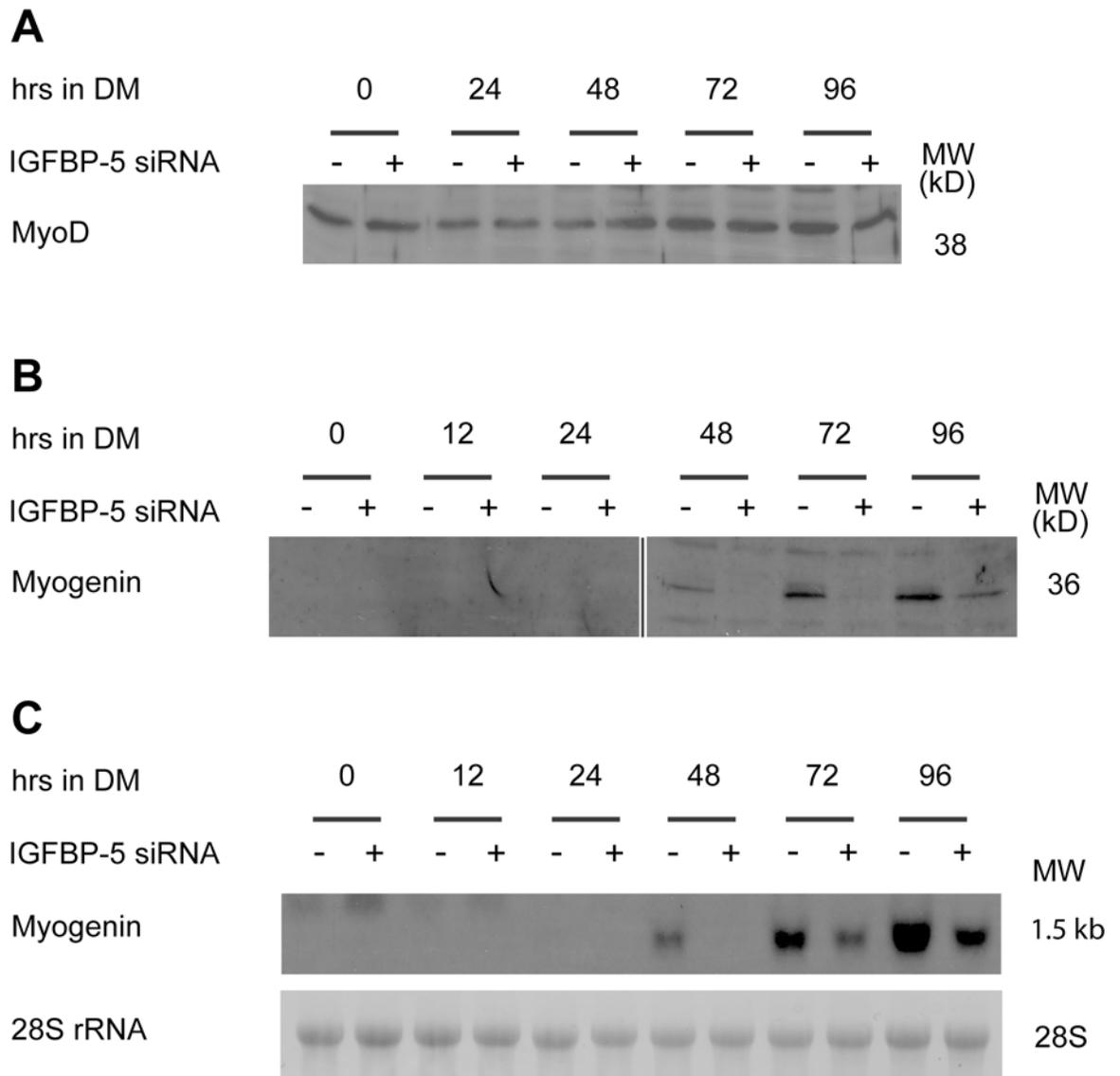
Figure 2.3. Knockdown of IGFBP-5 decreases Myogenin expression. After transfection with pSUPER or pSUPER-BP5, C2C12 myoblasts were switched to DM. Samples were collected at indicated time. A) Western immunoblot of MyoD. B) Western immunoblot analysis of Myogenin. C) Northern blot analysis of myogenin mRNA levels.

A

B

C

D
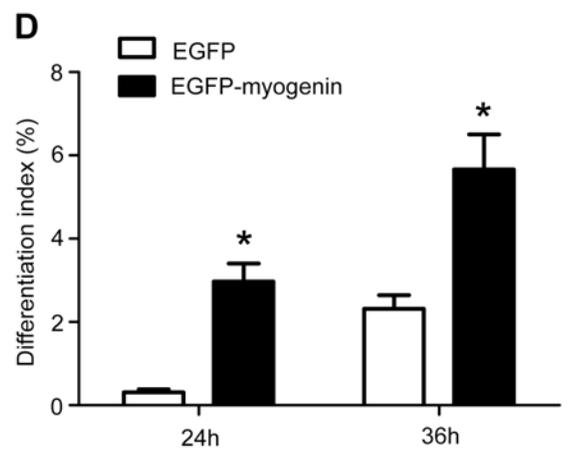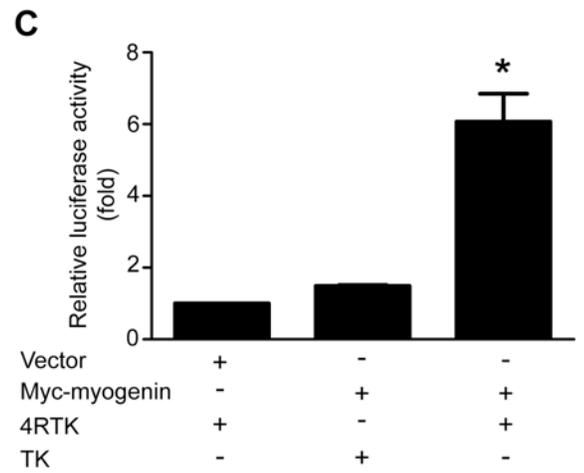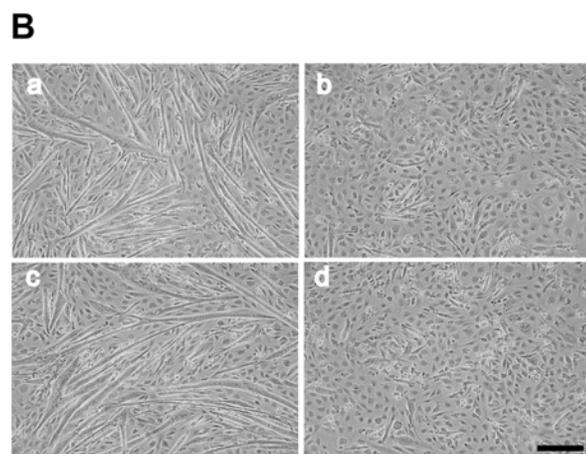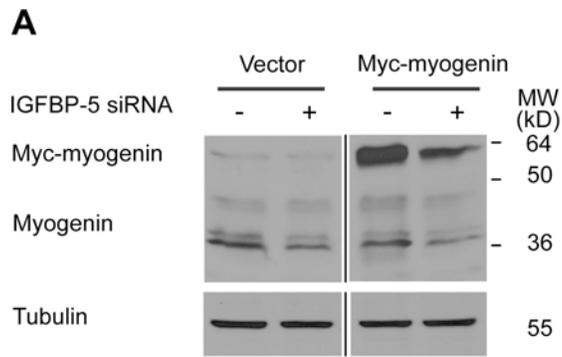
Figure 2.4. Forced expression of Myogenin does not rescue the myogenic defects in IGFBP-5 deficient cells. A) Western immunoblots analysis of C2C12 cells transfected with pSUPER + pCS2, pSUPER-BP5 + pCS2, pSUPER + Myc-tagged Myogenin, pSUPER-BP5 + Myc-tagged Myogenin 4 days after they were induced to differentiate. B) Phase-contrast images of cells transfected with pSUPER + pCS2 (a), pSUPER-BP5 + pCS2 (b), pSUPER + Myc-tagged Myogenin (c), and pSUPER-BP5 + Myc-tagged (d). These were representative images from four reproducible experiments. Bar = 200 µm. C) Luciferase activity was measured 2 days after differentiation was induced in C2C12 cells transfected with an empty vector and the Myogenin reporter construct 4RTK, Myc-tagged Myogenin and the reporter construct TK, and overexpression of Myogenin and 4RTK, respectively. Data are expressed as relative value to those of the empty vector and 4RTK transfected cells. Data shown are Means $\pm$ SE of three independent experiments each with duplicates. *$p < 0.05$. D) C2C12 cells were transfected with either EGFP or EGFP-tagged Myogenin and induced to differentiate for 24 or 36 hours and their differentiation indexes were determined. Data shown are Means $\pm$ SE of two independent experiments each performed in triplicates. *$p < 0.05$.

A

| hrs in DM	0		24		48		72		96		MW (bp)
IGFBP-5 siRNA	−	+	−	+	−	+	−	+	−	+	
IGF-II mRNA											372
IGF-I mRNA											246
IGF-IR mRNA											546
IGFBP-2 mRNA											556
IGFBP-4 mRNA											298
S17 mRNA											302
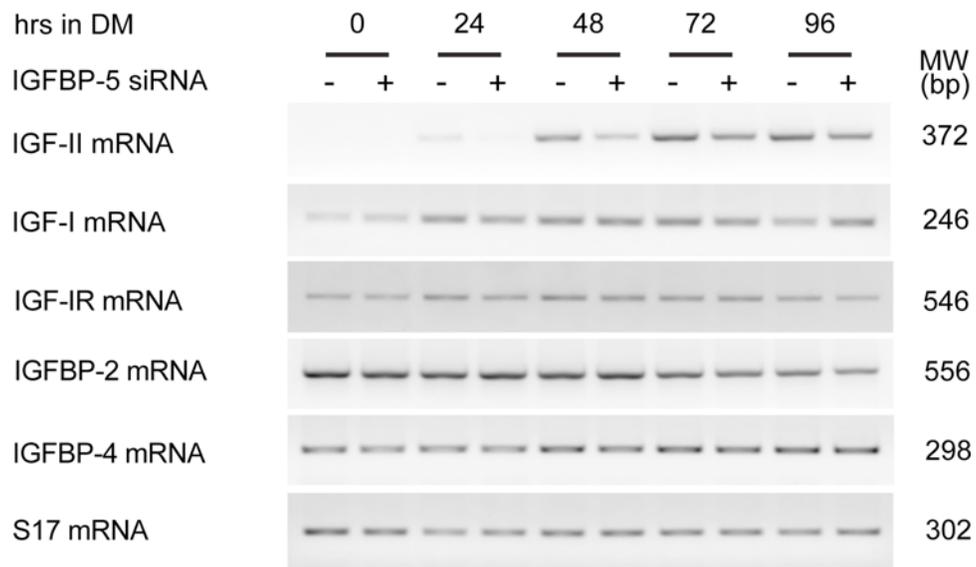
B
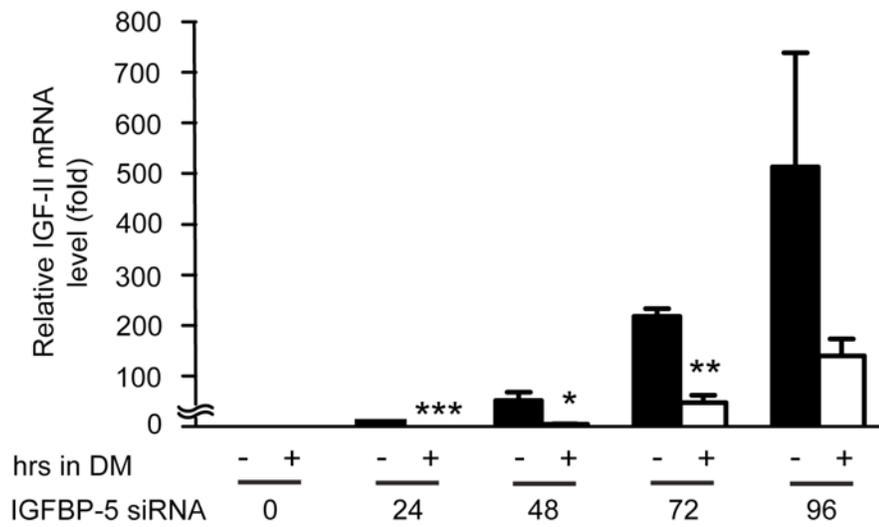
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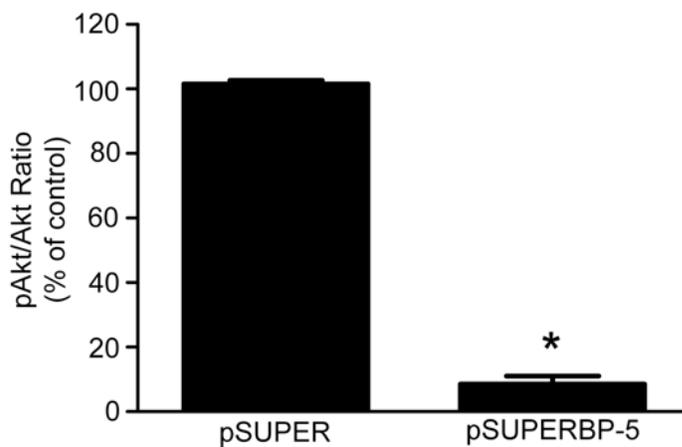
Figure 2.5. Knockdown of IGFBP-5 suppresses IGF-II gene expression and decreases IGF-IR-mediated signaling activity. A) C2C12 cells transfected with pSUPER or pSUPER-BP5 were induced to differentiate by switching to DM. RNA samples were prepared at 0, 24, 48, 72 and 96h later and subjected to RT-PCR. B) RNA samples described in A) were analyzed by qRT-PCR. Data are expressed as relative value to those of the pSUPER transfected cells at 24h after the induction of differentiation. Data shown are Means $\pm$ S.E. of three independent experiments. *p $<$ 0.05, **p$<$ 0.01, ***p$<$0.001 compared to the pSUPER transfected control groups at the same time point. C) Cells transfected with pSUPER or pSUPER-BP5 was induced to differentiate. 48 h later, cells were lysed and analyzed by Western immunoblot using antibodies against phospho-Akt and total Akt. The phospho-Akt/total Akt ratio was caculated by densitometry. Values are expressed as relative levels to pSUPER group. Data shown are Means $\pm$ S.E., n=4, *p $<$ 0.05.

A

a b
c d
e f

B

				MW (kD)
IGF-II	−	−	+	+
IGFBP-5 siRNA	−	+	−	+
MHC				200
Myogenin				36
Tubulin				55

C

				MW (kD)
IGF-II	−	−	+	+
IGFBP-5 siRNA	−	+	−	+
phospho-Akt				60
Total Akt				60

D

a b
c d

E

	Vector		myrAkt		MW (kD)
IGFBP-5 siRNA	−	+	−	+	
MHC					200
Myogenin					36
Tubulin					55
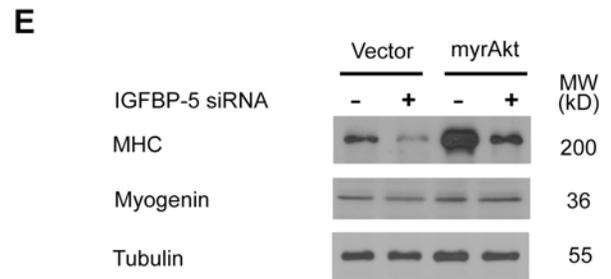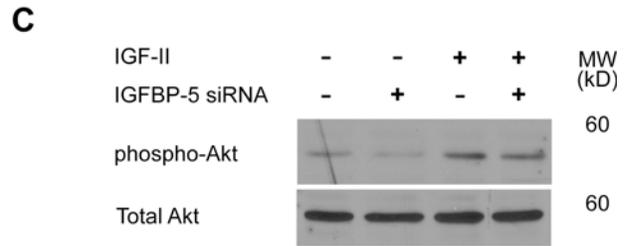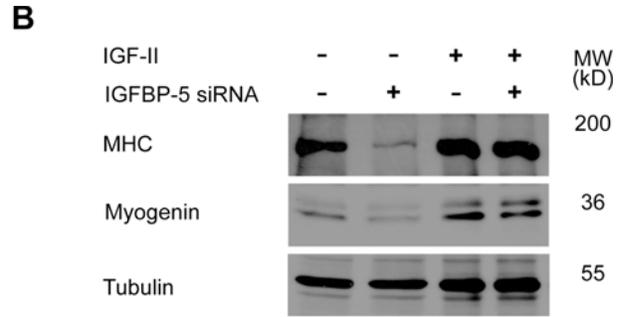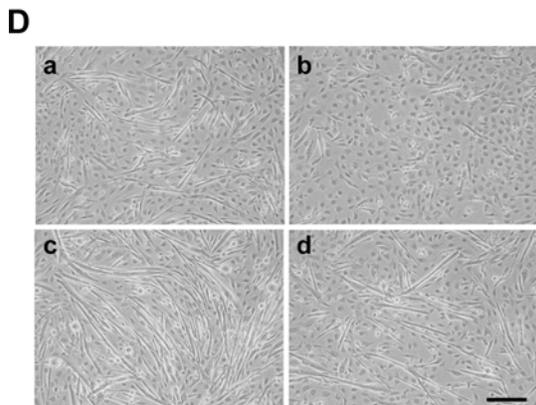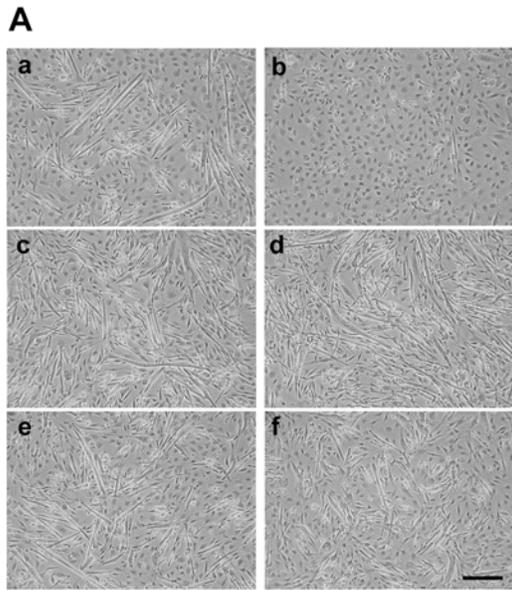
Figure 2.6. Exogenous IGFs or expression of a constitutively active Akt "rescues" the myogenic defects caused by IGFBP-5 knockdown. A) Addition of IGF-II or IGF-I promotes myotube formation in IGFBP-5 deficient cells. C2C12 cells transfected with pSUPER (a, c, and e) or pSUPER-BP5 (b, d, and f) were induced to differentiate by switching to the DM containing 0.5% horse serum without IGF-I (a and b), or with 400 ng/ml IGF-I (c and d), or 400 ng/ml IGF-II (e and f) for 4 days. Representative images from 4 reproducible experiments are shown. Bar = 200 μm. B) Western immunoblot analysis of MHC and Myogenin in cell lysates from the groups indicated. C) Western immunoblot analysis of Akt in cell lysates from the groups indicated. D) Forced expression of myrAkt increases myotube formation in IGFBP-5 deficient cells. C2C12 cells transfected with pSUPER + pCS2 (a), pSUPER-BP5 + pCS2 (b), pSUPER + pCS2+myr-Akt (c), and pSUPER-BP5 + pCS2+myr-Akt (d) were switched to the DM containing 0.5% horse serum for 4 days. Phase contrast images were representative images from 2 independent experiments. Bar = 200 μm. E) Forced expression of myrAkt increases MHC and Myogenin expression. Western immunoblot analysis of cell lysates from the groups indicated.
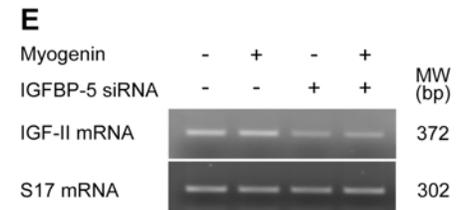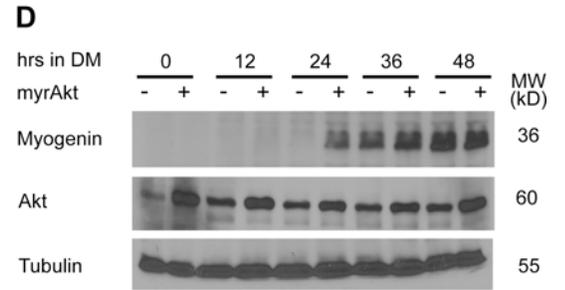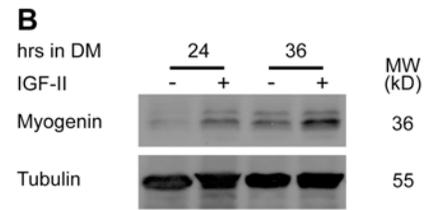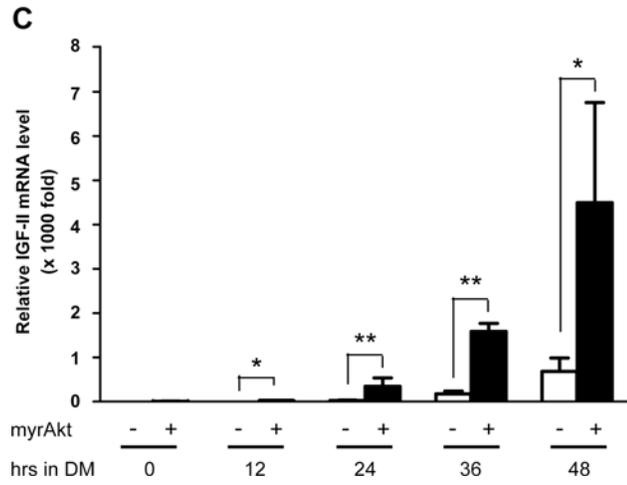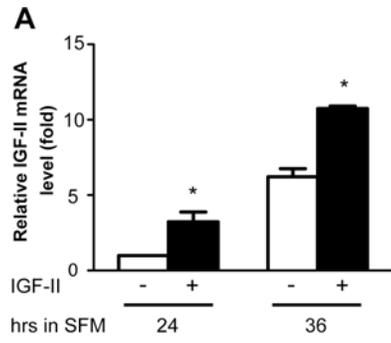
Figure 2.7. IGF-II up-regulates its own gene expression through the PI3K-Akt signaling pathway. A) Exogenous IGF-II increases IGF-II mRNA expression. Wild type C2C12 cells were switched to SFM supplemented with or without 300 ng/ml IGF-II. IGF-II mRNA levels were measured by qRT-PCR and normalized by cyclophilin mRNA levels. Data shown represent Means $\pm$ SE of two independent experiments. *$p < 0.05$ compared to the control group at the same time point. B) IGF-II increases Myogenin expression. Wild type C2C12 cells were switched to DM (containing 0.5% horse serum) supplemented with or without 300 ng/ml IGF-II. 24 and 36 h later, cells were lysed and subjected to Western blot analysis. C) Forced expression of myrAkt increases IGF-II mRNA expression. Cells transfected with the empty pCS2 (open box) or pCS2+myr-Akt plasmid (close box) were switched to DM. IGF-II mRNA levels were measured by qRT-PCR and normalized. The results were expressed as relative value to those of the empty vector transfected cells at 0 h. Data shown are Means $\pm$ S.E. of three independent experiments. *$p < 0.05$, **$p < 0.01$ compared to the control group at the same time point. D) Expression of myrAkt increases Myogenin expression. C2C12 cells transfected with the empty pCS2+ or pCS2+myr-Akt plasmid were switched to the DM containing 2% horse serum. Cells were lysed at the indicated time and subjected to Western blot analysis. E) Overexpression of Myogenin does not restore the reduced IGF-II expression in IGFBP-5 knocked down cells. Cells transfected with pSUPER + pCS2, pSUPER + Myc-Myogenin, pSUPER-BP5 + pCS2, and pSUPER-BP5 + Myc-Myogenin were induced to differentiate for four days. RNA samples were prepared and subjected to RT-PCR analysis. S17 was used as an internal control.
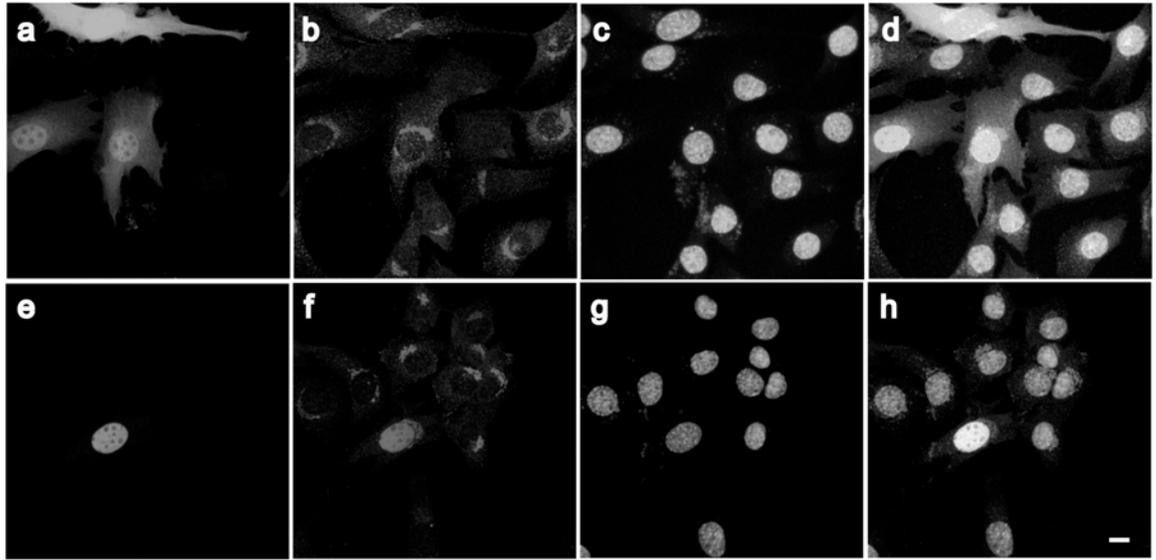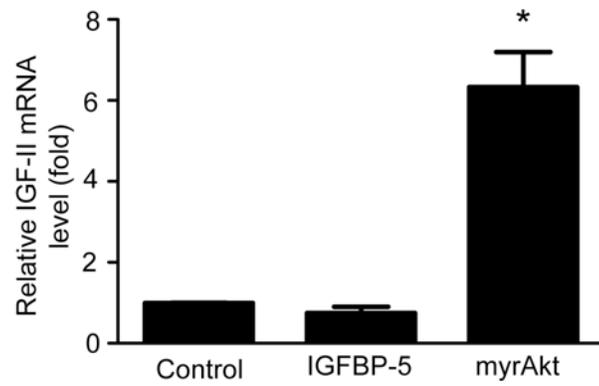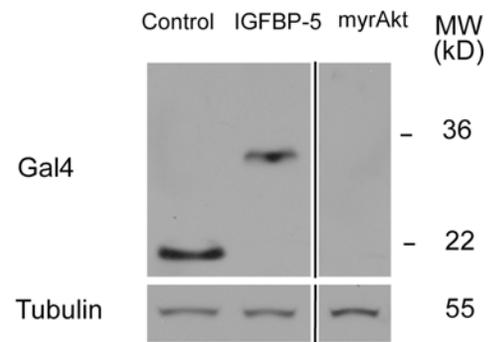
Figure 2.8. IGFBP-5 does not exert direct effect on IGF-II gene expression. A) IGFBP-5 is

