ROLES OF GROWTH HORMONE, INSULIN-LIKE GROWTH FACTOR I, AND SH3 AND CYSTEINE RICH DOMAIN 3 IN SKELETAL MUSCLE GROWTH

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Keywords: Myoblasts, Myotubes, Proliferation, Fusion, Differentiation, Signaling

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ABSTRACT

Three studies were conducted to achieve the following respective objectives: 1) to determine the cellular mechanism by which growth hormone (GH) stimulates skeletal muscle growth; 2) to identify the signaling pathways that mediate the different effects of insulin-like growth factor I (IGF-I) on skeletal muscle growth; and 3) to determine the role of a functionally unknown gene named SH3 and cysteine rich domain 3 (STAC3) in myogenesis. In the first study, the myogenic precursor cells, satellite cells, were isolated from cattle and allowed to proliferate as myoblasts or induced to fuse into myotubes in culture. GH increased protein synthesis without affecting protein degradation in myotubes; GH had no effect on proliferation of myoblasts; GH had no effect on IGF-I mRNA expression in either myoblasts or myotubes. These data suggest that GH stimulates skeletal muscle growth in cattle in part through stimulation of protein synthesis and that this stimulation is not mediated through increased IGF-I mRNA expression in the muscle. In the second study, the signaling pathways mediating the effects of IGF-I on proliferation of bovine myoblasts and protein synthesis and degradation in bovine myotubes were identified by adding to the culture medium rapamycin, LY294002, and PD98059, which are specific inhibitors of the signaling molecules mTOR, AKT, and ERK, respectively. The effectiveness of these inhibitors was confirmed by Western blotting. Proliferation of bovine myoblasts was stimulated by IGF-I, and this stimulation was partially blocked by PD98059 and completely blocked by rapamycin or LY294002. Protein degradation in myotubes was inhibited

by IGF-I and this inhibition was completely relieved by LY294002, but not by rapamycin or PD98059. Protein synthesis in myotubes was increased by IGF-I, and this increase was completely blocked by rapamycin, LY294002, or PD98059. These data demonstrate that IGF-I stimulates proliferation of bovine myoblasts and protein synthesis in bovine myotubes through both the PI3K/AKT and the MAPK signaling pathways and that IGF-I inhibits protein degradation in bovine myotubes through the PI3K/AKT pathway only. In the third study, the potential roles of STAC3 in myoblast proliferation, differentiation, and fusion were investigated. Overexpression of STAC3 inhibited differentiation of C2C12 cells (a murine myoblast cell line) and fusion of these cells into myotubes, whereas knockdown of STAC3 had the opposite effects. Either STAC3 overexpression or STAC3 knockdown had no effect on proliferation of C2C12 cells. Myoblasts from STAC3-deficient mouse embryos had a greater ability to fuse into myotubes than control myoblasts; the former cells also expressed more mRNAs for the myogenic regulators MyoD and myogenin and the adult myosin heavy chain protein MyHC1 than the latter. These results suggest that STAC3 inhibits myoblast differentiation and fusion.

Keywords: Myoblasts, Myotubes, Proliferation, Fusion, Differentiation, Signaling

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Chapter I

Literature Review

Introduction

Skeletal muscle is the most abundant tissue in animals, and it represents 40-50% of total body weight. Contraction of skeletal muscle enables locomotion and breathing, and supports posture and body position. Skeletal muscle produces 85% of the heat to maintain body temperature. Proper skeletal muscle mass and function are critical for life and health. Loss of muscle mass or strength results in impaired mobility, and is associated with many diseases, including cancer, diabetes, AIDS, congestive heart diseases, and renal failure. Loss of muscle mass and function is also associated with ageing. As meat, skeletal muscle is the primary product of animal agriculture.

A skeletal muscle is composed of a number of muscle fibers, with each fiber as a multinucleated muscle cell, or myofiber. Each myofiber develops from the fusion of single-nucleated-cells, or myoblasts. Skeletal muscle mass is, therefore, determined by both the number and size of muscle fibers. The number of muscle fibers is determined during prenatal development. The size of a muscle fiber is primarily regulated by the balance of protein synthesis and degradation during postnatal growth. In addition, skeletal muscle also has the capability of introducing additional DNA into existing muscle fibers during muscle maintenance or regeneration.

A great deal of understanding has been achieved about the mechanisms underlying skeletal muscle growth and regeneration. A complex and integrated network of signaling pathways dynamically modulates the activation and the subsequent differentiation of myogenic

progenitor cells, and controls the balance between anabolic and catabolic states in muscle. Modulation of these signaling pathways in animal models and in some human myopathies has shown considerable potential for increasing muscle mass and function, and for ameliorating some of the symptoms of myopathologies. In this review, I will discuss the cellular, molecular, and hormonal mechanisms that mediate the key processes of skeletal muscle growth, hypertrophy, and regeneration in postnatal animals, with the focus on the roles of growth hormone (GH) and insulin-like growth factor I (IGF-I) in these processes. I will also touch on the potential role of a novel gene named SH3 and cysteine rich domain 3 (STAC3) in skeletal muscle development and growth.

Postnatal Growth and Regeneration of Skeletal Muscle

Adult skeletal muscle has the ability to regulate size in response to environmental and physiological demands and to regenerate after injury (Glass, 2005b; Guttridge, 2004). Skeletal muscle growth in adult animals involves the increase of both nuclei and protein mass (Salleo et al., 1983; Schiaffino et al., 1972). Nuclei of muscle fibers (i.e. myonuclei) are provided by myogenic precursor cells, mainly satellite cells. Protein mass is a dynamic balance between protein synthesis and degradation. Most of these events are controlled by specific growth factors that are locally produced or transported from the bloodstream.

Precursor cells of skeletal muscle

Satellite cells: Satellite cells are stem cells located between sarcolemma of myofibers and surrounding basal lamina in adult skeletal muscle (Fig. 1.1). Satellite cells are Pax3 and Pax7 positive cells derived from the central dermomyotome (Gros et al., 2005). Pax3 and Pax7 are homeodomain transcription factors that play crucial roles in skeletal muscle development (Buckingham and Relaix, 2007; Sambasivan and Tajbakhsh, 2007). Mice carrying double mutant of Pax3 and Pax7 show loss of muscle progenitor cells (Relaix et al., 2005). During the postnatal stage, satellite cells proliferate to provide nuclei for both muscle growth (Macconnachie et al., 1964) and regeneration (Price et al., 1964; Shafiq and Gorycki, 1965). In newborn rodents, approximately 30% of muscle nuclei are satellite cells (Bischoff and Heintz, 1994). The number of satellite cells decreases to less than 5% in adult muscle (Bischoff and Heintz, 1994; Gibson and Schultz, 1983). Satellite cells are typically quiescent in mature muscles, but can be recruited to proliferate following injuries, physical activity and hormone levels. Activated satellite cells proliferate to give rise to more myoblasts, which fuse with growing myofibers to increase

myofiber size. Upon muscle injury, satellite cells also proliferate to repair muscle damage by fusing with existing myofibers or to form new myofibers when necessary (Grounds and Yablonka-Reuveni, 1993; Hawke et al., 2001).

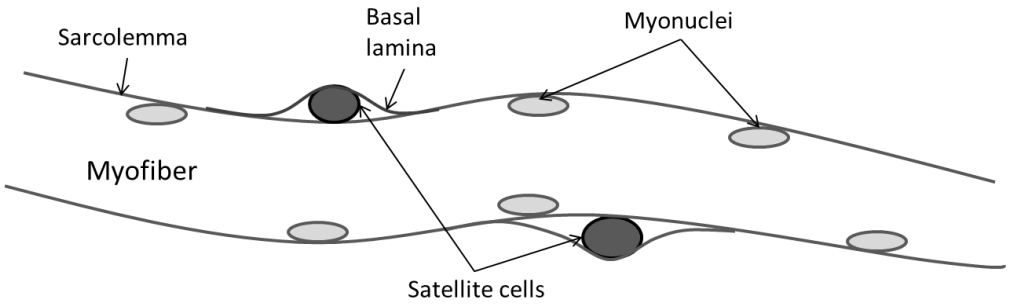


Fig. 1.1. Localizaton of satellite cells. In mature myofibers, myonuclei are located directly beneath the sarcolemma, and satellite cells reside between the sarcolemma and basal lamina surrounding myofibers.