not localized in the nucleus. C2C12 cells were transfected with either pcDNA3.1-EGFP (a-d)

or pcDNA3.1-EGFP-mIGFBP-5 (e-h). 24 h after the induction of differentiation, cells were

fixed, the membrane permeablized, and stained with an IGFBP-5 antibody (b and f) and

counter stained with DAPI (c and g). Panels a & e are GFP signal, and panels d & h are

overlays. B) Forced nuclear expression of the IGFBP-5 transactivation domain does not

increase IGF-II expression. C2C12 cells were transfected with either pBIND vector, pBIND-

BP5, or myrAkt and induced to differentiate. 36h later, total RNA samples were isolated and

IGF-II mRNA levels were determined by qRT-PCR. Values are relative to the control group

after normalized by cyclophilin levels. * $p < 0.05$ compared to the control group. C) Western

blot of cell lysates described in B) using a Gal4 antibody.

A

C

Differentiation index (%)

B

hrs in DM   0   24   MW (kD)

IGFBP-5   32

D

Relative MHC level

IGFBP-5 siRNA	-	+	+	+	+	+	+	+
IGF-II (ng/ml)	-	-	50	50	-	50	-	150
wt IGFBP-5 (ng/ml)	-	-	-	210	210	-	-	-
LBD IGFBP-5 (ng/ml)	-	-	-	-	-	210	210	-

76
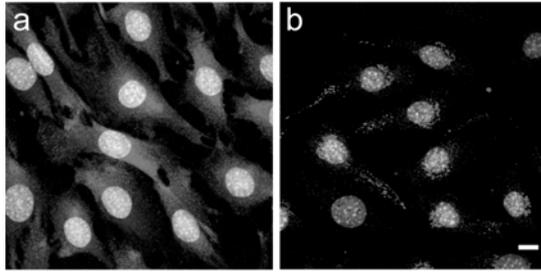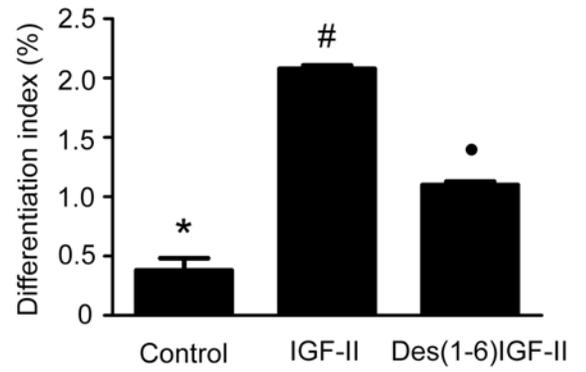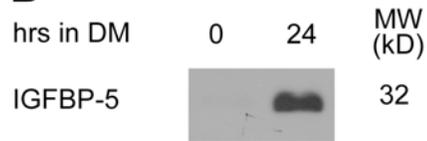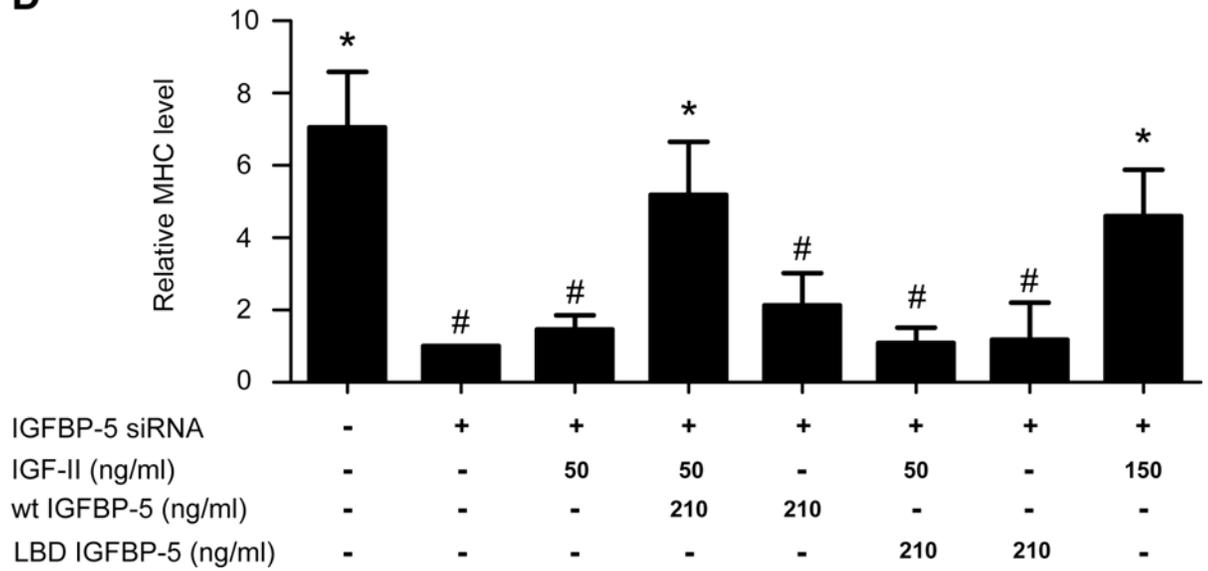
Figure 2.9. IGFBP-5 is localized on the cell surface and promotes myogenic differentiation by binding to and promoting IGF-II action. A) 24h after the induction of differentiation, C2C12 cells were fixed without (a) or with (b) membrane permeablization and stained with an IGFBP-5 antibody. B) 0 and 24 h after induction of differentiation, C2C12 cells were incubated with a high salt buffer to strip off membrane/ECM bound IGFBP-5. The released IGFBP-5 was detected by ligand blot. C) Des(1-6)IGF-II, an IGF-II analog with reduced binding affinity for IGFBPs, has a weaker effect in stimulating myogenic differentiation. C2C12 cells were switched to serum free medium containing 300 ng/ml IGF-II or Des(1-6)IGF-II. 36h later, the cells were subjected to MHC immunostaining and DAPI staining and the differentiation index determined as described in Materials and Methods. Data are Means $\pm$ SE of 3 independent experiments with duplicates. Group *, #, and ● are significantly different from each other at $p<0.05$. D) IGFBP-5 itself has no effect but it enhances the myogenic action of IGF-II. Cells transfected with pSUPER (a) or pSUPER-BP5 (b - h) were switched to DM (containing 0.5% horse serum) without (a - b), or with 50 ng/ml IGF-II (c), 50 ng/ml IGF-II + 210 ng/ml wild type IGFBP-5 (d), 210 ng/ml wild type IGFBP-5 (e), 50 ng/ml IGF-II + 210 ng/ml LBD-IGFBP-5 (f), 210 ng/ml LBD-IGFBP-5 (g), or 150 ng/ml IGF-II (h) for 4 days. MHC levels were measured by Western immunoblot and quantified by densitometry. Values are expressed as relative to the IGFBP-5 siRNA group. Data are Means $\pm$ SE of 3-4 independent experiments. Group * is significantly different from group # at $p<0.05$.
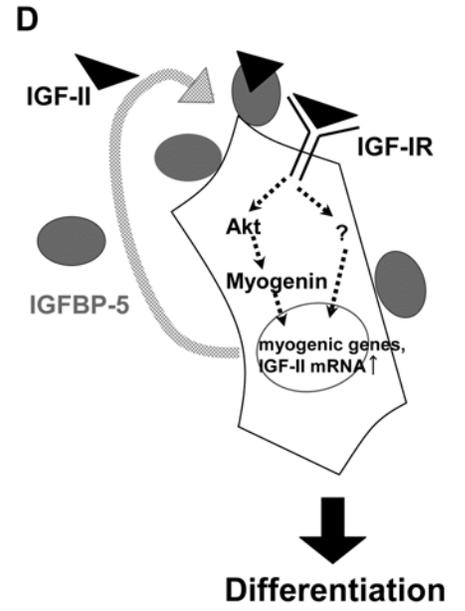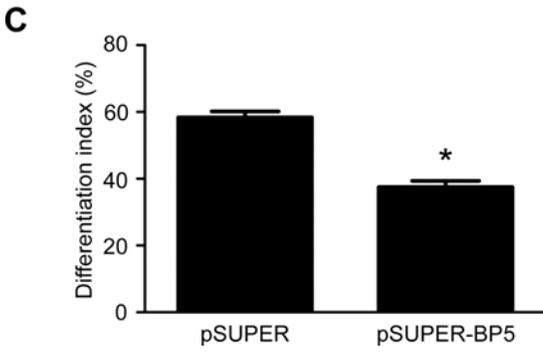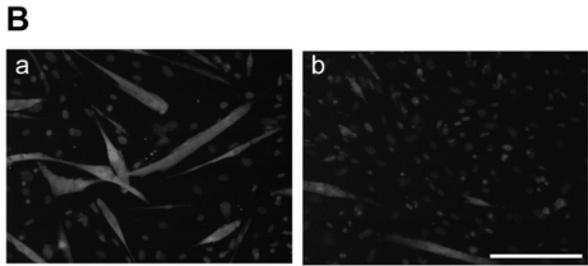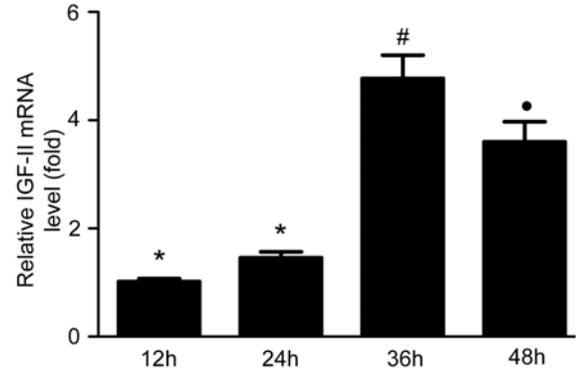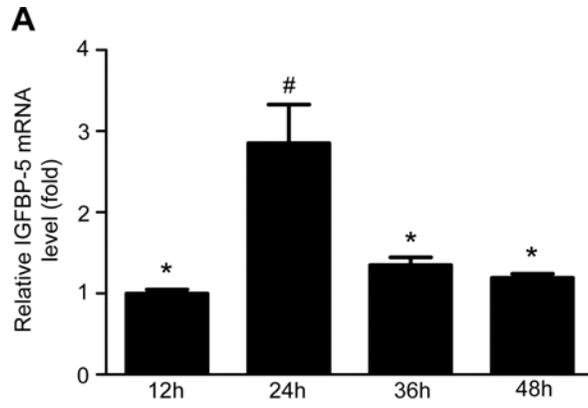
Figure 2.10. The IGF-II and IGFBP-5 genes exhibit similar induction patterns and IGFBP-5 knockdown inhibits myogenic differentiation in primary skeletal muscle cells. A) Total RNA was extracted from primary mouse skeletal myoblasts at the time indicated after the induction of differentiation. IGF-II mRNA and IGFBP-5 mRNA levels were measured by qRT-PCR. Values are expressed as relative levels to that of 12 h. Data shown are Means $\pm$ SE of three independent experiments each performed in duplicate. Groups labeled with different symbols are different from each other at $p<0.05$. B) Knockdown of IGFBP-5 inhibits muscle differentiation in primary skeletal muscle cells. Neonatal mouse myoblasts were transfected with the empty pSUPER vector (a) or pSUPER-BP5 (b). 30h after transfection, cells were induced to differentiate. Representative images from two independent groups are shown. Bar = 200μm. C) Cells described in A) were analyzed by MHC immunostaining and counterstained with DAPI. D) A proposed model on how IGFBP-5 and IGF-II act in concert to stimulate myogenic differentiation. IGFBP-5 is induced in early stages of myogenesis and is located on the cell surface. Cell-surface associated IGFBP-5 binds to IGF-II and targets IGF-II to the close proximity of the IGF-1R receptor, thereby enhancing IGF-1R-mediated signaling activity, leading to a further increase in IGF-II gene expression. This, in turn, results in an accelerated increase in the IGF-IR-PI3K-Akt signaling activity, leading to increases in Myogenin expression, and promoting myogenic differentiation.

CHAPTER III

HYPOXIA CONVERTS THE MYOGENIC ACTION OF IGF INTO MITOGENIC ACTION BY REPROGRAMMING GLUCOSE METABOLISM AND MULTIPLE SIGNALING PATHWAYS

Abstract

IGF stimulates both myoblast proliferation and differentiation. It remains elusive how those two mutually exclusive responses can be elicited by the same growth factor signaling through the same receptor. Here we show that while IGF promotes myoblast differentiation under normoxia, it stimulates proliferation under hypoxia. This conversion of IGF actions is HIF-1-dependent. Under normoxia, IGF activates the Akt-mTOR, p38, and Erk1/2 MAPK pathways, resulting in accelerated myogenesis. Hypoxia suppresses mTOR and p38 activities but enhances IGF-induced Erk1/2 activation. Under hypoxia, IGF preferentially activates Erk1/2, which stimulates proliferation and suppresses differentiation. Hypoxia also alters glucose metabolism by up-regulating several glycolytic enzymes and increasing lactate production. IGF stimulates proliferation by further enhancing the HIF-1-mediated increases in anaerobic glycolysis. These findings provide a mechanistic explanation for the paradoxical actions of IGFs and reveal a novel mechanism by which cells sense and integrate growth factor signals and oxygen availability in their microenvironments.

Introduction

Insulin-like growth factors (IGFs) are evolutionarily conserved polypeptides that play key roles in regulating animal development, growth, reproduction, and aging. At the cellular level, the pleiotropic IGFs regulate a variety of biological responses including, but not limited to, cell growth, proliferation, survival, migration, metabolism, and differentiation. IGFs are also key regulators of mammalian skeletal muscle differentiation and growth, as well as adult muscle regeneration and hypertrophy (Musaro et al., 2004; Musaro et al., 2001). During myogenesis, the IGF signal has been shown to promote both myoblast proliferation and differentiation - two mutually exclusive cellular events. These actions are mediated through the IGF-I receptor (IGF-IR), a transmembrane tyrosine kinase. The IGF-IR belongs to the receptor tyrosine kinase family and utilizes two major intracellular signaling pathways, namely the phosphatidylinositol 3-kinase (PI3K)-Akt cascade and the Raf- mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase (MEK)-extracellular signal-regulated kinase (Erk) cascade (White, 2003). Several laboratories have reported that activation of the PI3K-Akt-mTOR pathway promotes myogenic differentiation (Coolican et al., 1997; Engert et al., 1996; Jiang et al., 1999; Kaliman et al., 1996; Lawlor et al., 2000; Lawlor and Rotwein, 2000a; Lawlor and Rotwein, 2000b; Rommel et al., 2001; Rommel et al., 1999; Wilson and Rotwein, 2007; Wilson et al., 2004). Earlier studies also suggested that activation of the Raf-MEK1/2-Erk1/2 MAPK cascade has inhibitory effects on the myogenic differentiation induced by IGFs (Coolican et al., 1997). Despite extensive knowledge of the intracellular cascades that transmit the IGF signal to the nucleus, it remains unclear how the same IGF ligands,

acting through the same IGF-IR, stimulate two mutually exclusive cellular responses during myogenesis. This phenomenon is not unique to IGFs or skeletal muscle cells. Many growth factors and hormones exert pleiotropic actions in a wide variety of cell types. Understanding how specificity arises in connecting a given growth factor/hormone signal with the appropriate cellular responses is a fundamental question in cell biology.