Satellite cell-derived muscle cells express the myogenic regulatory factors (MRFs) Myf5, MyoD, myogenin and MRF4 (Fig. 1.2), which belong to the helix-loop-helix (HLH) transcription factor family (Fuchtbauer and Westphal, 1992; Grounds et al., 1992; Yablonka-Reuveni and Rivera, 1994). MRFs are master switches during myogenesis (Weintraub et al., 1991). They share the ability to induce myogenic differentiation when expressed in non-muscle cells (Pownall et al., 2002). Analysis of mouse mutants and over-expression in chick embryos showed that Pax3 and Pax7 control the expression of myogenic regulators (Bajard et al., 2006; Maroto et al., 1997; Sato et al., 2010). Previous experiments have defined the MRFs into two groups: the primary group and the secondary group. The primary group includes Myf5 and MyoD, which are required for myogenic determination. Myf5 deficient mice die perinatally due to severe rib defects, but show normal skeletal muscles (Braun et al., 1992). MyoD-null mice

show a normal skeletal muscle phenotype with reduced size (Macharia et al., 2010; Megeney et al., 1996; White et al., 2000). However, MyoD and Myf5 double mutant mice die at birth due to the absence of myoblasts and muscle (Rudnicki et al., 1993). The secondary group includes myogenin and MRF4 known as differentiation factors. Myogenin-null mice die at birth with normal number of myoblasts but no myofibers (Hasty et al., 1993). MRF4 inactivation results in normal muscle with a fourfold increase in myogenin expression (Zhang et al., 1995).

In addition, some studies indicated that some MRFs can functionally compensate for one another during muscle development. MyoD inactivation does not affect muscle generation but increases Myf5 expression (Rudnicki et al., 1992). Mice carrying mutations in Myf5, MyoD or MRF4 are viable, and severe muscle defects in the embryo are only observed when all three genes are knocked out (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). This suggests that there is redundancy in the role of Myf5, MyoD, and MRF4 in myogenesis.

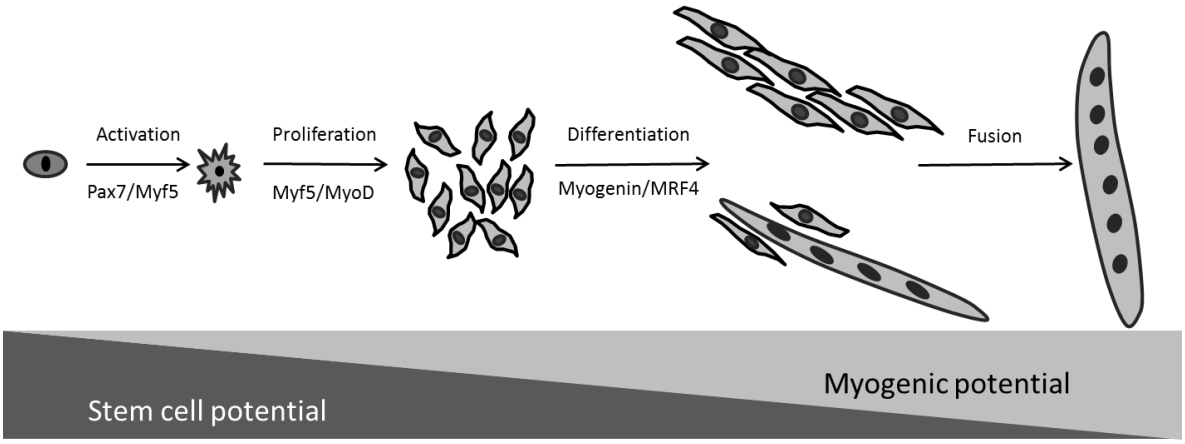


Fig. 1.2. Satellite cell proliferation, differentiation and fusion. Quiescent satellite cells can be activated to proliferate to increase the number of myoblasts, which are marked with Myf5 and MyoD. Myoblasts upregulate myogenin and MRF4 to differentiate and further fuse into myotubes for myogenesis.

In addition to myogenic-committed satellite cells, several studies found that cells from other tissues can give rise to myogenic cells, such as dermis, the neural tube, and the thymus (Cossu, 1997). Recent studies also suggest that other myogenic precursor cells might play a role in skeletal muscle growth and regeneration as well (Charge and Rudnicki, 2004; Peault et al., 2007).