In multicellular organisms, cell growth, proliferation, differentiation, and survival are not only regulated by hormonal or growth factor stimulation, but are also controlled by the availability of oxygen and nutrients in their microenvironments. Adaptation to hypoxia or low oxygen is a critical event for cells under numerous physiological and pathological situations, including tumor progression (Gordan et al., 2007; Le et al., 2004; Semenza, 2000). Hypoxia-inducible factor-1 (HIF-1) regulates many of the hypoxic responses. HIF-1 is composed of a stable β subunit and an oxygen-regulated α subunit (Bruick, 2003; Semenza, 2007). The HIF-1 complex binds to hypoxia-responsive element (HRE) in target genes and regulates their transcription. Recent studies have shown that hypoxia influences proliferation and differentiation of various stem/progenitor cell populations in mammals, in addition to its well-established roles in altering cellular energy metabolism and angiogenesis. It was demonstrated that neural crest stem cells growing at 5% O_2 showed increased proliferation and the formation of more multipotent clones as compared to those growing at 20% O_2 (Morrison et al., 2000). Hypoxia inhibits adipogenesis by repressing PPAR gamma 2 transcriptional activity via the HIF-1-regulated transcription repressor - DEC1/Stra13 (Yun et al., 2002). Nuclear HIF-1α accumulation under hypoxic conditions inhibits myogenic and neuronal differentiation and maintains cells in the undifferentiated state by directly binding to

intracellular Notch (ICN) (Gustafsson et al., 2005). In addition, HIFs have been shown to activate transcription factors such as Oct4 that control stem cell self renewal and multipotency (Keith and Simon, 2007).

The mechanisms integrating environmental cues and growth factor signals during myogenesis are largely unknown. In this study, we tested the hypothesis that oxygen tension plays a critical role in specifying the cellular responses to the pleiotropic IGF signal. We found that while IGF promotes muscle cell differentiation under normoxic conditions, it stimulates proliferation under hypoxic conditions. Our further analyses revealed that the activated HIF-1 complex and its transcriptional program coverts the myogenic action of IGF into mitogenic action, and it does so by reprogramming cellular glucose metabolism and by differentially regulating the PI3K-Akt-mTOR, p38, and Erk1/2 MAPK signaling activities. These data provide novel insights into our understanding of how muscle cell proliferation and differentiation are determined by signaling systems that sense and integrate growth factor/hormone, oxygen, and nutrient availability in their microenvironments.

Results

Hypoxia inhibits myoblast differentiation and represses the IGF-II gene expression

Cultured C2C12 myoblasts differentiated into morphologically distinct multinucleated myotubes after changing to differentiation medium (DM) (Fig. 3.1A). In contrast, they remained as mononucleated myoblasts under hypoxic conditions, as reported by Gustafsson et al. (2005). Consistent with the morphological changes, hypoxia repressed the expression of Myosin heavy chain (MHC), a terminal differentiation marker, and inhibited the expression of myogenic genes, such as MyoD, Myogenin, and p21 (Fig. 3.1B).

Since the endogenous IGF-II plays key roles in myogenesis under normoxic conditions

(Florini et al., 1996; Ren et al., 2008b; Wilson et al., 2004), we examined the effect of

hypoxia on IGF-II gene expression. As shown in Fig. 3.1C, hypoxia treatment reduced IGF-

II mRNA levels by 3.8 to 5.8 fold (p<0.01) at various time points after the induction of

differentiation. Likewise, $CoCl_2$, a chemical mimic of hypoxia, also reduced IGF-II

expression by 4 - 8 fold (data not shown). To explore the physiological relevance of the

above observation, we prepared primary skeletal myoblasts from neonatal mice and subjected

them to hypoxia. As shown in Fig. 3.1D, hypoxia treatment caused a 35%, highly significant

reduction (p < 0.001) in the differentiation index in these primary cells.

Hypoxia converts the myogenic action of IGF into mitogenic action

We next examined the impact of hypoxia on IGF-induced muscle differentiation. For

this, C2C12 myoblasts were induced to differentiate by adding IGF-II containing DM. As

shown in Fig. 3.1E, IGF-II caused a 2.5-fold, highly significant increase (p < 0.01) in the

differentiation index under normoxia. Hypoxia not only reduced the basal differentiation

index value by 3 fold (p <0.01), but also completely abolished the myogenic effect of IGF-II.

Similar results were obtained when the differentiation was assessed by the percentage of

Myogenin positive cells (Fig. 3.1F). While IGF-II significantly increased the percentage of

Myogenin-positive cells under normoxic conditions, there was essentially no difference

between the control and the IGF-II group under the hypoxic condition. Addition of NVP-

AEW541, a specific IGF-IR inhibitor (Garcia-Echeverria et al., 2004), abolished the IGF-

induced increase in differentiation (Fig. 3.1G), suggesting that the myogenic action of IGF-II is mediated by the IGF-IR.

We also examined whether hypoxia affects the mitogenic response of these cells to IGF stimulation by performing BrdU labeling experiments. Addition of IGF-II did not change the proliferation rate under the normoxic condition (Fig. 3.1H). Under the hypoxic condition, however, IGF-II caused a significant increase in the cell proliferation rate (Fig. 3.1H). A similar trend was found with the cell number. Compared to the control, IGF-II treatment caused a modest but statistically significant increase (11.2%, $p < 0.05$) in cell number under the normoxic condition (Fig. 3.1I). Under hypoxia, IGF-II caused a 45.7%, highly significant increase ($p < 0.001$) in cell number. Pharmacological blockade of the IGF-IR by NVP-AEW541 abolished the IGF-induced increases in cell number (Fig. 3.1J). These results indicate that while IGF stimulates myogenic differentiation under the normoxic condition, the same IGF signal promotes cell proliferation under the hypoxic condition. Both the myogenic and mitogenic actions of IGF-II are mediated by the IGF-IR.

Hypoxia alters the cellular responses to IGF through HIF-1-dependent mechanisms

We next examined the effect of hypoxia in activating the HIF-1 pathway in differentiating myoblasts by measuring nuclear HIF-1α protein abundance, HIF-1 transcriptional activity, and the mRNA levels of several known HIF-1 target genes. As shown in Fig. 3.2A, there was minimal HIF-1α protein accumulation in the nuclei of normoxic cells. Hypoxia greatly increased the nuclear HIF-1α levels. CoCl$_2$, a chemical that stabilizes HIF-1α, caused a similar increase in nuclear HIF-1α levels (Fig. 3.2A). The ability

of hypoxia to increase HIF-1-dependent transcription was measured using p2.1, a well-established HIF-1 reporter construct driven by human enolase promoter containing a functional HRE (Semenza et al., 1996). p2.4, which has a mutated HRE, served as negative control. As shown in Fig. 3.2B, hypoxia caused a 3 fold ($p < 0.01$) increase in the luciferase activity in p2.1 transfected cells, but did not change p2.4 activity. Hypoxia also caused marked increases in the mRNA levels of Glut1 and PGK1, two HIF-1 target genes (Fig. 3.2C). Collectively, these results demonstrated the activation of the HIF-1 transcriptional program in myoblasts cultured under hypoxia.

To explore the role of HIF-1α in determining the cellular responses to the IGF stimulation, we generated a pSUPER-HIF-1α siRNA construct targeting the same sequence reported by Lum et al. (2007). In good agreement with Lum et al. (2007), introducing of the pSUPER-HIF-1α construct into cultured myoblasts resulted in a marked reduction of nuclear HIF-1α protein under hypoxic conditions, whereas the control vector had no such effect (data not shown). As shown in Fig. 3.2D, hypoxia significantly reduced the basal differentiation levels. In control cells, IGF-II treatment increased differentiation under the normoxic condition, but it had no effect under the hypoxic condition. In the HIF-1α knockdown cells, IGF-II treatment caused a significant increase in the differentiation index under both normoxic and hypoxic conditions (Fig. 3.2D). The HIF-1α knockdown cells also exhibited altered mitogenic response to the IGF signal. In the control cells, IGF-II treatment caused a significant increase (38%, $p < 0.05$) in cell number only under hypoxia (Fig.3.2E). This effect was abolished in HIF-1α knockdown cells. These results suggest that hypoxia activates the HIF-1 complex and that the activated HIF-1 complex is required in converting the myogenic action of IGF into mitogenic action.

Hypoxia alters cellular glucose metabolism and this action is enhanced by IGF

It has been known for decades that rapidly proliferating tumor cells consume glucose at a much greater rate compared to normal cells and secrete most of the glucose-derived carbon as lactate rather than oxidizing it, a phenomenon known as the ''Warburg effect'' (Warburg, 1956). Thompson and colleagues (2008) recently proposed that metabolic reprogramming may fuel cell growth and proliferation: while the majority of ATP is generated by oxidative phosphorylation in quiescent cells, there is the large increase in glycolytic flux and most of the resulting pyruvate is converted to lactate in dividing cells (DeBerardinis et al., 2008). Growth factor signal transduction has been suggested to increase HIF-1α expression (Jiang et al., 1997; Richard et al., 2000; Semenza, 2003), which in turn reprograms the intracellular fate of glucose, resulting in decreased glucose-dependent anabolic synthesis and increased lactate production by regulating the expression of the glucose transporter and enzymes in the glycolytic pathway (Feldser et al., 1999; Liu et al., 2006; Lum et al., 2007).

We investigated whether hypoxia alters glucose metabolism in differentiating myoblasts and investigated the effect of IGF on the hypoxia-induced changes. Under the normoxic condition, the levels of nuclear HIF-1α were very low in the absence or presence of IGF-II (Fig. 3.3A). Hypoxia markedly increased HIF-1α levels and IGF-II increased it further (Fig. 3.3A). The possible effect of increased HIF-1α activity in altering glucose metabolism was examined by measuring the mRNA levels of several HIF-1 target genes that are involved in critical steps of glycolysis, including genes encoding glucose transporter 1 (Glut1) and

glycolytic enzyme PGK1. Hypoxia caused a significant increase in the Glut1 mRNA level (3.07 fold over the normoxia control group, $p<0.05$). IGF-II caused a further increase under hypoxia (1.9 fold, $p<0.05$) but it had no such effect under normoxic conditions (Fig. 3.3B). Likewise, PGK1 mRNA levels showed a 7.0 fold ($p<0.05$) increase in the hypoxia group. IGF-II caused a further 2.6 fold ($p<0.01$) increase over the hypoxia control group (Fig. 3.3C). Again, IGF stimulation did not increase PGK1 expression under the normoxic condition.

Recent studies suggest that the pyruvate dehydrogenase kinase 1 gene (PDK1) is a direct target of HIF-1 (Kim et al., 2006; Papandreou et al., 2006). PDK1 phosphorylates and inactivates the pyruvate dehydrogenase (PDH) enzyme complex that converts pyruvate to acetyl-coenzyme A, thereby limiting the entry of glycolytic carbon into the tricarboxylic acid (TCA) cycle (Holness and Sugden, 2003). As shown in Fig. 3.3D, hypoxia significantly increased the PDK1 mRNA levels and IGF-II enhanced the stimulatory effect of hypoxia on PDK1 expression. IGF-II had no such effect under the normoxic conditions (Fig. 3.3D). We next measured the end product – lactate. As shown in Fig. 3.3E, hypoxia caused a 2.3 fold increase ($p<0.001$) in lactate secretion. Addition of IGF-II resulted in a further increase (1.76 fold over the hypoxia group, $p<0.001$). IGF-II caused a modest but statistically significant increase in the lactate secretion under the normoxic condition. Taken together, these results suggest that hypoxia reprograms the glucose metabolism in myoblasts by increasing anaerobic glycolysis and lactate production and reducing oxidative phosphorylation - a metabolic profile resembling that of rapidly dividing cells. Stimulation with IGF further enhances these metabolic changes.

To test whether the reprogrammed glucose metabolism contributes to the mitogenic action of IGF observed under the hypoxia, cells were pretreated with 2-deoxy-D-glucose (2-

DG), which causes allosteric and competitive inhibition of hexokinase and limits glucose flux through the glycolytic pathway, prior to the IGF-II treatment under hypoxic condition. As shown in Fig. 3.3F, while IGF-II caused a 46.2% significant (p<0.001) increase in cell number, 2-DG abolished the mitogenic effect of IGF-II, indicating that the altered glucose metabolism caused by hypoxia favors the mitogenic action of IGF in myoblast cells.

Hypoxia inhibits the myogenic action of IGF by suppressing the Akt-mTOR signaling pathway

An important regulatory mechanism of hypoxic responses of mammalian cells is the inhibition of the mammalian target of rapamycin (mTOR) (Brugarolas et al., 2004; DeYoung et al., 2008; Kaper et al., 2006; Liu et al., 2006). As shown in Fig. 3.4A, in differentiating myoblasts, prolonged hypoxia strongly decreased S6 phosphorylation, an event downstream of the mTOR signaling (Fig. 3.4A). This inhibition was easily appreciated at 48 h. As time progressed, it became more evident. At 72 h and 96 h after the induction of differentiation, while the levels of phosphorylated S6 levels increased under normoxic condition, no phosphorylated S6 was detected in the hypoxia group (Fig. 3.4A). Addition of $CoCl_2$ to the differentiating myoblasts also completely repressed S6 phosphorylation (Fig. 3.4B). This suggests that hypoxia represses mTOR activity in differentiating myoblasts via HIF-1-dependent mechanism(s).

How does hypoxia inhibit mTOR activity in differentiating myoblasts? Studies in a number of cell types indicate that mTOR activity is regulated by the PI3K/Akt and AMPK pathways (Inoki et al., 2002; Inoki et al., 2003; Ma et al., 2005). These pathways converge at the tuberous sclerosis complex (TSC), which consists of TSC1 and TSC2 (Kwiatkowski,

2003; Li et al., 2004; Manning and Cantley, 2003). Phosphorylation of TSC2 by AMPK increases TSC complex activity (Inoki et al., 2002; Inoki et al., 2003), which in turn inhibits mTOR activity. In contrast, phosphorylation of TSC2 by Akt suppresses TSC2 GAP activity (Kwiatkowski, 2003; Ma et al., 2005), resulting in increased mTOR activity. Recent studies have reported that the HIF-1 target REDD1 inhibits mTOR signaling (Corradetti et al., 2005; Schwarzer et al., 2005). We carried out experiments to determine whether these mechanisms are involved in the repression of mTOR activity by hypoxia in differentiating muscle cells. As shown in Fig. 3.4C, the inhibition of mTOR activity by hypoxia was accompanied by an increase in REDD1 expression. While the REDD1 mRNA levels were not detectable by semi-quantitative RT-PCR under normoxic conditions, hypoxia resulted in a marked increase in REDD1 mRNA levels. Addition of IGF-II further increased REDD1 mRNA levels under hypoxic conditions. Hypoxia also caused a marked increase in the levels of phosphorylated AMPK (Fig. 3.4D). These results suggest hypoxia increases REDD1 expression and activates AMPK in C2C12 cells. To test whether the activated AMPK affects IGF actions, AICAR, an AMPK activator, was added to the cells in the presence or absence of IGF-II. The cells were induced to differentiate under the normoxic conditions. The results are shown in Fig. 3.4E. IGF-II increased myoblast differentiation by 2.9 fold ($p < 0.001$). Addition of AICAR not only caused a 3.8 fold reduction ($p < 0.05$) in basal differentiation, but also abolished the IGF-II-induced increase in myogenic differentiation, suggesting that the elevated AMPK activity can mimic the hypoxia effect and abolish the myogenic action of IGF.

To determine whether the reduced mTOR activity in hypoxia plays any role in converting the myogenic action of IGF into mitogenic action, cells cultured under normoxic and hypoxic conditions were treated with rapamycin in the absence and presence of IGF-II.

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In the condition of normoxia, IGF-II increased cell differentiation by 2.4 fold (p<0.001). Rapamycin treatment abolished the differentiation promoting effect of IGF-II. It also reduced the differentiation index value to 43% to that of the normoxia control group (p<0.001). Rapamycin also significantly reduced basal differentiation levels (p<0.001) under the normoxic and hypoxic conditions (Fig. 3.4F). Therefore, mTOR suppression is sufficient to abolish the myogenic action of IGF. Of note, the IGF-II plus rapamycin group had a significantly higher differentiation index value compared to the rapamycin alone group (0.61% vs. 0.03%, p<0.01), indicating the potential contributions by other signaling pathways. As shown in Fig. 3.4G, when added together with rapamycin, IGF-II caused a significant increase in cell number even under the normoxic condition. Under hypoxia, IGF-II increased the cell number significantly in the absence and presence of rapamycin. Rapamycin treatment resulted in a modest but significant decrease in basal cell number but it had no effect on the IGF-II-induced increase in cell number (Fig. 3.4G). These results suggest that mTOR signaling promotes the myogenic response to the IGF signal but it does not mediate the mitogenic action of IGFs.

We also examined the impact of oxygen tension on Akt signaling activity. As shown in Fig. 3.5A, under normoxic conditions, the phospho-Akt levels increased with the progress of differentiation. In contrast, the levels of phospho-Akt were extremely low or barely detectable under hypoxic conditions. $CoCl_2$ treatment had a similar effect in inhibiting Akt signaling in differentiating myoblasts (Fig. 3.5B), suggesting that hypoxia/HIF-1 strongly represses endogenous Akt activity during myogenesis. To determine the impact of hypoxia on the IGF-induced Akt activation, myoblasts were induced to differentiate under normoxic or hypoxic conditions and then exposed to IGF stimulation. As shown in Fig. 3.5C, IGF

stimulation resulted in a marked increase in Akt phosphorylation at 10 min and the activation

lasted for at least 30 min under normoxic conditions. Under hypoxia, the degree of Akt

activation caused by IGF stimulation was diminished. Quantitative results showed that

hypoxia significantly reduced the IGF-induced Akt phosphorylation ($p < 0.01$) (Fig. 3.5D).

Since IGF-II up-regulates its own gene expression via the PI3K-Akt pathway (Ren et al.,

2008) in these cells, we next measured the IGF-II mRNA levels as a functional test of the

PI3K/Akt signaling activity. As shown in Fig. 3.5E, while addition of IGF-II increased IGF-

II mRNA expression by 5.06 fold ($p<0.05$) under normoxic conditions, this effect of IGF-II

was abolished under hypoxia.

These results show that hypoxia represses basal and IGF-stimulated Akt signaling

activity. We postulated that this repression, together with the elevated levels of REDD1 and

AMPK activity, down-regulates mTOR activity, and thereby suppresses the myogenic action

of IGF. To test this idea, myrAkt, a constitutively active form of Akt, was introduced to

cultured C2C12 cells. If our hypothesis were correct, forced expression of the constitutively

active Akt should restore the reduced mTOR activity and rescue the differentiation defect

caused by hypoxia. As shown in Fig. 3.5F, expression of myrAkt increased the

differentiation under the hypoxic condition to a level comparable to the normoxia control

group. Western blot analysis showed that expression of myrAkt also restored the expression

of muscle specific genes, including MyoD, Myogenin, and MHC (data not shown). Further

quantitative analysis showed that while hypoxia decreased the differentiation index by 7.0

fold ($p<0.05$ compared with the normoxia control), expression of myrAkt caused a 12.0 fold

increase ($p<0.001$ compared with the hypoxia control) in the differentiation index, reaching a

level comparable to the normoxia control group. Expression of myrAkt increased the

differentiation index by 3.5 fold (p<0.001) under normoxic conditions (Fig. 3.5G). In contrast, overexpression of myrAkt did not result in any significant changes in cell number (Fig. 3.5H). Importantly, forced expression of myrAkt rescued the hypoxia-induced reduction in mTOR signaling activity as indicated by increased S6 phosphorylation levels (Fig. 3.5I). Rapamycin inhibited basal as well as myrAkt-induced S6 phosphorylation (Fig. 3.5I). These results support the notion that myrAkt rescues myoblast differentiation under hypoxic conditions via restoring mTOR activity.

Hypoxia promotes the mitogenic action but inhibits the myogenic action of IGF by altering the Erk1/2 and p38 MAPK activities

During the course of the above experiments, we noted that while hypoxia represses Akt activity, it did not decrease Erk1/2 MAPK signaling activity (Fig. 3.6A). To determine the precise impact of hypoxia on IGF-stimulated Erk1/2 activation, myoblasts were induced to differentiate under normoxic or hypoxic conditions and then exposed to IGF stimulation. Under the normoxic condition, IGF stimulation caused a rapid and transient increase in phospho-Erk1/2 levels. A greater and more sustained activation of Erk1/2 was observed under hypoxic condition (Fig. 3.6B). As shown in Fig. 3.6C, the levels of phosphorylated Erk1/2 in the hypoxia group were significantly higher than those of the normoxia control group at 15 min after the IGF stimulation (2.8 fold, p < 0.01). At 30 min, while the Erk1/2 activity returned to the basal levels under the normoxic condition, it remained significantly elevated under the hypoxic condition (Fig. 3.6C).

To determine the role of elevated Erk1/2 activation in mediating IGF actions, we used U0126 to inhibit MEK1/2 activity. As shown in Fig. 3.6D, while IGF-II significantly

increased myoblast differentiation under the normoxic condition, inhibition of Erk1/2 by

U0126 resulted in a further increase in differentiation in response to IGF stimulation (1.78

fold, p< 0.01). Under hypoxia, IGF-II did not increase myogenic differentiation by itself.