SPs (side population cells): The heterogeneous muscle SP cells are the population of myogenic precursor cells distinct from satellite cells. Muscle SP cells are present in Pax7 -/- mice, which exhibit a severe deficiency in SCs (Asakura et al., 2002). They were induced to express myogenic markers when co-cultured with primary myoblasts (Peault et al., 2007). They were considered a candidate for cell therapy in muscular diseases, because they gave rise to both muscle cells and satellite cells after intramuscular injection (Asakura et al., 2002; Peault et al., 2007; Uezumi et al., 2006).

MDSCs (muscle derived stem cells): MDSCs are a population of early myogenic progenitor cells (Peault et al., 2007). Compared to satellite cells, they have the advantage of prolonged proliferation, strong tendency for self-renewal, multi-lineage differentiation, and immune tolerance (Peault et al., 2007; Qu-Petersen et al., 2002).

CD133+ cells: $CD133^{+}$ cells circulate in the blood stream and are able to differentiate (Torrente et al., 2004). Injection of human CD 133^{+} cells into the circulation of scid/mdx mice improves skeletal muscle structure and function, and replenishes the satellite cell population (Torrente et al., 2004). Compared to human myoblast transplantation, CD 133+ showed better regeneration and repopulation of the satellite cells (Negroni et al., 2009).

Pericytes: Pericytes become myogenic in vitro when differentiation is induced and contribute to muscle regeneration in dystrophic mice after intra-arterial injection (Boldrin and Morgan, 2007; Dellavalle et al., 2007). Some pericytes were localized in a satellite cell position suggesting that these cells are able to replenish the satellite cell pool (Dellavalle et al., 2007). This makes them an interesting potential candidate for future cell therapy in patients.

Mesoangioblasts: Mesoangioblasts are vessel-associated stem cells derived from the embryonic dorsal aorta. They are able to differentiate into several mesodermal cell types including skeletal muscle cells (Charge and Rudnicki, 2004; Peault et al., 2007). As an alternative to myoblast transplantation, transplantation of mesoangioblasts into the blood circulation of dystrophic mice has recently shown great potential for skeletal muscle regeneration (Galvez et al., 2006; Sampaolesi et al., 2006).

HSCs (Hematopoietic stem cells): Hematopoietic stem cells are also the most important multipotent stem cells participating in skeletal muscle regeneration after the SC (Charge and Rudnicki, 2004; Corbel et al., 2003). HSCs transplantation resulted in the formation of myofibers, and they also contribute to the satellite cell pool (Bittner et al., 1999; Gussoni et al., 1997; LaBarge and Blau, 2002).

By now, most myogenic studies and clinical trials focus on satellite cells and satellite cell-derived myoblasts. As a good candidate for cell therapy of injured or diseased muscle, satellite cells are plentiful and easy to obtain. Recent studies have shown that freshly isolated satellite cells can not only efficiently regenerate skeletal muscles, but also replenish the satellite cell reservoir in the host muscle (Boldrin et al., 2009; Collins et al., 2005; Montarras et al., 2005; Sacco et al., 2008).

Myoblast proliferation

Cell proliferation is defined as the increase of cell number by division. Cell cycle is the process leading to that cell division. It consists of G1, S, G2, and M phases. Cell cycle progression involves a series of tightly regulated and coordinated events. Non-dividing cells stay at G0 phase before they enter the cell cycle. In S phase, DNA is replicated. In M phase, one cell divides into two daughter cells.

The molecular mechanisms that control the cell cycle occur in a sequential way, which makes the cell cycle irreversible. Cyclins and cyclin-dependent kinase (CDK) complex are the major components of these mechanisms (Nigg, 1995). Cyclin D is the first cyclin produced in the cell cycle, in response to extracellular signals. During the early stage of G1 phase, cyclin D interacts with cdk4 and cdk6 to trigger the molecular events to promote the cell cycle. Cyclin E binds cdk1 to form the complex, which pushes the cell from G1 to S phase (Sandhu and Slingerland, 2000). There are also inhibitors controlling the activities of cyclin-cdk complex during cell cycle. They are divided into two families: INK, which inhibits cdk4 and cdk6 (Sandhu and Slingerland, 2000; Sherr, 1995), and the KIP family, which appears to inhibit all cdk family members. The INK family includes p15 (CDKN2B), p16 (CDKN2A), p18 (CDKN2C) and p19 (CDKN2D), and the KIP family includes p21 (CDKN1A), p27 (CDKN1B), and p57 (CDKN1C).

Satellite cell proliferation is critical for providing myonuclei during postnatal muscle growth and regeneration. In growing muscle, satellite cells are shown to be able to undergo mitosis (Shafiq et al., 1968). After muscle injury, quiescent satellite cells migrate to the site of

injury and become proliferative (Beauchamp et al., 2000; Cooper et al., 1999; Cornelison and Wold, 1997; Smith et al., 1994a; Yablonka-Reuveni and Rivera, 1994).

Upon activating signals, satellite cells transit from G0 to G1 phase of the cell cycle, marked by expressing MyoD and Pax7 within hours of stimulation (Yablonka-Reuveni and Rivera, 1994; Zammit et al., 2004). As soon as the satellite cells enter their first cell cycle, they become highly proliferative (Conboy and Rando, 2002). From this stage, satellite cells are also known as myoblasts. Myoblasts up-regulate cyclin D1 when they proliferate (Rao et al., 1994; Skapek et al., 1995). Cyclin D1 expression is controlled by transcription factors c-jun and c-fos. In proliferating satellite cells, increased cyclin D1 expression promotes translocation of CDK4 to the nucleus. Cyclin D1–CDK4 complexes interact with MyoD to prevent its DNA binding (Li and Olson, 1992; Zhang et al., 1999). As a result of cyclin D1 degradation, MyoD is released from cyclin D1–CDK4 complex and collaborates with E-protein family members to induce myogenic gene transcription (Walsh and Perlman, 1997). In consequence, myoblasts exit cell cycle and start to differentiate. In addition to cyclin D1, increasing the CDK activity by overexpressing cyclins A, D, or E prevents myogenic differentiation by inhibiting MyoD as well (Guo and Walsh, 1997; Skapek et al., 1995). Moreover, cyclin E–CDK2 complexes phosphorylate MyoD and direct it to proteolytic degradation (Kitzmann et al., 1999; Song et al., 1998). Besides MyoD, activities of other MRF family members are also regulated by their phosphorylation status. For example, protein kinase C phosphorylates myogenin to inhibit its interaction with DNA (Li and Olson, 1992), and protein kinase A represses activity of both Myf-5 and MyoD (Winter et al., 1993).

Myoblast differentiation and fusion

Myoblasts remain in the cell cycle to proliferate when cultured in high serum concentrations. Upon serum withdrawal, myoblasts undergo ordered events to differentiate and fuse to form myotubes. During this process, myoblasts decrease expression of Pax7 (Olguin and Olwin, 2004; Zammit et al., 2004), and increase that of MRF4, myogenin and muscle-specific myosin heavy chain (MyHC) (Cornelison and Wold, 1997; Smith et al., 1994a; Yablonka-Reuveni and Rivera, 1994).

Initiation of myoblast differentiation is associated with permanent withdrawal from the cell cycle (Andres and Walsh, 1996). This permanent cell cycle withdrawal is featured as the loss of the ability to reenter the cell cycle in the presence of growth factor stimulation (Endo and Nadal-Ginard, 1986; Gu et al., 1993). Cell cycle regulators, such as cyclin D3, cyclin-dependent kinase inhibitors p21 and pRb, are essential for this terminal withdrawal (Andres and Walsh, 1996; Cenciarelli et al., 1999; Halevy et al., 1995; Kiess et al., 1995). In myogenic cells, MyoD regulates p21 and pRb transcription. Overexpression of cyclin D3 increases expression of negative cell cycle regulators such as p21, and muscle-specific genes such as myogenin or MyHC. On the other hand, lack of cyclin D3 leads to the decrease in p21, MyHC and α-actin (De Santa et al., 2007). Ectopic expression of cyclin D3 was shown to correct the defects in myogenic differentiation associated with myotonic dystrophy type 1 (Salisbury et al., 2008).