However, inhibition of Erk by U0126 restored its myogenic effect. In fact, the IGF-II +

U0126 group under hypoxia had a differentiation index value comparable to that of the IGF-

II group under normoxic condition (Fig. 3.6D). An opposite trend was observed with the

mitogenic action. IGF-II caused a significant increase in cell number under normoxic and

hypoxic conditions but the increase was greater under hypoxia (14% vs. 56% increase, p<

0.01). Inhibition of Erk by U0126 not only reduced basal cell number, but it completely

abolished the mitogenic action of IGF-II (Fig. 3.6E). These data suggested that Erk1/2

MAPK mediates IGF-induced cell proliferation but it inhibits the myogenic action of IGF.

p38 MAPK has also been shown to play an essential role in myogenesis by activating

myogenic transcription factors such as MEF2 and MyoD (Keren et al., 2006). As shown in

Fig. 3.6F, there was a significant reduction in p38 MAPK activity in differentiating

myoblasts under hypoxic conditions (55%, $p < 0.0001$). IGF stimulation increased p38

MAPK activity in myoblasts at the early stages of myogenesis (Fig. 3.6G). Pharmacological

blockade of the p38 kinases α and β was achieved by the continuous exposure of the cells to

the inhibitor SB203580 (SB). Under the normoxic condition, SB203580 inhibited the basal

differentiation (Fig. 3.6H) and blocked the myogenic action of IGF-II. Inhibition of p38 by

SB203580 alone did affect the cell number but it increased the mitogenic effect of IGF-II

under the normoxic condition. As shown in Fig. 3.6I, the IGF-II+ SB203580 group had

significantly more cells compared to those in the IGF-II alone group ($p < 0.05$). Consistent

with the low p38 MAPK activity under hypoxic conditions, addition of SB203580 did not

change basal or IGF-II-induced changes in differentiation or cell number under hypoxic conditions (Fig. 3.6H and I). Taken together, the data showed that hypoxia strongly represses p38 MAPK activity during myogenesis and the reduced p38 MAPK contributes to the conversion of myogenic action of IGF into mitogenic action under hypoxia.

Discussion

It has been known for decades that IGFs not only stimulate myoblast proliferation but also promote myogenic differentiation (Florini et al., 1996; Rosenthal and Cheng, 1995). Several groups have shown that activation of PI3K, Akt, and FoxO promotes myogenic differentiation and survival (Coolican et al., 1997; Engert et al., 1996; Jiang et al., 1999; Kaliman et al., 1996; Lawlor et al., 2000; Lawlor and Rotwein, 2000a; Lawlor and Rotwein, 2000b; Rommel et al., 2001; Rommel et al., 1999; Wilson and Rotwein, 2007; Wilson et al., 2004). We have recently shown that locally produced IGF binding protein-5 promotes muscle cell differentiation by binding to IGF-II and switching on the IGF-II auto-regulation loop (Ren et al., 2008a). Despite these advances, it remains puzzling how the same IGF signal elicits these opposite biological responses. In this study, we show that oxygen tension plays a pivotal role in specifying IGF actions in muscle cells. While the IGF signal promotes muscle cell differentiation under normoxic conditions, it stimulates proliferation under hypoxia. Both actions are mediated by the IGF-IR, as indicated by the fact the IGF-IR specific inhibitor NVP-AEW541 abolishes both the myogenic and mitogenic actions of IGF. Mechanistically, hypoxia activates the HIF-1 complex in muscle cells, and the activated HIF-1 complex coverts the myogenic action of IGF into mitogenic action by reprogramming intracellular glucose metabolism and by differentially regulating multiple signal transduction

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pathways downstream of the IGF-IR. These findings have provided a mechanistic explanation for the pleiotropic actions of IGFs during myogenesis.

Myoblast cells produce endogenous IGF (primarily IGF-II in the case of the C2C12 model) that promotes myogenesis under normoxic conditions. We show that hypoxia inhibits myoblast differentiation and disrupts the IGF-II autocrine regulation loop. Under hypoxia, IGF only stimulates cell proliferation. These findings suggest that the interaction between two important signals, the IGFs and hypoxia or its effector HIF-1, determines whether a myoblast cell will divide or undergo terminal differentiation. We provide strong evidence that the effect of hypoxia in converting the myogenic action of IGF into mitogenic action is HIF-1α-dependent. First, hypoxia activates the HIF-1 complex in differentiating muscle cells, as indicated by the increased HIF-1α levels in the nucleus, increased HIF-dependent transcription activity, and elevated mRNA expression of HIF-1 target genes. Second, $CoCl_2$, which stabilizes HIF-1α, had similar effect. Most importantly, knockdown of HIF-1α reversed the cellular responses to IGF stimulation. Intriguingly, IGF stimulation increases HIF-1 accumulation and its target gene expression under hypoxic conditions, but not normoxic conditions. This functional interaction between the hypoxia and IGF signaling unraveled in this study is in good agreement with previous studies. Growth factor signal transduction has been suggested to enhance HIF-1α expression in various cancer cells (Jiang et al., 1997; Richard et al., 2000; Semenza, 2003). Activation of the PI3K and MAPK pathways is associated with an increase in HIF-1α protein synthesis (Bardos et al., 2004; Fukuda et al., 2002; Jiang et al., 2001; Kasuno et al., 2004; Laughner et al., 2001; Richard et al., 1999; Zhong et al., 2000). A recent study by Lum et al. (2007) suggest that IL3-dependent HIF-1α expression reprograms the intracellular fate of glucose, resulting in

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decreased glucose-dependent anabolic synthesis and increased lactate production in hematopoietic cells, an effect that is enhanced when HIF-1α protein is stabilized by hypoxia. In this study, we have found that hypoxia leads to a change in the energy metabolism that shifts from oxidative phosphorylation to glycolysis by affecting the expression of Glut1, PGK1 and PDK1 gene in differentiating muscle cells. The IGF-dependent induction of HIF-1α appears to partially contribute to the ability of IGF to induce a metabolic conversion to aerobic glycolysis. The glycolysis inhibitor, 2-DG, inhibited IGF-II induced cell proliferation under hypoxia. Knockdown of HIF-1α reversed the cellular response to IGF-II.

In addition to reprogramming glucose metabolism, hypoxia induces adaptive changes in multiple signaling pathways. In differentiating myoblasts, hypoxia inhibits mTOR activity. Pharmacological blockade of mTOR activity results in compromised myogenic differentiation even in the presence of IGF. Therefore, mTOR suppression in hypoxic environments is sufficient to inhibit the myogenic action of IGF. The inhibition of mTOR signaling, however, did not affect the mitogenic activity of IGF. It has been reported that hypoxia exerts its effect on mTOR signaling by inducing negative regulatory factors, such as REDD1 and AMPK (Brugarolas et al., 2004; DeYoung et al., 2008; Kaper et al., 2006; Liu et al., 2006). In good agreement with these previous reports, we have found that hypoxia increases REDD1 and AMPK levels in differentiating myoblasts. Moreover, the AMPK activator, AICAR, can mimic the hypoxia effect. Another major pathway regulating mTOR activity is the PI3K-Akt pathway, which acts downstream of the IGF-IR. There is convincing evidence that IGF activates the PI3K-Akt-mTOR signaling pathway to promote myogenesis under normoxic conditions (Ren et al., 2008a; Wilson et al., 2004). In the present study, we have shown that hypoxia strongly represses the basal and the IGF-stimulated Akt signaling

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activity. We have provided convincing genetic and pharmacological evidence to support the notion that down-regulation of Akt, combined with the elevated AMPK activity and increased REDD1 levels leads to the inhibition of mTOR activity under hypoxia, which in turn inhibits the myogenic action of IGFs. We show that forced expression of the constitutively active Akt restored hypoxia-caused reduction in mTOR signaling activity and rescued the differentiation action of IGF under hypoxia. This effect is specific to the myogenic action of IGFs, as overexpression of myrAkt did not result in any significant changes in cell number. Inhibition of mTOR activity with rapamycin abolished the differentiation promoting effect of IGF under normoxia, while rapamycin treatment had no effect on the IGF-II-induced increases in cell number.

Hypoxia also strongly inhibits p38 MAPK activity in differentiating muscle cells. Although the p38 MAPK was discovered based on its activation by stresses and by pro-inflammatory cytokines, there is now convincing evidence for the myogenic action of p38 MAPK during myogenesis (Keren et al., 2006; Perdiguero et al., 2007). In the present study, we have discovered that IGF stimulation activates p38 MAPK activity at the early stages of myogenesis. Pharmacological blockade of the p38 kinases α and β decreased the basal differentiation and blocked the myogenic action of IGF. Importantly, inhibition of p38 MAPK by SB203580 increased the mitogenic effect of IGF under the normoxic condition, suggesting that p38 MAPK plays dual roles during myogenesis - it promotes the myogenic actions of IGF while inhibits mitogenic actions. Consistent with the low p38 MAPK activity under hypoxia, pharmacological blockade of the p38 kinases α and β by SB203580 did not change basal or IGF-induced changes in differentiation or cell number under hypoxic conditions. Taken together, our results indicate that IGF activates the Akt-mTOR signaling

pathway and the p38 MAPK pathway under normoxic conditions. While both Akt-mTOR

and p38 MAPK positively contribute to myogenesis, activation of the p38 MAPK also

inhibits myoblast proliferation. Hypoxia strongly represses Akt-mTOR and p38 MAPK

activities during myogenesis and the reduced activities of Akt-mTOR and p38 MAPK

contribute to the altered cellular responses to IGF stimulation (Fig. 3.7). At present, there are

contradictory views about the relationship between the PI3K-Akt and p38 MAPK pathways

in muscle cells. Many studies suggested that a positive feedback loop may exist between

these two pathways (Cabane et al., 2004; Conejo et al., 2002; Cuenda and Cohen, 1999;

Gonzalez et al., 2004). Other studies, however, have found that inhibition of the PI3K did not

affect the phosphorylation and activity of p38 MAPK (Li et al., 2000; Sarker and Lee, 2004;

Serra et al., 2007; Tamir and Bengal, 2000), indicating that that they maybe two parallel

pathways that regulate common myogenic genes. Regardless of what their precise

relationship is, our studies have clearly demonstrated that these two pathways have

overlapping yet distinct roles during myogenesis: while both Akt and p38 MAPK are

required for myogenic differentiation, p38 MAPK but not Akt affects proliferation.

Another important finding made in this study is that hypoxia increases and/or

prolongs the IGF-stimulated Erk1/2 activation in differentiating myoblasts. While IGF

stimulation caused a rapid and transient increase in phospho-Erk1/2 levels under the

normoxic condition, the same IGF stimulation caused a greater and more sustained activation

of Erk1/2 under hypoxic condition. There are several possible mechanisms by which hypoxia

may alter the IGF-stimulated activation of Erk1/2. A recent study by Carracedo et al. (2008)

have shown that inhibition of mTOR by rapamycin leads to the activation of Erk1/2 through

a IRS1/PI3K-dependent feedback loop in human cancer cells in vitro and in vivo.

Intriguingly, these authors also reported that rapamycin, when added together, enhanced the ability of IGF-1 and insulin to activate Erk1/2 MAPK in MCF7 human breast cancer cells (Carracedo et al., 2008). Koyama et al. (2008) has shown that IGF-I activates Erk1/2 via the formation of the Gab1 and SHP2 complex in C2C12 myoblasts under normoxia. Importantly, the Gab1-SHP2-Erk1/2-signaling pathway inhibits IGF-dependent myogenic differentiation in C2C12 myoblasts (Koyama et al., 2008). It is plausible that hypoxia may alter the expression of Gab1 directly or indirectly. Regardless the precise biochemical mechanisms by which hypoxia alters the IGF-stimulated activation of Erk1/2, It is clear that IGF preferentially activates the Erk1/2 MAPK signaling pathway in the hypoxic environments because both the Akt-mTOR and p38 MAPK pathways are repressed under hypoxia. This rewiring of signaling network has important functional significance. In agreement with the prevailing view that activation of Erk1/2 MAPK leads to cell proliferation, inhibition of Erk1/2 activity by U0126 abolishes the mitogenic action of IGFs in myoblasts. Unexpectedly but importantly, inhibition of Erk1/2 activity not only resulted in an increase in the myogenic action of IGF stimulation under normoxic conditions, but also restored the myogenic action of IGF under hypoxia. These data suggested that the activation of the Erk MAPK signaling pathway not only stimulates myoblast proliferation, but also suppresses myogenic differentiation (Fig. 3.7). How hypoxia alters the IGF's ability to activate Erk1/2 MAPK and how the activated Erk1/2 inhibits muscle cell differentiation is not clear at this movement. It has been shown that inhibition of Erk activity resulted in increased p38 MAPK activity and vice versa in L6E9 myoblasts (Khurana and Dey, 2002). This interaction between p38 and Erk1/2 MAPK activities could explain the results of this study. It is also possible that the Erk1/2 MAPK pathway may directly affect muscle differentiation.

Based on these and other findings, we propose a model of how oxygen tension determines muscle cell responses to the IGF signal (Fig. 3.7). The in vivo importance of this mechanism cannot be directly demonstrated at present because of the technical difficulty in manipulating local oxygen tensions in vivo in a mammalian embryo and the fact that HIF-1 also has hypoxia-independent actions (Bruick, 2003; Semenza, 2007). However, the fact that hypoxia has similar effect in primary mouse skeletal myoblasts strongly argues that this mechanism is physiologically relevant. This model is also supported by recent findings in non-mammalian vertebrate model organisms, such as zebrafish. It has been reported that hypoxia decreases the rate of somitogenesis in zebrafish embryos through the inhibition of IGF signaling (Kajimura et al., 2005b). Therefore, the link between hypoxia and IGF signaling unraveled in this study may represent a normal developmental program by which muscle stem/precursor cells respond to different oxygen tensions in their microenvironments. The interplay between hypoxia/HIF-1 and IGF signaling pathway also have important implications in muscle hypertrophy, muscle atrophy, and muscle regeneration. There is in vitro and in vivo evidence that the activation of the PI3K/Akt signaling pathway by IGFs increases skeletal muscle hypertrophy and prevents muscle atrophy (Bodine et al., 2001; Rommel et al., 2001). Muscle regeneration occurs when the adult myogenic progenitors (e.g., satellite cells) are activated in response to myofiber injury (Wagers and Conboy, 2005). Both pathways are activated by regeneration cues and influence the ability of muscle progenitors to execute different stages of the regeneration program. Hypoxia blocks myogenic satellite cell differentiation (Gustafsson et al., 2005). The PI3K/Akt/mTOR signaling, on the other hand, mediates satellite cell response to IGFs that promote critical events in the regeneration process, such as proliferation, muscle gene expression, myoblast fusion, survival, and

postmitotic growth of myotubes (Lawlor and Rotwein, 2000a; Musaro and Rosenthal, 1999; Rommel et al., 2001). The functional importance of the IGF1/ PI3K/Akt pathway in muscle regeneration is supported by in vivo studies (Barton et al., 2002; Musaro et al., 2001). Likewise, the p38 MAPK pathway is also activated in satellite cells in response to locally released soluble inflammatory factors or cell-to-cell interactions, and promotes cell-cycle arrest (Perdiguero et al., 2007; Puri et al., 2000) and terminal differentiation (Wu et al., 2000; Zetser et al., 1999). IGFs are mitogens and potent survival factors for a variety of cancer cells and the up-regulation of IGFs are found to be positively correlated with tumor progression and metastasis (Clemmons, 2007). Drugs targeting IGF signaling have been developed and are under clinical trials. One aspect of the microenvironment that differs in normal tissue versus tumor tissue is oxygen tension. For example, solid tumors often have poorly formed vasculature, which is associated with local hypoxia. The presence of HIF-1 often associates with poor prognosis and has been proposed to link to tumor cell survival, proliferation, and migration (DeBerardinis et al., 2008; Gordan et al., 2007; Keith and Simon, 2007). Many HIF-1 target genes are involved in crucial aspects of cancer biology (Semenza, 2003) and efforts are underway to develop specific molecules targeting the HIF pathway as anticancer therapeutics. Therefore, the control of IGF actions by oxygen tension unraveled in this study may also have implications in cancer biology.

Material and Methods

Materials- Monoclonal MHC antibody (MF20) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). Antibodies against MyoD (M-318), Myogenin (M-225), and p21 (C-19) were purchased from Santa Cruz Biotechnology

(Santa Cruz, CA). Antibodies for Akt, phospho-Akt (Ser473), phospho-S6 (Ser235/236), and pAMPK were from Cell Signaling Technology (Danvers, MA). Monoclonal anti-tubulin antibody and 2-DG were from Sigma (St. Louis, MO). The HIF-1α antibody (NB100-449) was from Novus Biologicals (Littleton, CO). Histone H3 antibody was from Abcam (Cambridge, MA). The secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Recombinant human IGF-II was purchased from GroPep (Adelaide, Australia). Rapamycin, SB 203580, U0126, and AICAR were purchased from Calbiochem (Gibbstown, NJ). The IGF-1R inhibitor, NVP-AEW541, was kindly provided by Norvatis (Basel, Switzerland). Trypsin, fetal bovine serum (FBS), horse serum (Wilson et al.), Dulbeco's Modified Eagle's Medium (DMEM), OPTI-Minimum Essential Medium, and penicillin-streptomycin were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). The BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL). The Dual-luciferase reporter assay kit was from Promega (Madison, WI). TriPure Isolation Reagent was from Roche Molecular Biochemicals (Nutlet, NJ). Oligonucleotide primers for PCR were purchased from Invitrogen. iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA). All other chemicals were reagent grade and were purchased Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

Plasmid construction- Mouse IGF-II, PGK1, GLUT1, and PDK1 partial cDNA were amplified by RT-PCR and cloned into the pGEM-T Easy vector (Promega). The pCS2+myr-Akt construct, which expresses a constitutively active, membrane-localized full-length mouse Akt1 (Kohn et al., 1998), was provided by Dr. Anne Vojtek (University of Michigan). pSUPER vector was kindly provided by Dr. Reuven Agami, Netherlands Cancer Institute.

pSUPER-HIF-1α was constructed based published sequence (Lum et al., 2007). All of the plasmids were verified by DNA sequencing.

Cell culture and transfection- Mouse C2C12 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS in a humidified air atmosphere containing 5% CO_2. For transfection, 6×10^4 cells were seeded in 6-well plates (Falcon, Corning, NY). 2 μg plasmid DNA was transfected into cells as previously reported (Yin et al., 2004). For differentiation experiments, thirty hours after transfection, the cells were washed with SFM and then transferred to differentiation medium (DM) consisting of DMEM plus 2% - 0.5% horse serum. Simultaneously, cells were subjected to hypoxia (1% O_2) or normal oxygen (20% O_2) in a humidified modular incubation chamber (Billups-Rothenberg Inc., Del Mar, CA). IGF-II was used at the dose of 300 ng/ml unless otherwise stated. The final concentration of aminoimidazole carboxamide ribonucleotide (AICAR), rapamycin, SB 203580, U0126, and NVP-AEW541 were 500 μM, 200 nM, 20 μM, 10 μM, and 1 μM, respectively. For experiments with inhibitor treatment, cells were pretreated with inhibitor for 1h, and subjected to IGF treatment. For 2-DG treatment, cells were pretreated with 10 mM 2-deoxy-D-glucose (2-DG) at 37°C for 1h before switching to differentiation medium. Primary mouse skeletal myoblasts were isolated from 2-5 day old mice, grown, and differentiated as previously reported (Tang and Goldman, 2006). Fugene6 (Roche) was used for transfection of these primary cells. Differentiation was induced by switching to DMEM medium containing 5% HS. All experiments were conducted in accordance with guidelines approved by the University Committee on the Use and Care of Animals, University of Michigan.

Western immunoblot analysis - Equal amounts of protein were separated by SDS-PAGE and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA) and subjected to Western blot following published procedures (Duan et al., 1996). The antibodies were used at concentrations recommended by the commercial sources.

Reverse transcription (RT)-PCR and quantitative real-time RT-PCR (qRT-PCR)- Total RNA was extracted from cells using TriPure Reagent. After treated with DNase, RNA was subjected to reverse-transcription using SuperScript II reverse transcriptase (Invitrogen) according to the supplier's instructions. S17 was used as control for RT-PCR (Wilson et al., 2003). REDD1 primers were 5'-AACTCTTCCTTGGTCCCTGGTTAC-3' (forward), 5'-TCCTGGAGAACACTTCCTTCCC-3' (reverse). The linear range of product amplification was established in pilot studies for each primer pair, and the cycle number representing the approximate midpoint was used in final experiments.

qRT-PCR was carried out using an iCycler iQ Multicolor real-time PCR detection system (Bio-Rad). qPCR was performed using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. 4 μl of the cDNA product (1:20 dilution) was used as a PCR template. Plasmid cDNA for cyclophilin (Hasel and Sutcliffe, 1990), a housekeeping gene, was provided by Dr. Gregor Sutcliffe (Scripps Research Institute). Serial dilutions of the plasmids ranging from 10^8 to 10 molecules/2 μl were used for standard curve. The number of molecules of particular gene transcript was calculated based on the standard curve and normalized to the cyclophilin RNA level. The specificity of the PCR was verified by denaturing curve analysis and direct sequencing of the products. IGF-II Primer sequences were reported by others (Boutinaud et al., 2004). The primers are PGK1: 5'-TGGTGTGAAGATTACCTTGCCTG-3' (forward), 5'-ATGAGTGACTTGGTTCCCCTGG-

3' (reverse); GLUT1: 5'-ACTGTGGTGTCGCTGTTTGTTG-3' (forward), 5'-

GAAGATGAAGAAGAGCACGAGGAG-3' (reverse), and PDK1 primers: 5'-

AGACCTCGTTTATGTTTCTGCGAC-3' (forward), 5'-

TCTCCTTGTATTCAGTCACACCCTG-3' (reverse).

Immunohistochemistry- Cells cultured in 6-well plates were washed twice with 1x

PBS before fixation in 4% paraformaldehyde. Cells were permeablized for 5 min in 1xPBS

containing 0.2% Triton X-100 (PBST), washed twice with PBS, and incubated with primary

antibodies at 4°C overnight. After washing three times with PBST, they were incubated with

Cy3 conjugated secondary antibodies for 2 h at room temperature. Immunofluorescence or

phase contrast images were obtained at room temperature using a Nikon Eclipse E600

microscope with Plan Fluor 10x 0.3 or 20x 0.5 dry objectives. Images were processed with

Photoshop for overlay. Cell number was quantified as the number DAPI-stained nuclei per

microscopic field of view. Differentiation index (%) is defined as the percentage of MHC

positive nuclei over total nuclei number.