The differentiated myoblasts gain the ability to fuse. Myoblast fusion can be divided into two stages. At the first stage, myoblasts fuse to generate the nascent myotubes. At the second stage, myoblasts fuse with the nascent myotubes to add nuclei and size to myotubes. Myoblast fusion is a complicated process requiring collaboration of different factors, including the extracellular signals, cell surface metalloproteases, membrane receptors and channels, calcium signaling pathways and factors that regulate myoblast motility (Horsley and Pavlath, 2004). One

of the extracellular signals is interleukin-4 (IL-4), which recruits myoblasts to growing myofiber for fusion. The nuclear factor of activated T-cells cytoplasmic 2 (NFATC2) transcription factor regulates the secretion of IL-4 (Horsley et al., 2001; Horsley et al., 2003). In NFATC2 null mice, myofibers showed reduced size and lower myonuclear numbers (Horsley et al., 2001). Similar to NFATC2, the membrane protein myoferlin is also important for myoblast fusion. Its membrane location helps to identify the actual site of fusion between myoblasts. Myoferlin deficient mice had no problem with forming myofibers, but the myofibers showed reduced cross sectional area, and had defects in regeneration after muscle injury (Doherty et al., 2005). Although genes important for fusion have been identified, the underlying mechanisms through which they exactly regulate the process are still under investigation.

Skeletal muscle hypertrophy and atrophy

Skeletal muscle hypertrophy is defined as an increase in protein mass, and atrophy is a decrease in protein mass. The protein content in a myofiber is determined by the balance between protein synthesis and degradation. The net balance between these two processes leads the muscle to go hypertrophy or atrophy (Fig. 1.3).

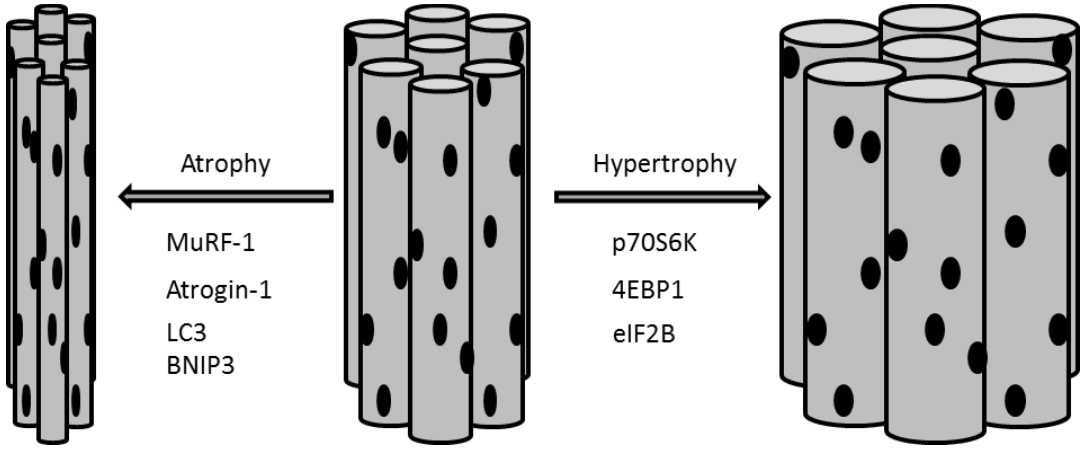


Fig. 1.3. Myofiber atrophy and hypertrophy. The balance between protein synthesis and degradation determines the increase or the decrease in myofiber size and muscle mass. Both processes are tightly regulated by the signals from the extracelluar environment. Distinct sets of proteins are involved in muscle atropy (MuRF-1, Atrogin-1, LC3 and BNIP3) and muscle hypertrophy (p70S6K, 4EBP1 and eIF2B).

Under anabolic conditions, the AKT-mediated pathway is one of those important signal cascades promoting protein synthesis (Bodine, 2006; Guttridge, 2004). Signaling through several receptors activates the phosphoinositide 3-kinase (PI3k)-AKT pathway to stimulate protein synthesis by phosphorylating and thus activating mammalian target of rapamycin (mTOR) (Bodine, 2006; Bodine et al., 2001b; Sandri, 2008). Blocking mTOR-dependent signaling by rapamycin resulted in repression of exercise-induced muscle hypertrophy (Bodine et al., 2001b). Upon activation, mTOR enhances protein translational efficiency by activating the positive regulator of protein synthesis p70S6K and inhibiting the negative regulator 4EBP1 (Glass, 2005b). Phosphorylated mTOR also inhibits glycogen synthase kinase 3 β (GSK3β), which has been implicated in inhibiting the translation initiation factor eIF-2B (Bodine, 2006; Glass, 2005b). Dominant negative overexpression of GSK3β induces myotube hypertrophy in vitro (Rommel et al., 2001). These pathways are highly conservative among different species (Sakamoto et al., 2004).

Under catabolic conditions, skeletal muscle adapts to the environment by reducing muscle mass (Glass, 2005a; Guttridge, 2004). At least three levels of degradative systems are involved in protein degradation in skeletal muscle: lysosomal degradation system, ubiquitin proteasome system (Ciechanover, 2005; Reinstein and Ciechanover, 2006), and calpain 3-coupled ubiquitin-mediated degradation, which target cytoskeleton and sarcomeric components

(Duguez et al., 2006; Kramerova et al., 2005; Kramerova et al., 2006). The ubiquitin proteosome system is a major and widely studied system in muscle atrophy. Proteins to be degraded by proteosomes are marked with E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligating enzyme (Glickman and Ciechanover, 2002). Two E3 enzymes were identified specifically in skeletal muscle, the muscle-upregulated RING finger (MuRF-1) and atrogin-1, also known as MAFbx1 (Bodine et al., 2001a; Gomes et al., 2001). It was recently found that the transcription factor forkhead box O3 (FoxO3) regulates the expression of both MuRF-1 and MAFbx1 (Sandri et al., 2004; Stitt et al., 2004). It has also been reported that FoxO3 regulates the expression of microtubule-associated protein light chain 3 (LC3) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), both of which are critical in lysosomal degradation system (Mammucari et al., 2007). Thus, FoxO3 controls both lysosomal and ubiquitin proteasome systems of protein degradation in skeletal muscle.

Inflammation-induced mechanisms are also involved in muscle atrophy (Dehoux et al., 2007; Mourkioti et al., 2006; Mourkioti and Rosenthal, 2005; Spate and Schulze, 2004). Upon the stimulation of TNFα and IL-1, NF-κB is activated to transfer into nucleus. Several studies in muscle tissue cultures suggested that activation of NF-κB is necessary for cytokine-induced skeletal muscle loss (Cai et al., 2004; Mourkioti et al., 2006). Mice overexpressing IκB kinase, which activates NF-κB, showed enhanced ubiquitin-dependent proteolysis mediated by MuRF1 (Cai et al., 2004).

It has been recently discovered that the AKT pathway controls not only protein synthesis but also protein degradation in skeletal muscle. On one hand, AKT phosphorylates mTOR to increase protein translational efficiency by activating 4EBP1 and S6 kinase (Carrera, 2004), but on the other hand, Akt phosphorylates FOXO transcription factors and represses the transcription

of atrogenes, which are muscle atrophy-related ubiquitin ligases. They enhance the AKT-mediated increase of protein mass by shutting down protein degradation. However, stimulation of this one pathway cannot counteract all modes of muscle atrophy, such as the one induced by pro-inflammatory cytokines.

Skeletal muscle regeneration

Skeletal muscle constantly experiences injury during daily activity. There are also muscle diseases called muscular dystrophies characterized by progressive muscle wasting and weakness leading to a variable degree of mobility limitation and heart and/or respiratory failure (Emery, 2002). Because of the large amount of satellite cells, skeletal muscle has the remarkable ability to regenerate after injury or diseases (Carlson, 1973). It has been demonstrated that muscle regeneration requires activation of the mononuclear satellite cells (Bittner et al., 1999; Ferrari et al., 1998; Parrish et al., 1996; Patel et al., 2002).

Muscular dystrophies are some of the most difficult diseases to treat. Satellite cells are considered candidates to treat muscular dystrophy, because of their abilities to repair the damaged myofiber and replenish the satellite cell population in host skeletal muscle. As such, it is important to understand the cellular and molecular mechanisms underlying skeletal muscle regeneration.