Luciferase reporter assay- The transcription activity of HIF-1 was determined using

the dual luciferase reporter assay kit (Promega) following manufacturer's instruction. Briefly,

myoblasts were co-transfected with p2.1, a human enolase promoter harboring hypoxia

responsive element (HRE), or p2.4 with HRE mutated, and an internal control vector

encoding *Renilla* luciferase. (Semenza et al., 1996). Differentiation was induced 1 day after

transfection. Two days after inducing differentiation, cells were washed, and cell lysates

were used to measure the firefly and *Renilla* luciferase activities. Transfection efficiency was

normalized by *Renilla* luciferase activity. The result was expressed as fold change over the

control.

Lactate assays – Cells were incubated in serum-free medium for 24h under various experimental conditions. Lactate content in the medium was measured by a lactate assay kit purchased from BioVision Inc. (Mountain View, CA).

Proliferation Assays— Cell proliferation was determined by bromo-2-deoxyuridine (BrdU) incorporation assay (Pozios et al., 2001). Briefly, cells were exposed to BrdU (20 µM) for 1h after they were incubated in DM under various conditions for 24 h. BrdU positive cells were identified by immunocytochemistry. Proliferation index is defined as the percentage of BrdU-labeled nuclei over total nuclei.

Statistical analysis- Differences among groups were analyzed by Student's *t* test or One-Way Analysis of Variance followed by Fisher's protected least significance difference test, using Prizm (GraphPad Software, Inc., San Diego, CA). Significance was accepted at $p < 0.05$ or higher.

Acknowledgements

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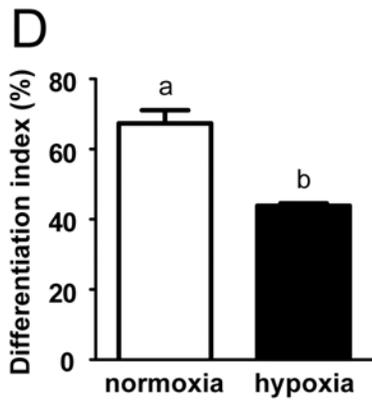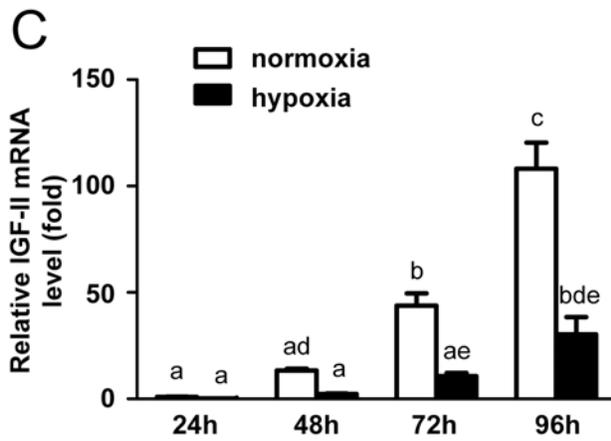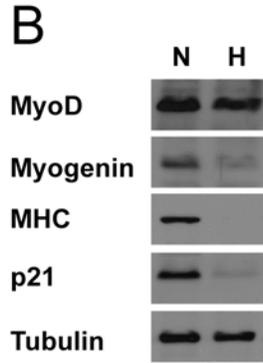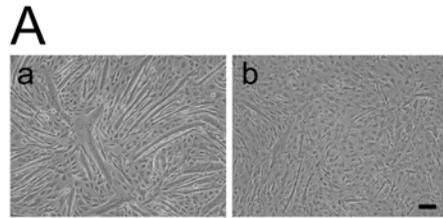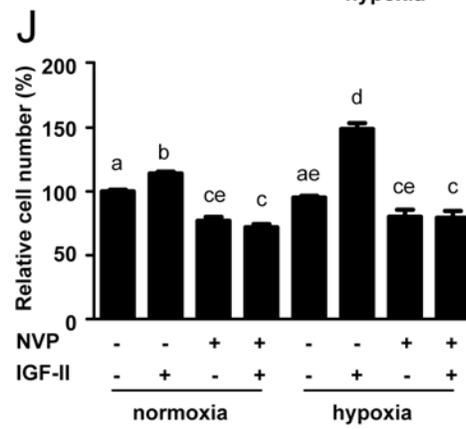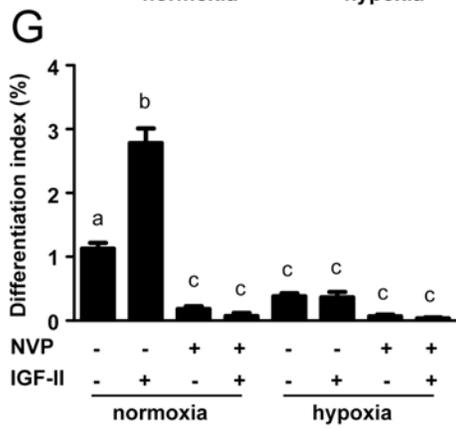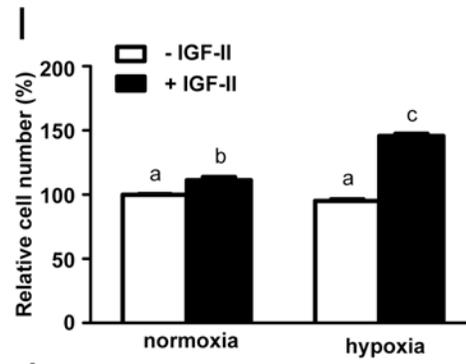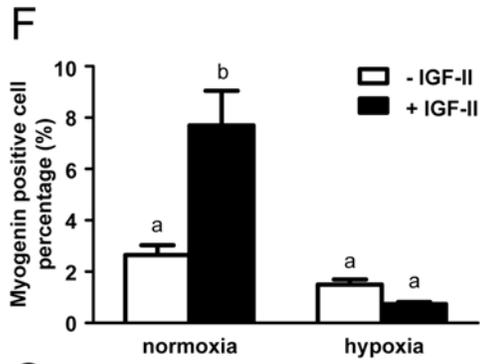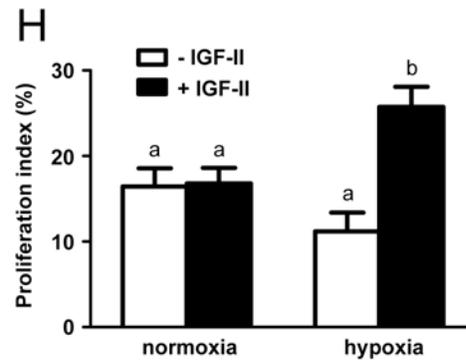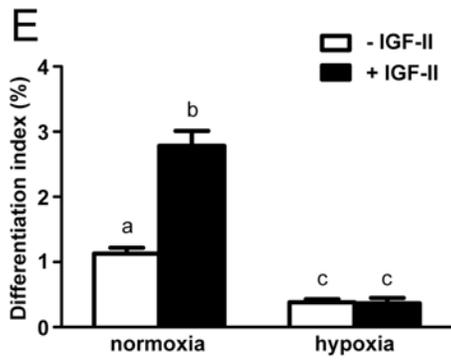
Figure 3.1. IGF stimulates differentiation under normoxic conditions but promotes proliferation under hypoxic conditions through the activation of the same receptor. (A) Hypoxia inhibits myogenic differentiation. C2C12 myoblasts were induced to differentiate by switching to the horse serum containing differentiation medium (DM) under normoxic or hypoxic conditions. Representative images 4 days after the induction of differentiation. Bar = 100μm. (B) Western blot analysis of the cells described in (A). (C) Hypoxia represses IGF-II expression during myogenesis. IGF-II mRNA levels were measured by qRT-PCR at the indicated time points after the induction of differentiation. Values are expressed as relative levels to that of the normoxia 24h control group after normalized to cyclophilin levels. Data shown are Means ±S.E. n = 3. Values marked with different letters are significantly different from each other ($p < 0.01$). (D) Primary myoblasts were isolated from newborn mice and induced to differentiate in DM. After 2 days, cells were fixed and immunocytochemistry was performed with a MHC antibody and counter stained with the nuclear dye DAPI. Differentiation index (%) is defined as the percentage of MHC positive nuclei over the total nuclei. Data shown are means $\pm$ SE of 4 independent samples. Values marked with different letters are significantly different from each other ($p < 0.001$). (E – F) IGF stimulates myogenic differentiation under the normoxic but not hypoxic condition. C2C12 myoblasts were induced to differentiate in the presence or absence of IGF-II (300 ng/ml) under normoxic or hypoxic conditions. 36 h later, cells were fixed and the differentiation index was determined (E). Data shown are Means ±S.E., n=7~9. Values marked with different letters are significantly different from each other ($p < 0.01$). (F) Cells were treated as described in (E) and stained with a Myogenin antibody and counter stained with DAPI. The percentage of Myogenin positive cells was quantified. Data shown are Means ±S.E., n=3. Values marked

with different letters are significantly different from each other ($p < 0.01$). (G) The IGF-IR inhibitor NVP-AEW541 abolishes the myogenic action of IGF. Cells were induced to differentiate in the presence or absence of IGF-II (300 ng/ml) and NVP-AEW541 (1 μM) under normoxic or hypoxic conditions. Differentiation index was measured 36 h later. Data shown are Means ±S.E. n = 4~9. Values marked with different letters are significantly different from each other ($p < 0.001$). (H – I) IGF stimulation promotes cell proliferation under the hypoxic but not the normoxic condition. (H) Cells were induced to differentiate in the presence or absence of IGF-II (300 ng/ml) under normoxic or hypoxic conditions for 24 h. After pulse labeling with BrdU, cells were fixed and subjected to immunocytochemistry using a BrdU antibody and counter stained with DAPI. Proliferation index is defined as the percentage of BrdU positive cells. Data shown are Means ±S.E., n=4. Values marked with different letters are significantly different from each other ($p < 0.05$). (I) IGF stimulation causes a greater increase in cell number under hypoxic condition only. Cells were allowed to differentiate as stated in (E). 36h later, cell number was quantified. Values are expressed as relative levels to that of the normoxia control group. Data shown are Means ±S.E., n=4. Values marked with different letters are significantly different from each other ($p < 0.01$). (J) The IGF-IR inhibitor NVP-AEW541 abolishes IGF-induced cell number increase. Cells were induced to differentiate in the presence or absence of IGF-II (300 ng/ml) and NVP-AEW541 (1 μM) under normoxic or hypoxic condition. Cell number was determined. Data shown are Means ±S.E. n = 4~9. Values marked with different letters are significantly different from each other ($p < 0.05$).

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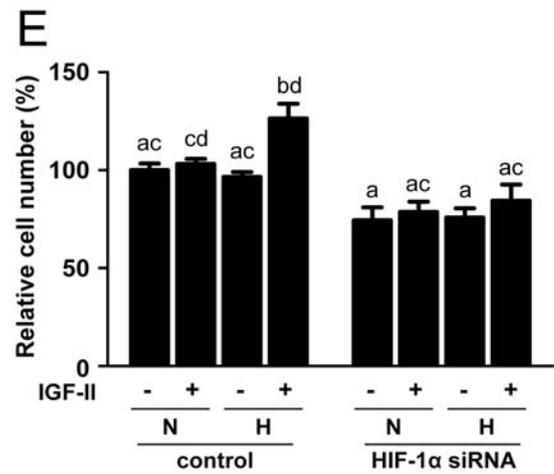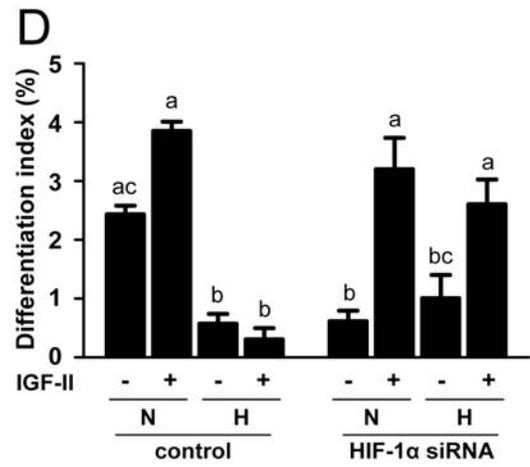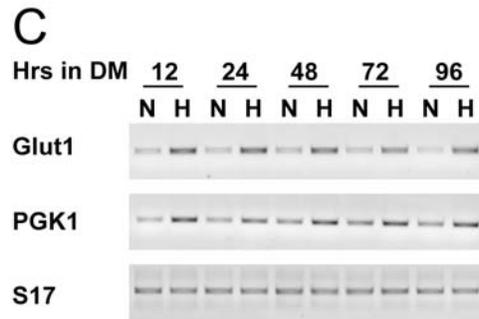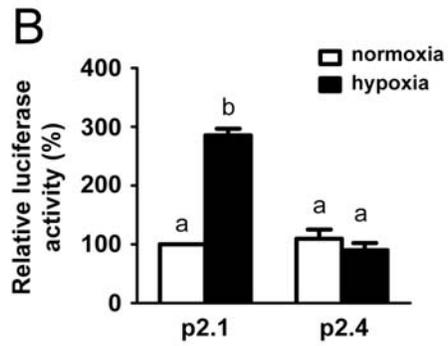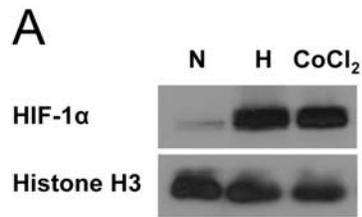
Figure 3.2. Hypoxia converts the myogenic action of IGF into mitogenic action through HIF-1-dependent mechanisms. (A) Hypoxia increases nuclear HIF-1α accumulation. C2C12 myoblasts were subjected to normoxia, hypoxia, or $CoCl_2$ (100 μM) treatment for 8 h. The nuclear fraction was prepared and analyzed by Western immunoblot using the antibodies indicated. (B) Hypoxia induces HIF-1-dependent transcription. C2C12 myoblasts were transfected with the HIF-1 reporter gene, p2.1 and control p2.4 with the HRE mutated. Cells were induced to differentiate under normoxic or hypoxic conditions for 2 days. Luciferase activities were measured. Transfection efficiency was normalized by *Renilla* luciferase activity. Data shown are Means ±S.E. n=4. Values marked with different letters are significantly different from each other ($p < 0.01$). (C) Hypoxia increases the expression of HIF-1 target genes. C2C12 cells were induced to differentiate under normoxic or hypoxic conditions. Semi-quantitative RT-PCR analysis of PGK1 and Glut1 was performed at the indicated time points. (D) Knockdown of HIF-1α restores the myogenic response to IGF-II under hypoxic condition. C2C12 cells transfected with control or HIF-1α targeting siRNA plasmid were induced to differentiate in the presence or absence of IGF-II (300 ng/ml) under normoxic or hypoxic conditions. The differentiation index was determined after 36 h. Data shown are Means ± S.E., n=4. Values marked with different letters are significantly different from each other ($p < 0.05$). (E) Knockdown of HIF-1α abolishes the mitogenic action of IGF-II under the hypoxic condition. Cell number was determined at the end of the experiments described in (D). Data shown are Means ± S.E., n=4. Values marked with different letters are significantly different from each other ($p < 0.05$).
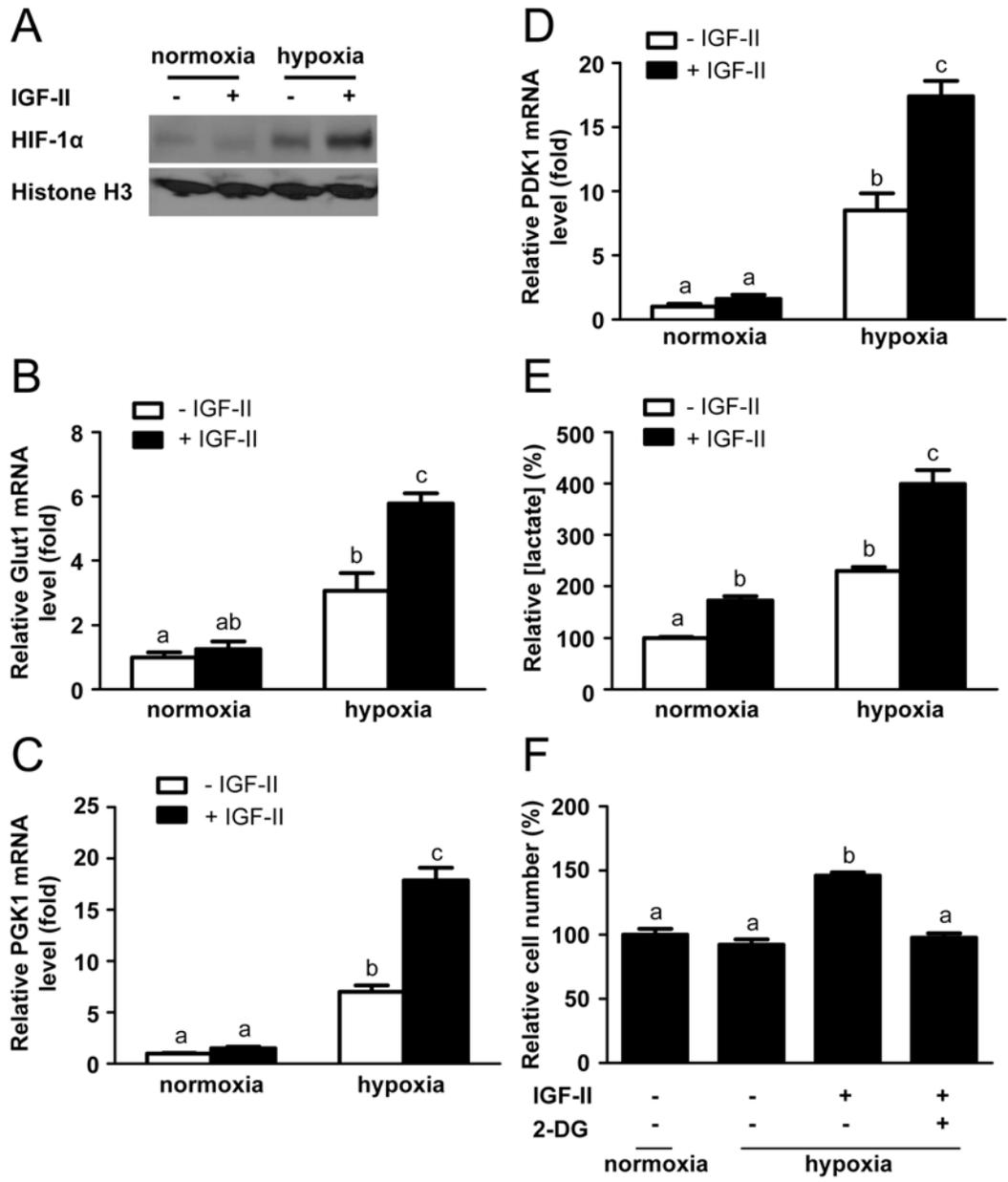
Figure 3.3. Hypoxia reprograms glucose metabolism and this action is enhanced by IGF stimulation. (A) IGF stimulation increases nuclear HIF-1α levels under hypoxia. C2C12 cells were serum starved overnight and subjected to the indicated treatment for 8 h. The nuclear fraction was prepared and analyzed by Western blot. (B-D) IGF stimulation enhances the hypoxia-induced increases in target gene expression. C2C12 cells were induced to differentiate in the presence or absence of IGF-II (300 ng/ml) under normoxic or hypoxic conditions for 36 h. RNA was isolated and Glut1 (B), PGK1 (C), and PDK1 (D) mRNA levels were determined by qRT-PCR. Values are expressed as relative levels to those of the normoxia control group. Data shown are means ±S.E., n=2. Values marked with different letters are significantly different from each other ($p < 0.05$). (E) IGF enhances the hypoxia-induced increase in lactate production. C2C12 cells were subjected to the indicated treatments in serum free medium. After 24 h, the conditioned medium was collected and the lactate concentration was measured. Values are expressed as relative levels to that of the normoxia control group. Data shown are Means ±S.E., n=4. Values marked with different letters are significantly different from each other ($p < 0.05$). (F) Inhibition of glucose uptake by 2-deoxy-D-glucose (2-DG) abolishes the mitogenic action of IGF. C2C12 cells were treated with or without 2-DG (10 mM) for 1h before being subjected to treatments with or without IGF-II. After 36 h, cell number was quantified and expressed as relative levels to that of the normoxia control group. Data shown are Means ±S.E., n=5. Values marked with different letters are significantly different from each other ($p < 0.001$).
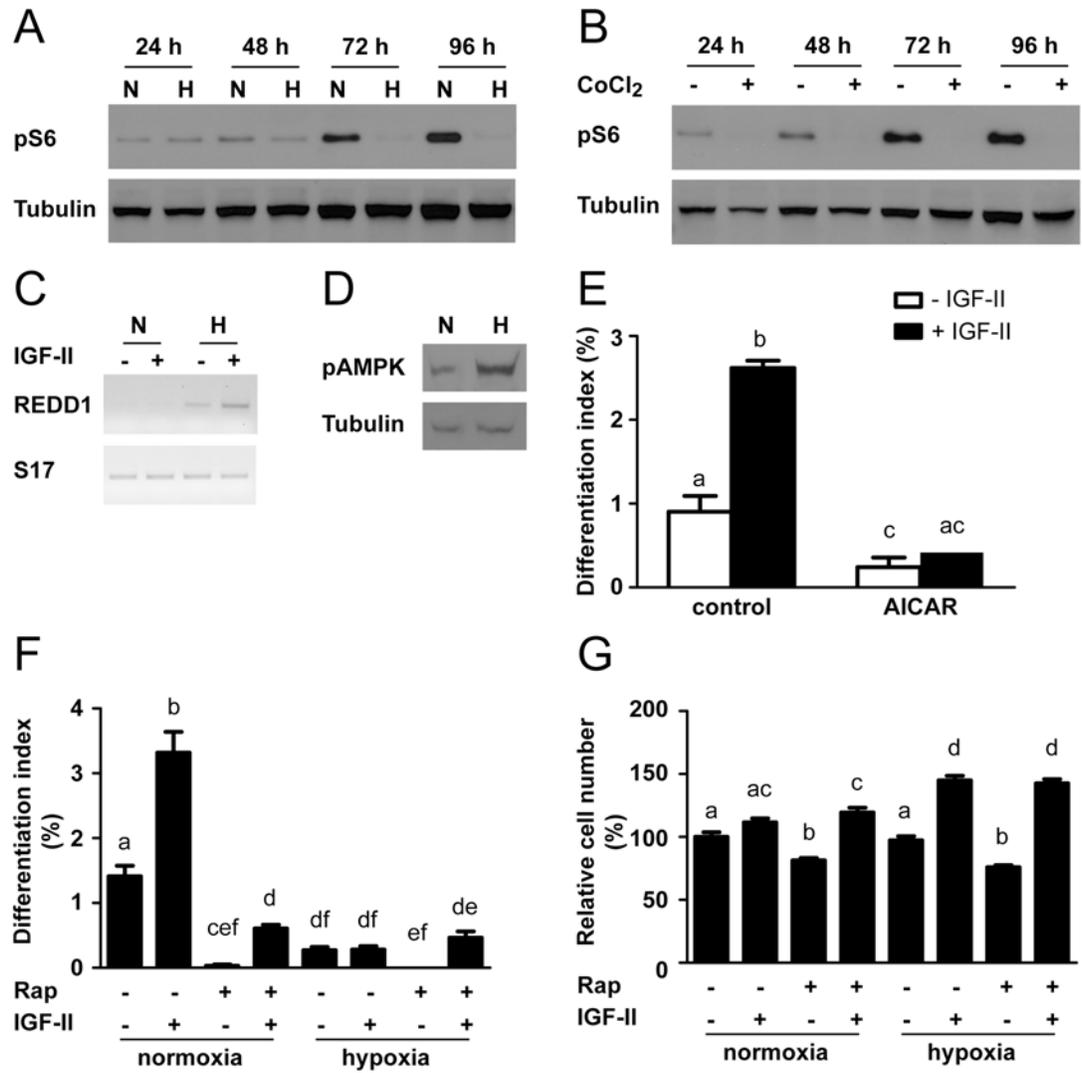
Figure 3.4. Hypoxia represses mTOR signaling during myogenesis and the inhibition of mTOR activity abolishes the myogenic action but not the mitogenic action of IGF. (A) Hypoxia represses mTOR signaling activity. C2C12 myoblasts were induced to differentiate under normoxic or hypoxic conditions. Phosphorylated S6 levels were determined by Western immunoblot at the time points indicated. (B) $CoCl_2$ treatment inhibits mTOR signaling activity. C2C12 myoblasts were induced to differentiate in the presence or absence of $CoCl_2$ (100 μM) under normoxic conditions. Phosphorylated S6 levels were determined by Western immunoblot at the time points indicated. (C) Hypoxia induces REDD1 expression in C2C12 myoblasts. 36 h after the cells were induced to differentiate under conditions indicated, RNA samples were collected and subjected for RT-PCR analysis. (D) Hypoxia increases the levels of phosphorylated AMPK. C2C12 cells were induced to differentiate under normoxic or hypoxic conditions for 48 h. pAMPK levels were determined by Western immunoblot analysis. (E) AICAR, an AMPK activator, abolishes the myogenic action of IGF. C2C12 cells were induced to differentiate in the presence or IGF-II (300 ng/ml) and/or AICAR (500 μM) under normoxic conditions. The differentiation index was measured 36 h later. Data shown are Means ±S.E., n=4. Values marked with different letters are significantly different from each other ($p < 0.001$). (F-G) Inhibition of mTOR activity decreases the myogenic action but not the mitogenic action of IGF. C2C12 myoblasts were induced to differentiate in the absence or presence of IGF-II (300 ng/ml) and/or rapamycin (200 nM) under normoxic or hypoxic conditions. After 36 h, cells were fixed and differentiation index (F) and cell number (G) were determined. Data shown are Means ±S.E., n = 4~6. Values marked with different letters are significantly different from each other ($p < 0.05$).
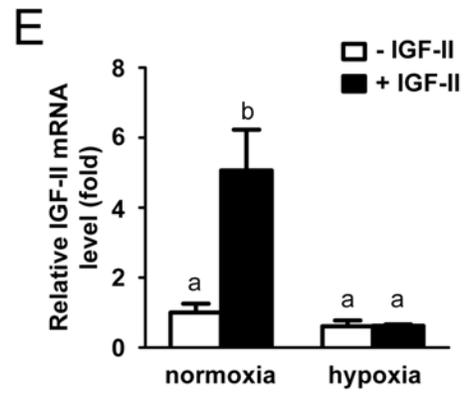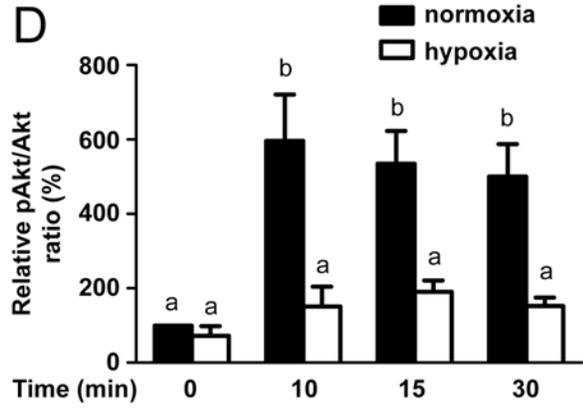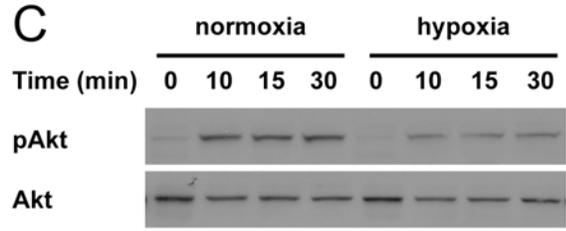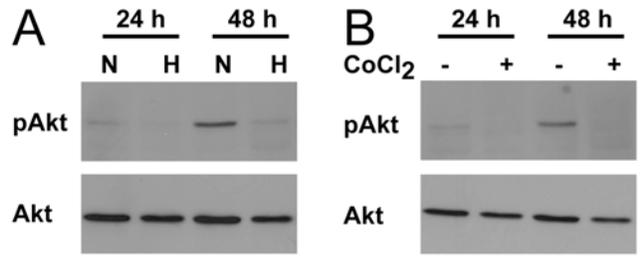
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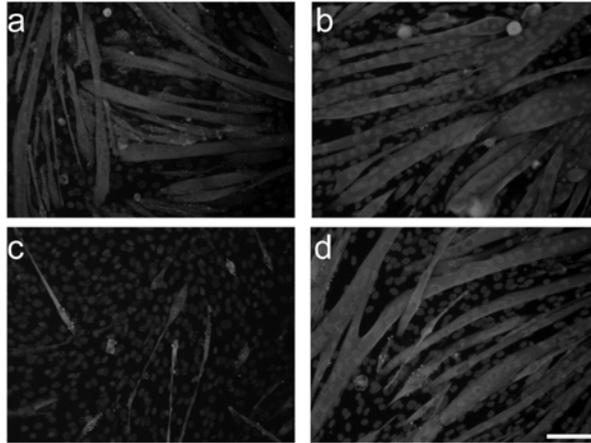
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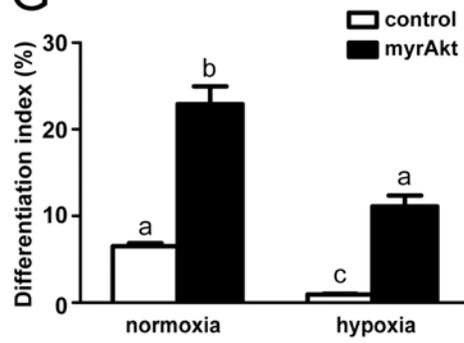
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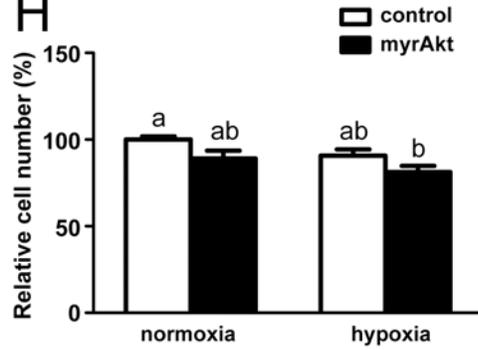
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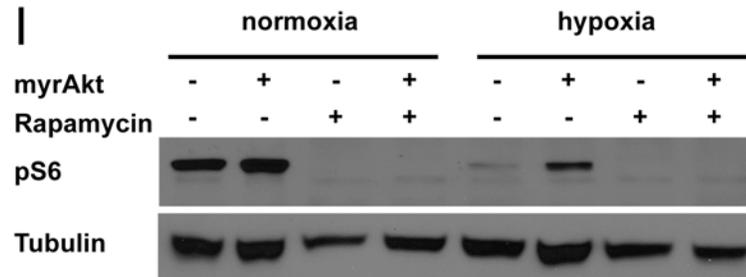
Figure 3.5. Hypoxia represses Akt signaling activity, and expression of constitutively active Akt restores the mTOR activity and myogenic action of IGF under hypoxic conditions. (A) Hypoxia represses Akt signaling activity. C2C12 myoblasts were induced to differentiate under normoxic or hypoxic conditions. Total and phosphorylated Akt levels were determined by Western immunoblot at the time points indicated. (B) $CoCl_2$ inhibits Akt signaling activity. C2C12 myoblasts were induced to differentiate in the presence or absence of $CoCl_2$ (100 μM) under normoxia. Total and phosphorylated Akt levels were determined by Western immunoblot at the time points indicated. (C) Hypoxia decreases IGF-induced Akt activation. After switching to DM for 72 h under normoxic or hypoxic conditions, 50 ng/ml IGF-I was added. Cell lysates were harvested at the times points indicated, and subjected to Western immunoblot analysis. (D) The phospho-Akt/total Akt ratio in (C) was measured by densitometry. Values are expressed as relative to that of the 0 h normoxia group. Data shown are Means $\pm$S.E., n = 6. Values marked with different letters are significantly different from each other ($p < 0.01$). (E) Hypoxia disrupts the IGF-II autocrine regulatory loop during myogenesis. C2C12 myoblasts were switched to DM with or without 300 ng/ml IGF-II under normoxic or hypoxic conditions. After 36 h, RNA was isolated and IGF-II mRNA levels were measured by qRT-PCR. Values are expressed as relative levels to that of the normoxia control group after normalized to cyclophilin levels. Data shown are Means $\pm$S.E. n = 2. Values marked with different letters are significantly different from each other ($p < 0.05$). (F) C2C12 cells were transfected with the control plasmid (a & c) or the constitutively active Akt (myrAkt) plasmid (b & d) and induced to differentiate under normoxic (a & b) or hypoxic (c & d) conditions. Cells were fixed after 4 days. Immunocytochemistry was performed using a

MHC antibody and counter stained with DAPI. Bar = 100µm. (G and H) 24h after

differentiation was induced, differentiation index (G) and cell number (H) were measured.

Data shown are Means ±S.E., n=4, Values marked with different letters are significantly

different from each other (p< 0.05). (I) Effect of myrAkt and rapamycin on mTOR signaling

activity. Cells were transfected with the control or the myrAkt plasmid and induced to

differentiate in the presence or absence of rapamycin (200 nM) under normoxic or hypoxic

conditions. After 4 days, cells were lysed and analyzed by Western immunoblot using the

indicated antibodies.
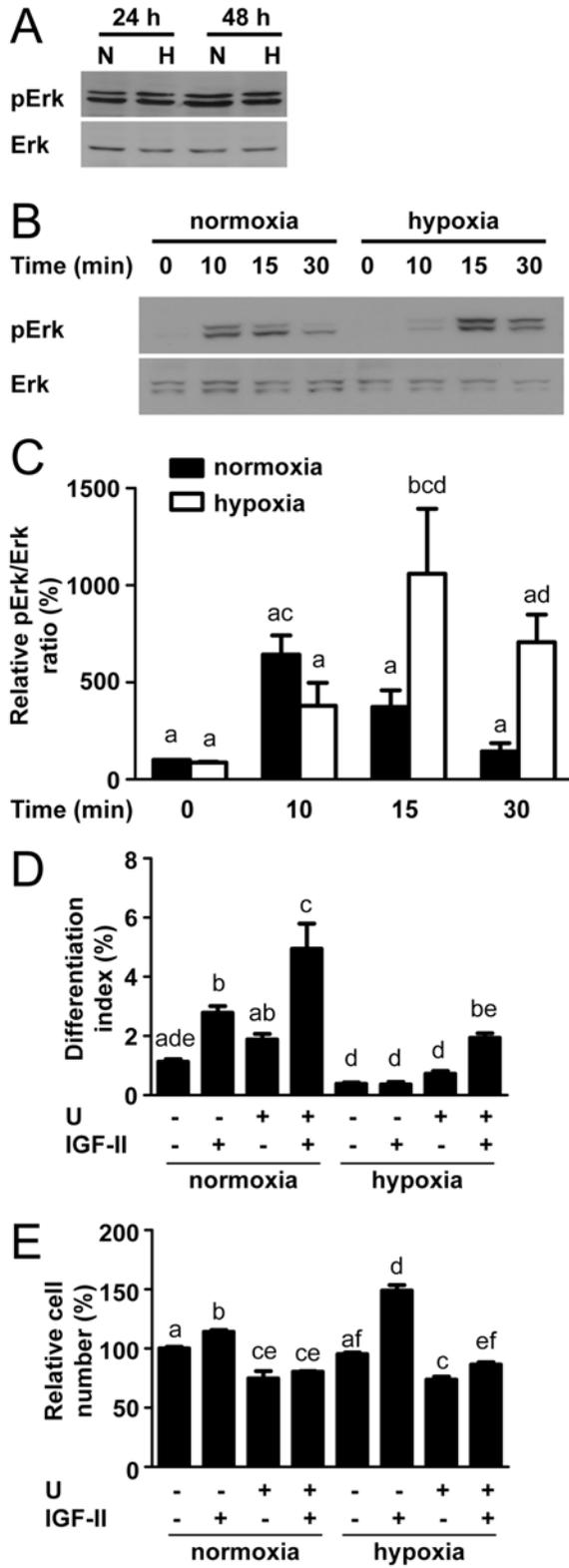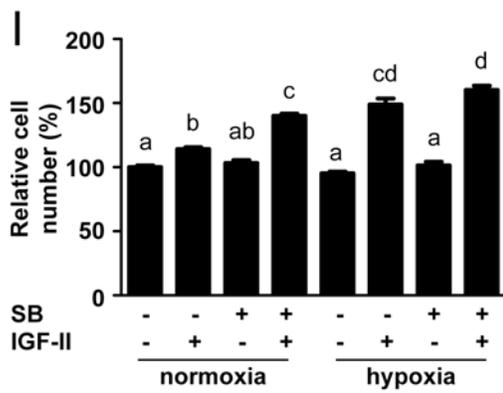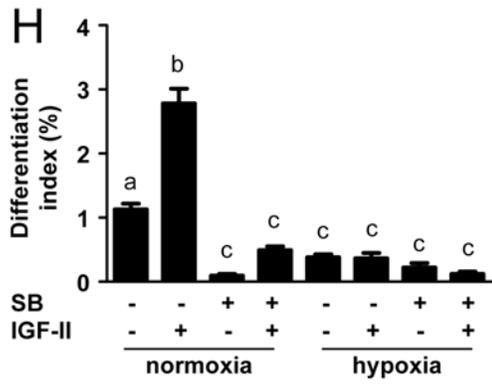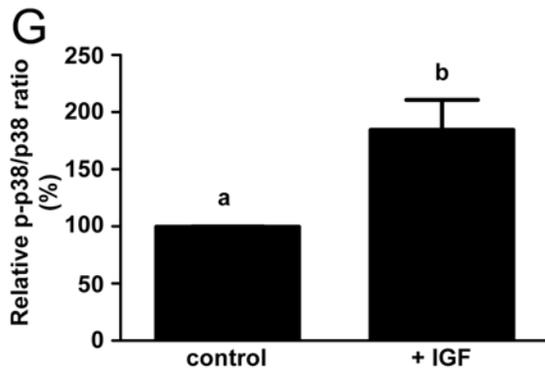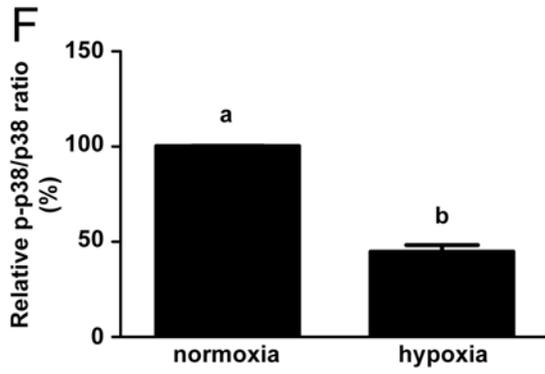
Figure 3.6. Hypoxia alters IGF actions by differentially regulating Erk1/2 and p38 MAPK pathways. (A – E) Hypoxia increases and prolongs IGF-induced Erk1/2 activity and inhibition of Erk1/2 enhances the myogenic action of IGF but abolishes its mitogenic action. (A) Hypoxia does not repress Erk activity. C2C12 myoblasts were induced to differentiate under normoxic or hypoxic conditions. Total and phosphorylated Erk1/2 levels were determined by Western immunoblot at the time points indicated. (B) Hypoxia alters IGF-induced Erk1/2 activation. After switching to DM for 72 h under normoxic or hypoxic conditions, 50 ng/ml IGF-I was added. Cell lysates were harvested at the times points indicated, and subjected to Western immnoblot analysis. (C) The phospho-Erk/total Erk1/2 ratio in (B) was measured by densitometry. Values are expressed as relative to that of the 0 h normoxia group. Data shown are Means ±S.E., n = 4. Values marked with different letters are significantly different from each other (p < 0.05). (D and E) Inhibition of the Erk1/2 activity by U0126 (10 μM) enhances the myogenic action but abolishes the mitogenic action of IGF. Data shown are means ±S.E., n = 4~6. Values marked with different letters are significantly different from each other (p <0.05). (F – I) Hypoxia represses p38 MAPK activity and inhibition of p38 MAPK abolishes the myogenic action but increases the mitogenic action of IGF. (F) Hypoxia represses p38 MAPK activity. C2C12 myoblasts were induced to differentiate under normoxic or hypoxic conditions for 4 days. Phosphorylated and total p38 MAPK levels were determined by Western immunoblot. The ratio of phospho/total p38 MAPK was measured by densitometry. Values are expressed as relative to that of the normoxia group. Data shown are Means ±S.E., n = 6. Values marked with different letters are significantly different from each other (p < 0.0001). (G) IGF activates p38 MAPK in myoblasts. C2C12 myoblats growing in growth medium were serum starved overnight and

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exposed to IGF-I (50 ng/ml). After 10 min, cells were lysed and subjected to Western

immunoblot analysis. The ratio of phospho- and total p38 MAPK was measured by

densitometry. Values are expressed as relative to that of the control group. Data shown are

Means ±S.E., n = 5. Values marked with different letters are significantly different from each

other (p < 0.01). (H and I) Inhibition of p38 MAPK activity by SB203580 (20 µM) abolishes

the myogenic action but enhances the mitogenic action of IGF. Data shown are Means ±S.E.,

n = 4~6. Values marked with different letters are significantly different from each other (p

<0.05).

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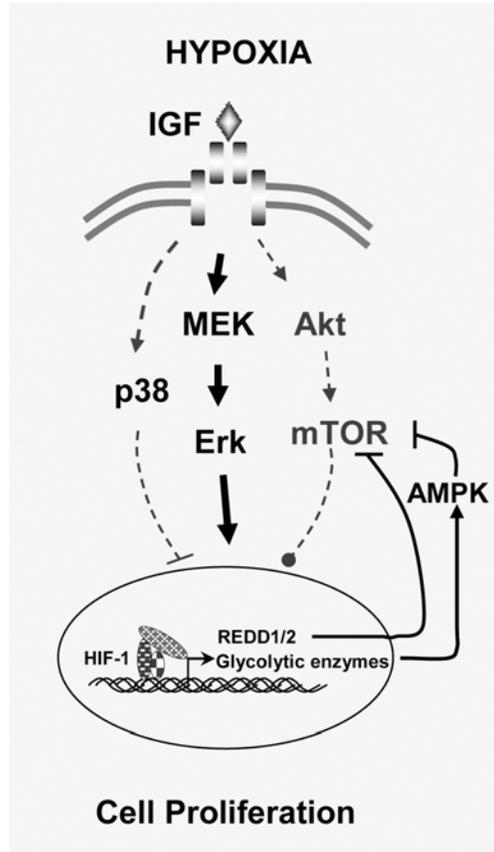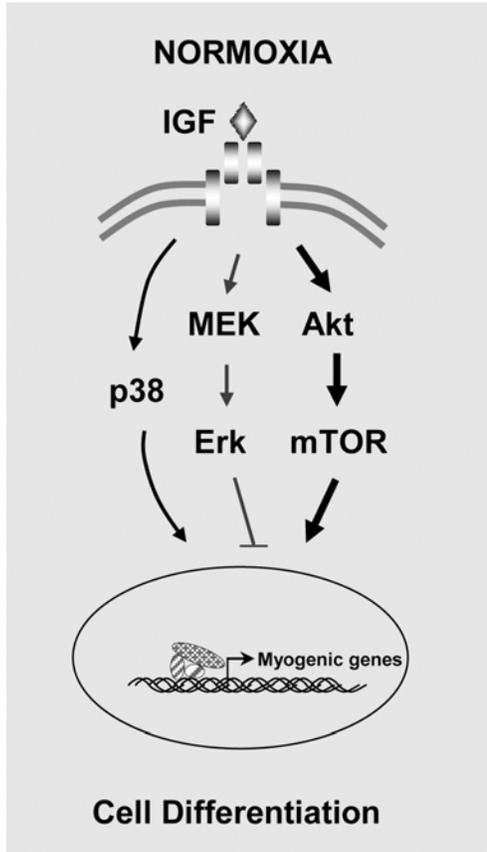
Figure 3.7. Schematic diagram illustrates the mechanisms by which hypoxia alter the myogenic and mitogenic actions of IGF during myogenesis. In differentiating muscle cells, there are at least three signaling pathways downstream of the IGF-IR: the Akt-mTOR, p38 MAPK, and Erk1/2 MAPK. Activation of the Akt-mTOR pathway strongly promotes myogenic differentiation (indicated by an arrow) but has little effect on proliferation (indicated by a circle). Activation of the p38 MAPK promotes differentiation but inhibits proliferation (indicated by a line). In contrast, activation of the Erk1/2 MAPK stimulates proliferation but inhibits differentiation. In normoxic environments, binding of the IGF-IR by IGF-II or -I strongly activates the Akt-mTOR signaling pathway and the p38 MAPK pathway (bold line). Both Akt-mTOR and p38 MAPK positively contribute to myogenesis by up-regulating myogenic genes. The Erk1/2 MAPK signaling pathway is also activated by IGF stimulation in normoxic environments, which results in a modest increase in cell number. Hypoxia alters the cellular response to IGF stimulation by reprogramming glucose metabolism and by suppressing the Akt-mTOR and the p38 MAPK signaling activities (broken lines). Hypoxia, through the activation of HIF-1 complex, up-regulates REDD gene expression, which inhibits mTOR activity. Hypoxia also activates AMPK, which in turn inhibits mTOR activity. Hypoxia/HIF-1 increases glycolysis by up-regulating several glycolytic enzymes. Hypoxia may also inhibit Akt activity via HIF-1-dependent and independent mechanisms. Under the hypoxic conditions, the binding of the IGF-1R by the IGF ligand preferentially activates the Erk1/2 MAPK signaling pathway. Activation of the Erk1/2 MAPK signaling pathway stimulates cell proliferation and suppresses differentiation in hypoxic microenvironments.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

IGFBP-5 potentiates IGF actions during myogenesis

In order to investigate the physiological role(s) of IGBP-5 during myoblast differentiation, I took a loss-of-function approach by using IGFBP-5 siRNA. The specificity and efficiency of IGFBP-5 siRNA have been validated. IGFBP-5 knockdown inhibits myoblast differentiation. IGFBP-5 siRNA disrupts myotubes formation and reduces MHC expression. It also suppresses the expression of myogenic transcription factor – Myogenin. Interestingly, knockdown IGFBP-5 also causes significantly reduced IGF-II transcription during differentiation. As a result, IGF-II signaling activity is significantly inhibited as measured by phospho-Akt levels. It is then hypothesized that IGFBP-5 positively regulates myogenesis by potentiating IGF-II function.

Several lines of evidence are provided in order to support the hypothesis. First, either adding back exogenous IGF ligands or introducing constitutively active Akt rescues the differentiation defects caused by IGFBP-5 siRNA. Second, IGF-II transcription exhibits positive autocrine regulation during differentiation. Both exogenous IGF-II treatment and constitutively active Akt can increase IGF-II expression. Third, IGFBP-5 is localized at the cell surface upon induction of differentiation. Forth, IGF-II analog, des(1-6)IGF-II, with much reduced affinity for IGFBP, is significantly less efficient in stimulating myogenesis. Last, IGFBP-5 mutant with deficient IGF binding capacity is unable to potentiate IGF-II actions compared with wild type IGFBP-5. Based on these, I propose the following model to

explain how IGFBP-5 potentiates IGF actions during myogenesis. Upon induction of differentiation, IGFBP-5 expression is quickly increased and precedes IGF-II expression. Secreted IGFBP-5 binds to IGF-II with high affinity and targets it to the close proximity of IGF-IR on the cell surface, which in turn initiates and magnifies IGF-II signaling cascades.

Determining the molecular mechanism of how IGFBP-5 potentiates IGF action

Our model is sufficient to explain the data generated in Chapter II. However, more experiments can be done to further test the model. The ability of IGFBP-5 to associate with cell surface is a critical point in the model. IGFBP-5 is capable of binding to cell surface via association with ECM components. A heparin binding motif in the C-domain of IGFBP-5 mediates its binding to cell surface (Schneider et al., 2002). Binding of IGFBP-5 to ECM or glycosaminoglycans (GAG) at the cell surface reduces its binding affinity for IGFs, which as a result releases IGF ligand to IGF-IR (Arai et al., 1996; Arai et al., 1994; Parker et al., 1998). Parker et al. has demonstrated that critical residues (K211, R214, R217, and R218) in the C-domain of IGFBP-5 confer its ability to associate with cell surface (Parker et al., 1998). Epitope-tagged IGFBP-5 mutant (K211N/R214A/R217A/R218A) can be used to further test the hypothesis. First, whether these critical amino acid residues contribute to cell surface association will be tested in C2C12 myoblasts. C2C12 myoblasts will be transfected with either wild type or the mutant IGFBP-5. The cell surface associated proteins will be collected by washing with high salt solution and subjected to western blot analysis with antibody against epitope tag. If the tagged mutant IGFBP-5 is only detected in the medium fraction, it will indicate that this IGFBP-5 mutant loses its cell surface association capability in myoblasts. Next, whether the membrane association is indeed attributed to the potentiating

effect of IGFBP-5 will be tested. C2C12 myoblasts will be transfected with IGFBP-5 siRNA and induced to differentiate. When combined with equal molar ratio of low dose of IGF-II, wild type IGFBP-5 should show significant rescue effect. If the hypothesis is correct, IGFBP-5 mutant (K211N/R214A/R217A/R218A), losing its cell surface association capability, should not potentiate IGF-II myogenic actions when added at equal molar ratio to low dose of IGF-II.

An alternative hypothesis is cell surface associated IGFBP-5 may be subjected to proteolytic cleavage, which leads to release of IGF ligand. Potential proteolytic sites have been identified in the L-domain of IGFBP-5 (Schneider et al., 2002). In order to test this alternative hypothesis, IGFBP-5 with epitope tag at its C terminus will be constructed. The tagged IGFBP-5 can be purified from conditioned medium of cultured mammalian cells. Then, purified IGFBP-5 can be incubated with C2C12 myoblasts in differentiation medium. Both the medium fraction and the cell surface associated fraction will be collected and examined by western blot with antibodies against the epitope tag. The presence of smaller fragments with molecular mass approximately either one third or two thirds of the full-length IGFBP-5 will suggest that IGFBP-5 is indeed proteolysed during myoblast differentiation. If the results are consistent with the prediction, the hypothesis can be further tested by using IGFBP-5 constructs with these proteolytic sites mutated and with epitope tag at the C terminus. IGFBP-5 mutant proteins can be purified from the conditioned medium of the cells stably expressing these constructs at high levels. The purified epitope-tagged IGFBP-5 mutant proteins will be incubated with differentiating myoblasts, in order to validate whether these mutants are indeed resistant to proteolysis. If the alternative hypothesis is correct,

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IGFBP-5 mutant resistant to proteolysis should not potentiate IGF-II actions, and rather exert inhibitory effects.

Investigating how IGF-I and IGF-II gene expression is regulated during myoblast differentiation

Another novel finding came out from this study is that IGF-II stimulates its own gene expression in differentiating myoblasts. I demonstrated *IGF-II* gene expression is under the IGF-II autocrine regulation via IGF-IR-Akt signaling cascade. Interestingly, this autocrine regulation is unique to *IGF-II* gene and cannot be extended to *IGF-I* gene. My preliminary data showed that exogenously added IGF-II decreased *IGF-I* gene expression. As shown in Fig. 4.1, exogenous IGF-II increases endogenous IGF-II transcription but suppresses endogenous IGF-I transcription. This indicates that *IGF-I* and *IGF-II* gene expression is subjected to distinct regulatory mechanisms during myogenesis. Interestingly, a recent study in adipocytes showed that IGF-I expression is under the negative regulation of IGF-I (Kloting et al., 2008). Conditional deletion of IGF-IR in adipose tissue leads to significantly increased IGF-I mRNA. Conversely, IGF-I stimulation of wild type adipocytes significantly decreases IGF-1 mRNA expression (Kloting et al., 2008). Future studies may reveal the different molecular mechanism of *IGF-I and IGF-II* gene transcription and how each is regulated by IGF-II autocrine function. I propose the following experiments. First, the results from the semi-quantitative RT-PCR need to be further validated. C2C12 myoblasts will be induced to differentiate in presence or absence of exogenous IGF-II. After 36 hours, cells will be harvested. Real-time quantitative RT-PCR will be used as a measurement for IGF-I and IGF-II mRNA in these samples. Second, IGF-I, instead of IGF-II, will be used to

stimulate differentiating C2C12 myoblasts. Although IGF-I and IGF-II mainly signal through the same receptor – IGF-IR, it is reported recently that IGF-IIR may possess signaling activity in addition to its role in IGF-II turnover (El-Shewy et al., 2007). C2C12 myoblasts will be induced to differentiate in presence or absence of exogenous IGF-I. After 36 hours, cells will be harvested. Real-time quantitative RT-PCR will be used as a measurement for IGF-I and IGF-II mRNA in these samples. If the stimulation with exogenous IGF-I yields similar results, i.e. increased IGF-II transcription and decreased IGF-I transcription, then which downstream signaling pathways mediate these effects will be determined. For example, PI3K/Akt pathway can be inhibited by PI3K inhibitor LY294002. C2C12 myoblast will be induced to differentiate with or without exogenous IGF in the presence or absence of LY294002. IGF-I and IGF-II transcripts will be measured by real-time quantitative RT-PCR. Based on the working model from Chapter II, the presence of LY294002 will abolish the increase in IGF-II transcription in response to exogenous IGF stimulation. Whether the same pathway mediates the suppression of IGF-I transcription in response to exogenous IGF stimulation will be determined by measuring IGF-I transcripts. The contribution of other signaling pathways, including Erk MAPK, can be assessed using a similar approach. If certain pathways mediate the different changes in IGF-I and IGF-II transcription after exogenous IGF stimulation, the downstream nuclear transcription factors binding to IGF-I and IGF-II promoter can be identified. IGF-I and IGF-II signal through the same IGF-IR, therefore I predict exogenous IGF-I or IGF-II stimulation will lead to the same results. However, there is a small chance that the results may be different. In this case, the future study will be focused on IGF receptors – IGF-IR, IGF-IIR, and hybrid receptors formed by IGF-IR and insulin receptor (IR). Specific siRNA can be designed to target IGF-IR, IGF-IIR,

IRa, and IRb. Whether IGF-I and IGF-II induce different effect through different receptor complexes can be tested by transfecting cells with the specific siRNA targeting individual receptor.

Assessing the temporal function of IGFBP-5 during myoblast differentiation

Although I consider loss-of-function approach may yield more interpretable data on IGFBP-5 function in physiologically relevant conditions, more detailed analysis of IGFBP-5 function during myogenesis needs to be carried out. For example, siRNA vector used in the studies in Chapter II constitutively expresses IGFBP-5 siRNA. Therefore, IGFBP-5 expression is probably already down-regulated prior to differentiation induction. IGFBP-5 has been reported to act as a survival factor during osteoblasts and neuronal cell differentiation (Tanno et al., 2005; Yin et al., 2004). C2C12 myoblasts were transfected with or without IGFBP-5 siRNA and induced to differentiate. My data showed that IGFBP-5 siRNA caused a 40% reduction in total cell number during myoblast differentiation (Fig. 4.2A). This suggests either decreased cell proliferation or increased cell apoptosis. When apoptosis was quantified by measuring TUNEL positive cells, IGFBP-5 siRNA caused a 4-fold increase in apoptosis at early phase of myoblast differentiation (Fig. 4.2B). Therefore, whether IGFBP-5 promotes cell survival during early myoblast differentiation needs to be addressed.

In addition, our analysis of the IGFBP-5 expression profile showed that IGFBP-5 expression peaked early during differentiation and then decreased at later stages. Whether IGFBP-5 plays different role(s) at different stages of myogenesis needs to be determined. To this purpose, siRNA construct with inducible promoter can be engineered. The precise

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temporal control of IGFBP-5 knockdown can be achieved, thus IGFBP-5 can be acutely knocked down at different stages of differentiation.

Hypoxia converts IGF actions by reprogramming its signaling cascades and cellular metabolism

As discussed in Chapter II, IGF-II is the major IGF produced by differentiating C2C12 cells. IGF promotes myoblast differentiation under normoxia but stimulates proliferation under hypoxia.

Hypoxia exerts pleiotropic effects on cells, including cell metabolism. In the scarcity of oxygen, cells switch to glycolysis for ATP generation as opposed to oxidative phosphorylation. This creates multiple challenges, including less-efficient ATP generation per molecule of glucose and availability of metabolite for biosynthetic pathway. Yet, strikingly, myoblasts incubated in differentiation medium under hypoxic respond to exogenous IGF-II with massively increased proliferation. Therefore, in contrast to promoting differentiation under normoxia condition, IGF-II acts as a strong mitogen under hypoxia condition. IGF-II stimulated cell proliferation in hypoxia condition is probably mediated by enhanced HIF-1α activity and increased glycolytic metabolism. Knockdown HIF-1α under hypoxia restored IGF's pro-differentiation activity and reversed its pro-proliferation function.

Under normoxia, autocrine IGF-II promotes cell differentiation via positive regulatory mechanism through the IGF-IR – Akt – mTOR signaling pathway (Erbay et al., 2003; Ren et al., 2008a). Hypoxia inhibits myoblast differentiation by suppressing the Akt – mTOR signaling pathway. Thus, the IGF-II autocrine regulation is disrupted by hypoxia.

Endogenous Akt activity in differentiating myoblasts is suppressed by hypoxia. Moreover, Akt activation in differentiating myoblasts in response to acutely administered IGF is significantly diminished after prolonged incubation in hypoxia. Hypoxia also induces negative regulators for mTOR signaling, including REDD1 expression and phosphorylated AMPK. Akt, REDD1 and pAMPK activities converges on mTOR signaling through TSC1/2 complex. As a result, mTOR signaling activity is reduced during myogenesis in hypoxia shown by suppressed phospho-S6 levels. The inhibited Akt – mTOR signaling pathway, however, does not contribute to IGF-stimulated cell proliferation in hypoxia. First, pharmacological inhibition of IGF – Akt – mTOR signaling pathway by rapamycin not only reduces the basal differentiation but also abolishes the IGF-induced differentiation. Second, overexpressing constitutively active Akt rescues the differentiation effects caused by hypoxia, but did not significantly change the cell number.

Another major IGF downstream signaling pathway is Erk MAPK pathway. As opposed to compromised Akt signaling activity in hypoxia, basal Erk activity is not changed. Furthermore, Erk activation in differentiating myoblasts in response to acutely administered IGF is significantly enhanced after prolonged incubation in hypoxia. Pharmacological blockade of Erk1/2 signaling pathway by MEK1/2 specific inhibitor U0126 not only rescued the differentiation defects in hypoxia but also decreased cell proliferation in response to IGF stimulation. Therefore, Erk MAPK pathway and Akt-mTOR pathway exert opposite effects on cell proliferation versus differentiation, and the two pathways are differentially regulated by hypoxia treatment.

How hypoxia differentially regulates two major IGF downstream pathways remains unclear. In order to elucidate the molecular mechanism, it is important to characterize the

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effect on each individual component of the IGF signaling pathway. I will discuss the possible scenarios as following.

Analyzing whether receptor phosphorylation is altered under hypoxia

IGF-IR is a heterotetrameric receptor tyrosine kinase (RTK). Ligand binding activates IGF-IR and causes auto-phosphorylation of the kinase domains (Ullrich and Schlessinger, 1990). The IGF and insulin signaling is negatively regulated by protein tyrosine phosphatases (PTPs). Among the PTPs, PTP1B has been extensively studied for its direct regulation on insulin signaling. PTP1B reduces insulin signaling activity (Cicirelli et al., 1990). PTP1B directly binds to activated insulin receptor and dephosphorylates critical tyrosine residues (Seely et al., 1996). Seely et al. demonstrated the direct interaction between PTP1B and the activated insulin receptor using glutathione S-transferase (GST) pull down and co-immunoprecipitation methods (Seely et al., 1996). Phosphopeptides mimicking the receptor's kinase domain disrupts the interaction between PTP1B and activated IR. Nevertheless, mutant IR with kinase domain deleted still bind to GST-tagged PTP1B. Elchebly et al. showed PTP1B negatively regulates insulin signaling by generating PTP1B null mouse (Elchebly et al., 1999). These mutant mice showed increased IR phosphorylation in the liver and improved insulin sensitivity (Elchebly et al., 1999). Given the sequence homology between IGF-IR and IR, PTP1B is of particular interest to study how hypoxia affects IGF signaling. First, whether PTP1B expression is regulated by hypoxia will be determined. C2C12 myoblasts will be induced to differentiate under normoxia or hypoxia. Cell lysates will be harvested and subjected to western blot analysis using PTP1B specific antibody. Second, whether hypoxia directly regulates PTP1B activity will be determined. Affinity

purified PTP1B will be incubated with phosphopeptide mimicking the activated tyrosine

kinase motif of IGF-IR under normoxia or hypoxia. Whether the enzymatic activities of

PTP1B are regulated can be determined by directly measuring the changes of

phosphorylation status of the polypeptide. Although being a prominent candidate, PTP1B is

not the only PTP (Xu and Qu, 2008). If PTP1B is not regulated by hypoxia, other PTPs will

be screened using similar approaches. If a particular PTP is regulated by hypoxia, whether it

interacts with activated IGF-IR will be tested by standard approaches detecting protein-

protein interaction, such as GST pull down and co-immunoprecipitation. Phosphorylated

tyrosine residues on the activated IGF-IR present important docking sites for downstream

signaling effectors. Therefore determining whether the PTPs are regulated by hypoxia is

critical to reveal if the IGF signaling is differentially regulated at the receptor level.

Investigating whether IRS proteins are regulated by hypoxia

Activated IGF-IR signals through the associated IRS proteins. There are six members

in the family of IRS proteins (Cai et al., 2003; Fantin et al., 1998; Lavan et al., 1997b; Sun et

al., 1991; Sun et al., 1995). IRS-1 and IRS-2 are widely expressed, while the other IRS genes

have limited tissue distribution. Therefore the following proposed experiments will focus on

IRS-1 and IRS-2.

IGF receptor tyrosine kinase activates PI3K – Akt and Erk MAPK pathway through

adaptor proteins – IRS proteins (Haeusler and Accili, 2008). IRS binds to activated receptor

with high affinity through pleckstrin-homology (PH) domains and phosphotyrosine-binding

(PTB) domains. IRS proteins are critical components of the IGF signaling pathways.Their

activities are tightly regulated by phosphorylation – activated by tyrosine residue

phosphorylation and inhibited by serine residue phosphorylation. Therefore, the phosphorylation status of these proteins may be critical to determine how IGF signaling is diverged under different physiological conditions. First, IRS could be the factor that integrates IGF signaling with cell metabolism and microenvironment. Tzatsos et al. reported that hypoxia inhibits PI3K-Akt signaling via AMPK-dependent phosphorylation of IRS-1 (Tzatsos and Tsichlis, 2007). Second, the potential changes of IRS phosphorylation could be linked with hypoxia-altered downstream signaling pathway. For example, Erk MAPK has been reported to be in the negative-feedback loop of insulin signaling by phosphorylating IRS-1 on serine residues (Bouzakri et al., 2003). Using cultured human skeletal muscle myotubes in primary culture, Bouzakri et al. found myotubes established from patients with type 2 diabetes have reduced IRS-1 tyrosine phosphorylation but increased serine phosphorylation after insulin stimulation (Bouzakri et al., 2003). Increased Erk MAPK activity correlates with IRS-1 serine phosphorylation, and pharmacological inhibition of Erk activity by PD98059 reduced IRS-1 serine phosphorylation (Bouzakri et al., 2003). Together, these results suggest that activated Erk MAPK pathway can inhibit IRS-1 phosphorylation by a negative feedback mechanism, which could in turn inhibit other IGF downstream signaling pathways, such as PI3K-Akt. In order to test this possibility, C2C12 myoblasts will be induced to differentiate under normoxia or hypoxia with or without exogenous IGF stimulation. Cell lysates will be collected. The phosphorylation status of IRS proteins will be analyzed by western blot using various antibodies detecting specific phosphorylated amino acid residues, such as Ser302, Ser307, Ser332/336, Ser789, Ser1101, Tyr895, and Tyr1222, etc.

In addition to phosphorylation, IRS proteins are also regulated at the level of protein expression (Hirashima et al., 2003; Rui et al., 2002; Shimomura et al., 2000). For example, in certain cell types, insulin suppresses the expression of both IRS-1 and IRS-2 proteins through different mechanisms. IRS-1 is regulated at post-translational level by proteasomal degradation, while the decrease in IRS-2 transcription parallels with the decrease in IRS-2 protein (Hirashima et al., 2003). In mice, hyperinsulinemia down-regulates IRS-2 transcription and stimulates fatty acid synthesis by increasing the expression of serum responsive element binding protein 1-c (SREBP-1c) (Shimomura et al., 2000). This suggests that under certain physiological condition, receptor tyrosine kinase downstream signaling pathways can be differentially regulated by IRS protein levels. Whether hypoxia changes IRS-1 or IRS-2 protein abundance will be examined. C2C12 myoblasts will be induced to differentiate under normoxia or hypoxia with or without exogenous IGF. Under these various conditions, total IRS-1 and IRS-2 proteins will analyzed by western blot. If there are any detectable changes in total proteins, their roles in mediating IGF signaling under normoxia and hypoxia will be further analyzed. For example, if certain IRS protein is reduced in response to IGF stimulation under hypoxia compared with normoxia, construct expressing that IRS protein will be introduced into cells in order to determine whether the phenotype can be reversed to that under normoxia. Alternatively, each individual IRS protein can be knocked down by siRNA in differentiating myoblasts. Thus, the function of each IRS protein can be determined by assaying cellular responses to exogenous IGF treatment under normoxia versus hypoxia.

Investigating how PI3K-Akt and Erk MAPK pathways are regulated by hypoxia

IRS proteins also contain multiple potential tyrosine-phosphorylation sites. The phosphorylation of these sites mediates the interaction between IRS and downstream signaling molecules that contain Src-homology-2 (SH2) domains. Two well-studied SH2 domain-containing proteins are the regulatory subunit of PI3K and the adaptor Grb2. Next, I will discuss the possible mechanism(s) of how hypoxia differentially regulates the two downstream signaling pathways – PI3K-Akt and Erk MAPK pathways.

Hypoxia inhibits Akt activation in response to IGF stimulation. Besides potential regulation at the levels of receptor and IRS proteins, hypoxia may affect PI3K activation. PI3K activates downstream effectors by generating lipid second messenger PIP_3 (Engelman et al., 2006). Proteins containing PH domains bind to PIP_3 and become localized to the same region, which leads to their activations (Hawkins et al., 2006). A well-studied example is 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Hawkins et al., 2006). Activated PDK1 in turn phosphorylates and activates Akt (Burgering and Coffer, 1995; Franke et al., 1995). Therefore, down-regulation of PIP_3 by phospholipids phosphatases could account for reduced Akt activation. Two well-studied phospholipids phosphatases are phosphatases and tensin homologue (PTEN) and SH2-containing inositols 5′-phosphatase-2 (SHIP2), which suppress PI3K-Akt signaling pathway (Vinciguerra and Foti, 2006). C2C12 myoblasts will be induced to differentiate under normoxia or hypoxia with or without exogenous IGF. Under these various conditions, PTEN and SHIP2 expression will be analyzed by western blot. If PTEN and/or SHIP2 expression is up-regulated under hypoxia, this will provide an explanation for reduced Akt activation in response to IGF stimulation under hypoxia. Next, PTEN and/or SHIP2 siRNA can be designed and introduced into cells differentiating under

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normoxia or hypoxia. Then, it can be tested whether IGF induces different cellular responses under normoxia and hypoxia is caused by increased phosphatases expression under hypoxia.

Akt activity is also tightly regulated by protein phosphatases, including protein phosphatases-2A (PP2A) and the PH-domain leucine-rich repeat-protein phosphatases (PHLPP) (Brazil et al., 2004; Gao et al., 2005; Meier et al., 1998). Gao et al. identified PHLPP as Akt specific phosphatase, which in turn triggers apoptosis and suppresses tumor growth (Gao et al., 2005). Other negative regulators also regulate Akt activity. For example, carboxyl-terminal modulator protein (CTMP) binds specifically to the C terminal regulatory domain of Akt at the plasma membrane (Maira et al., 2001). Binding of CTMP directly reduces Akt activity by inhibiting phosphorylation on critical Ser/Thr residues (Maira et al., 2001). Another negative regulator of Akt, tribbles-3 (TRB3) was initially identified from yeast two-hybrid screen as an Akt binding protein (Du et al., 2003). TRB3 binds to unphosphorylated Akt and inhibits its phosphorylation and activation (Du et al., 2003). Whether hypoxia regulates Akt activity via the negative regulators needs to be tested. C2C12 myoblasts will be induced to differentiate under normoxia or hypoxia with or without exogenous IGF stimulation. Protein expression of PP2A, PHLPP, CTMP, and TRB3 will be analyzed under various treatments. It is possible that hypoxia may increase the expression of the negative regulator(s), which directly leads to reduced Akt activation. If the expression of certain Akt negative regulator(s) is up-regulated under hypoxia, it will be examined whether the changes are happened at the transcription or translation level. In order to determine whether the gene is a direct target of HIF, the promoter region will be scanned for potential HRE sites. If the regulation does not occur at the transcriptional level, whether hypoxia affect the stability of the mRNA or protein will be tested. Next, specific siRNA construct can be

engineered and introduced into cells differentiating under normoxia or hypoxia. If the hypothesis is correct, knocking down specific negative regulator(s) of Akt will restore Akt activation in response to IGF stimulation under hypoxia.

One possible explanation for the up-regulated Erk MAPK downstream signaling pathways under hypoxia is that with PI3K-Akt signaling activity toned down under hypoxia, Erk MAPK signaling becomes more prominent by default. This hypothesis can be tested by manipulating the negative regulators of PI3K-Akt, such as PTEN and SHIP2. If the hypothesis is correct, overexpression of these negative regulators should mimic the effect of hypoxia on IGF signaling, i.e. decreased PI3K-Akt signaling and increased Erk MAPK signaling. Conversely, knockdown of these negative regulators will reverse hypoxia's effect on IGF signaling, i.e. restored Akt phosphorylation and concomitantly decreased Erk MAPK activation in response to IGF stimulation under hypoxia.

An alternative explanation for the differentially regulated IGF downstream signaling pathways is that certain adaptor protein critical for PI3K-Akt and Erk MAPK pathways is altered under hypoxia. One candidate is Grb2-associated binder 1 (Gab1). Gab1 acts as an adaptor protein and coordinates various receptor tyrosine kinase signaling pathways (Sarmay et al., 2006). Gab1 undergoes tyrosine phosphorylation after growth factor stimulation (Gu and Neel, 2003; Nishida and Hirano, 2003). The phosphorylated tyrosine residues present docking sites for SH2 domain-containing molecules, such as SH2-containing protein-tyrosine phosphatases 2 (SHP2) (Gu and Neel, 2003). In addition, Gab1 constitutively associated with PI3K regulatory subunit p85 (Dance et al., 2006; Schaeper et al., 2007). Interestingly, a recent study showed that Gab1-SHP2 interaction in response to IGF-I stimulation is important for Erk MAPK-mediated inhibitory effect on myoblast differentiation (Koyama et

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al., 2008). Koyama et al. engineered Gab1 mutant defective in binding to SHP2 (Gab1$^{\Delta SHP2}$) and Gab1 mutant defective in binding to p85 (Gab1$^{\Delta p85}$) (Koyama et al., 2008). They showed that IGF-induced myogenic differentiation was enhanced in myoblasts overexpressing Gab1$^{\Delta SHP2}$, but inhibited in those overexpressing either Gab1 or Gab1$^{\Delta p85}$. Gab1 knockdown by siRNA enhanced myogenic differentiation. Blocking Erk MAPK pathway reversed the inhibitory effect of Gab1 overexpression on myogenic differentiation. Moreover, Gab1 is critical mediating IGF-stimulated PI3K-Akt and Erk MAPK signaling activities. IGF-induced Erk MAPK activation was significantly repressed in myoblasts overexpressing Gab1$^{\Delta SHP2}$ but enhanced in those overexpressing either Gab1 or Gab1$^{\Delta p85}$. We postulate that hypoxia may differentially regulating IGF downstream signaling pathways by altering Gab1 expression and/or phosphorylation. C2C12 myoblasts will be induced under normoxia or hypoxia. Gab1 expression will be analyzed by western blot. Next, Gab1 phosphorylation in response to IGF stimulation under normoxia or hypoxia will be investigated. C2C12 myoblasts will be induced under normoxia or hypoxia with or without exogenous IGF. Gab1 phosphorylation will be determined by phospho-tyrosine specific Gab1 antibodies. In order to determine whether Gab1 mediates the differentially regulated PI3K-Akt and Erk MAPK signaling pathways under hypoxia, Gab1 siRNA will be employed to knock down Gab1 in differentiating myoblasts. Investigating Gab1 expression and phosphorylation under hypoxia may elucidate the molecular mechanism underlying the differentially regulated IGF downstream signaling pathways.

Examining how p38 MAPK pathway is regulated by IGF and hypoxia

A third pathway that may contribute to the conversion of IGF actions in hypoxia is p38 MAPK pathway. p38 MAPK pathway has been reported as an important myogenic pathway (Keren et al., 2006; Perdiguero et al., 2007). Hypoxia also strongly inhibits p38 MAPK activity in differentiating muscle cells. We have discovered that IGF stimulation activates p38 MAPK activity at the early stages of myogenesis. Pharmacological blockade of the p38 kinases decreased the basal differentiation and blocked the myogenic action of IGF. Importantly, inhibition of p38 MAPK by SB203580 significantly increased the mitogenic effect of IGF under the normoxic condition, suggesting that p38 MAPK plays dual roles during myogenesis - it promotes the myogenic actions of IGF while inhibits mitogenic actions. More questions regarding p38 MAPK signaling in hypoxia need to be answered.

p38 MAPK is activated in response to extracellular stimuli such as UV light, heat, osmotic shock, inflammatory cytokines, and growth factors (Freshney et al., 1994; Han et al., 1994; Lee et al., 1994; Raingeaud et al., 1995; Rouse et al., 1994). Although p38 MAPK pathway is commonly associated cytokine signaling, more recent studies show that it can be activated by receptor tyrosine kinase. For example, insulin stimulates glucose transporter 4 (Glut4) activation in adipocytes and myoblasts by activating p38 MAPK pathway, which is abolished p38 MAPK specific inhibitor SB203580 (Furtado et al., 2002; Sweeney et al., 1999). However, it remains to be determined whether IGF directly activate p38 MAPK. And, if so, how does IGF activate p38 MAPK pathway? p38 MAPK is activated by MAP kinase kinase (MKKs). MKK3 and MKK6 are two main MKKs to activate p38 (Zarubin and Han, 2005). The further upstream activators of p38 are MKK kinases (MAP3K), which includes a diverse range of kinases (Zarubin and Han, 2005). Whether IGF activates p38 MAPK via a particular MKK and MAP3K can be determined by stimulating cells with IGF and analyzing

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phosphorylated MKK and MAP3K. Alternatively, whether a certain MKK or MAP3K

mediates IGF actions on p38 MAPK can be tested by siRNA knockdown or overexpressing

dominant negative form of the upstream kinase.

My study showed that p38 activation is inhibited by hypoxia. How does hypoxia

inhibit p38 activation? Whether it is a direct or rather indirect inhibition remains unclear. I

will discuss the several possible answers as following. First, whether hypoxia regulates p38

activators, such as MKK and MAP3K, needs to be determined. C2C12 myoblasts will be

induced to differentiate under normoxia or hypoxia. MKK and MAP3K activation will be

analyzed and compared between samples subjected to normoxia or hypoxia treatment.

Second, reduced p38 activation under hypoxia may be a result of crosstalk between the

PI3K-Akt and the p38 MAPK pathway. Both PI3K-Akt and p38 MAPK pathways have been

shown to play important roles during myogenesis. It is yet to be definitely shown if the two

pathways crosstalk with each other or not. In my study, either rapamycin or SB203580

inhibits basal myoblast differentiation and significantly reduces IGF-induced myoblast

differentiation under normoxia. However, IGF-induced differentiation is not completely

abolished by the presence of a single inhibitor. This indicates that PI3K-Akt and p38 MAPK

pathways may have interdependent crosstalk and yet some distinct functions during

myogenesis. Studies from other labs have similar results. SB203580 not only inhibits p38

activation but also inhibits the activation of PI3K-Akt-mTOR target p70 S6 kinase; likewise

rapamycin also prevents the activation of p38 endogenous target (Cuenda and Cohen, 1999).

Gonzalez et al. reported that the reciprocal crosstalk and activation between the PI3K-Akt

and p38 MAPK pathways is essential for efficient myoblast differentiation (Gonzalez et al.,

2004). Inhibition or activation of p38 with SB203580, dominant-negative p38, or

constitutively active MKK6 regulates Akt kinase activity. And, activation or inhibition of

PI3K regulates p38 activity upstream of MKK6, indicating reciprocal communication and

positive feedback characteristic of myogenic regulation (Gonzalez et al., 2004). Furthermore,

Serra et al. showed that the functional interdependence between p38 and IGF-PI3K-Akt

pathways is converged on the chromatin of muscle genes to target distinct components of the

muscle transcriptosome (Serra et al., 2007).

In addition, I would like to propose one potential mechanism by which PI3K-Akt and

p38 MAPK crosstalk. Ueki et al. reported that PI3K regulatory subunit p85α is required for

Jun N-terminal kinase (JNK) signaling pathway, which is a stress-kinase pathway similar to

p38 MAPK pathway (Ueki et al., 2003). p85α null cells have reduced PI3K-Akt signaling

activity showing a 50% decrease in PI 3-kinase activity and a 30% decrease in Akt activity.

p85α null cells have decreased insulin-stimulated JNK activity, which can be restored by

expression of p85α or a p85 mutant that does not bind to PI3K catalytic subunit p110 (Ueki

et al., 2003). In my study, I have observed the reduced activation of both PI3K-Akt and p38

MAPK pathways. Therefore, I propose to examine the expression of PI3K regulatory subunit

p85α. C2C12 myoblasts will be induced to differentiate under normoxia or hypoxia. p85α

expression will be analyzed by western blot using sample under different treatment. If p85α

expression is specifically reduced under hypoxia, it would provide an explanation for the

reduction in both PI3K-Akt and p38 MAPK signaling activities.

Future study to establish the physiological relevance

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Besides the detailed mechanistic studies of IGF signaling pathways, the *in vivo* biological significance of the findings outlined in Chapter III needs to be established. To this purpose, I propose the following experiments. First, whether the effect of hypoxia on IGF signaling will be studied in primary cultures. Primary myoblasts from neonatal mice can be isolated based on established protocol (Tang et al., 2004). My study has shown that hypoxia inhibits primary myoblast differentiation. Whether primary myoblasts respond to exogenous IGF under different oxygen tensions will be further investigated. Primary myoblasts will be induced to differentiate under normoxia or hypoxia with or without exogenous IGF. Then, cell proliferation and differentiation can be quantified using the methods outlined in previous chapters. Furthermore, the activation of downstream signaling pathways will be analyzed by western blot using the primary myoblast cell lysates. Second, primary myoblasts will be used to examine whether the differential response to IGF stimulation under different oxygen tensions is indeed HIF-1-dependent. HIF-1α knockout is embryonic lethal, which presents challenges for direct isolation of HIF-1α null primary myoblasts. As an alternative approach, primary myoblasts from neonatal mice with HIF-1α floxed allele can be isolated. HIF-1α null myoblasts will be generated by transfecting with Cre-expressing vectors. Therefore, whether HIF-1 is the critical switch mediating different cellular responses to IGF can be determined by using HIF-1α wild type primary myoblasts and HIF-1α null primary myoblasts. Third, the findings can be further tested in transgenic mouse models. HIF-1α conditional knockout mice will be used because of the embryonic lethality associated with HIF-1α null. Mice with muscle specific deletion of HIF-1α can be generated by crossing mice carrying floxed HIF-1α alleles with mice expressing Cre under the control of muscle specific promoter, for example muscle creatine kinase promoter. Histological analysis can be

performed using muscles from HIF-1α conditional knockout mice. Thus, whether HIF-1 affects myoblast proliferation and differentiation *in vivo* can be examined.

Results from this part of the thesis may provide answers to a broad range of questions, which may not be limited to IGF system and myogenesis. Tissues in normal physiological or pathological states may vary dramatically in the perspectives of oxygen tension, nutrient supply and growth factor accessibility. For example, solid tumors often have poorly formed vasculature. As a result, hypoxia occurs. The presence of HIF often associates with poor prognosis. HIF as potential cancer therapy target has been extensively studied. Various hypotheses have been proposed linking HIF with tumor cell survival, proliferation, and migration (DeBerardinis et al., 2008; Gordan et al., 2007; Keith and Simon, 2007). IGF has long been associated with cancer. IGF overexpression positively correlates with tumor progression and metastasis (Clemmons, 2007). Drugs targeting IGF signaling have been developed and gone on clinical trials. Our finding that IGF signaling in differentially oxygenated tissue cultures could elicit dramatically different outcomes may provide novel cues to cancer therapy. To our best knowledge, the question how the IGF signaling specificity is established under different oxygen tension has not been addressed. Our study will not only fill the gap in the field but also generate innovative information applicable to questions in cancer and developmental biology.
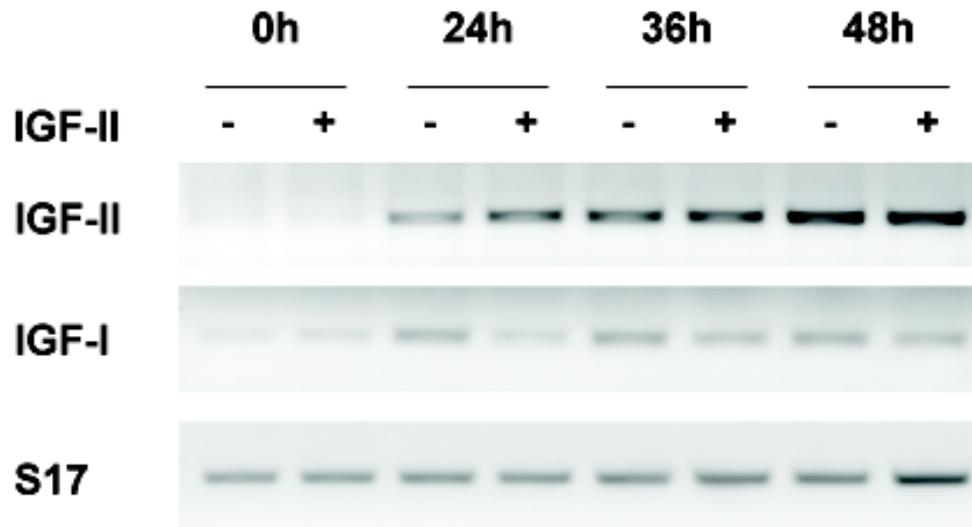
Fig. 4.1. IGF-I and IGF-II transcription is differentially regulated by IGF-II stimulation during myoblast differentiation. A) C2C12 myoblasts were induced to differentiate with or without 300ng/ml IGF-II supplemented in differentiation medium. Samples were collected at different time points indicated in the figure. IGF-I and IGF-II transcription was analyzed by semi-quantitative RT-PCR. S17 was used as an internal control. Data shown are the representative of 2 – 3 repeated experiments.
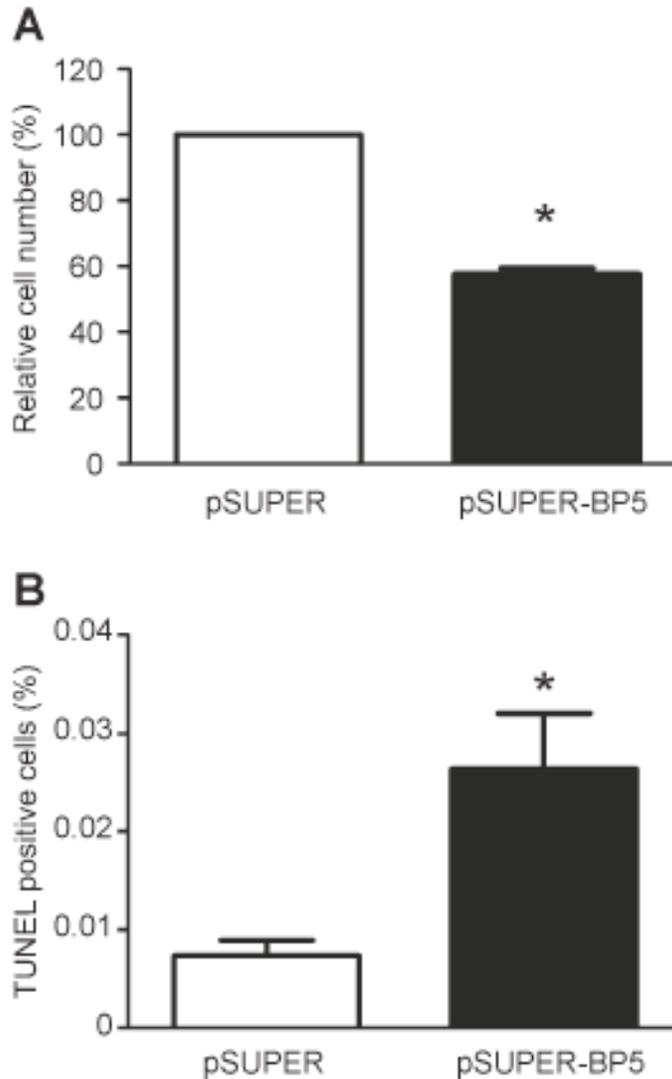
Fig. 4.2. IGFBP-5 siRNA decreases cell number with a concomitant increase in apoptosis in

at early phase of differentiation. A) C2C12 myoblasts transfected with pSUPER or pSUPER-

BP5 construct were induced to differentiate. Cells were fixed and stained with DAPI. Cell

number was counted as nuclei number. Data are means ± SE of 4 independent experiments.

* $p < 0.05$. B) C2C12 myoblasts transfected with pSUPER or pSUPER-BP5 construct were

induced to differentiate. Cells were subjected to TUNEL assay 12 – 24h later. Values are

expressed as percent of TUNEL positive nuclei number over total nuclei number. Data are

means ± SE of 3 independent experiments. * $p < 0.05$.

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