The most common and severe muscle dystrophy disease is Duchenne muscular dystrophy (DMD), in which satellite cells exhaust their proliferation ability to repair the damaged myofiber. Mdx mouse is the disease model for human DMD. In mdx mice, intramuscular injection of normal myoblasts led to very limited fusion into host myofiber (Partridge et al., 1989). Freshly isolated satellite cells showed a much greater capacity to fuse into fibers of mdx mice than the

same cells after in vitro expansion (Montarras et al., 2005). However, no success has been reported in clinical trials. There are mainly three problems with the intramuscular injection to treat muscle dystrophy: firstly, most injected cells die within the first 72 h after injection (Fan et al., 1996; Guerette et al., 1997); secondly, intramuscularly-injected cells only stay where they are injected (Huard et al., 1992); finally, immune responses toward the injected SCs have been described, even in the case of major histocompatibility locus coincidence (Cossu, 2004). Those three problems are inevitable to make advance in muscular dystrophy treatments with satellite cells. Experiments using other myogenic precursor cells showed limited improvement in those aspects. Further exploration of the cellular and molecular mechanisms of satellite cell regeneration procedure in vivo will help to make the muscular dystrophy treatment more efficient in the future.

Role of GH and IGF-I in Skeletal Muscle Growth

Growth hormone

Growth hormone is a cytokine polypeptide secreted from the anterior pituitary into the circulating system. Its secretion is tightly regulated by two hypothalamic factors, the stimulating GH releasing hormone (GHRH) (Ling et al., 1984; Spiess et al., 1983) and the inhibitory hormone, somatostatin (SS) (Brazeau et al., 1973). The synthesis of GH is also detected in other tissues, which suggests that GH may also have paracrine/autocrine effects (Waters et al., 1999). GH is a major hormone regulating growth and metabolism of multiple organs and systems, such as liver, fat, mammary gland, and skeletal muscle (Guler et al., 1988).

GH binds to GH receptors (GHR) on the target cell membrane to activate intracellular signals. The GHR is a transmembrane glycoprotein. It spans the membrane once and contains an extracellular region, a single hydrophobic transmembrane domain and an intracellular region. The sequence of GHR is highly conservative between different species (Leung et al., 2000). It has been shown that GHR is expressed in a variety of tissues, including liver, fat, muscle, kidney, heart, and prostate, with liver, muscle and fat showing the highest expression levels (Ballesteros et al., 2000). The expression of GHR mRNA was also detected in some human malignant tissues (Fisker et al., 2004; Wei et al., 2006; Wu et al., 2006).

During posttranscriptional and posttranslational modifications of GHR expression, GH binding protein (GHBP) is generated, which corresponds to the extracellular ligand-binding domain of the GHR (Baumann et al., 1986; Leung et al., 1987). In humans, GHBP is the product of GHR proteolytic cleavage (Baumann, 1995). It is the truncated product from mRNA splicing

in rodents (Baumbach et al., 1989). It is believed that the binding of GHBP stabilizes GH and elongates its half-life in the circulating system.

Upon GH binding to its receptors, GHRs form homodimers and recruit intracellular protein kinases, such as Janus Kinase 2 (JAK2) (Fig. 1.4). Although the GHRs do not possess intrinsic tyrosine kinase activity, this association results in auto-phosphorylation of JAK2 (He et al., 2003). Once activated, JAK2 phosphorylates the GHR on multiple tyrosine residues and further triggers several downstream signaling pathways (Wang et al., 1996). Activation of JAK2 is believed to be critical for the initiation of most of these signaling pathways, including Ras/Raf/mitogen-activated protein kinases (MEKs)/ extracellular signal-regulated kinases (ERKs), the insulin related substrate (IRS)/phosphatidylinositol-3'-kinase (PI3-kinase)/protein kinase B (PKB), and signal transducers and activators of transcription (STATs) (Carter-Su et al., 1996; Zhu et al., 2001). In hepatocytes, STAT5b responds to the GH signal for regulating gene expression, such as IGF-I. Upon phosphorylation of GHR-JAK2, STAT5b proteins in the cytoplasm form homodimers and translocate into the nucleus where they activate transcription of the target genes (Takeda and Akira, 2000).

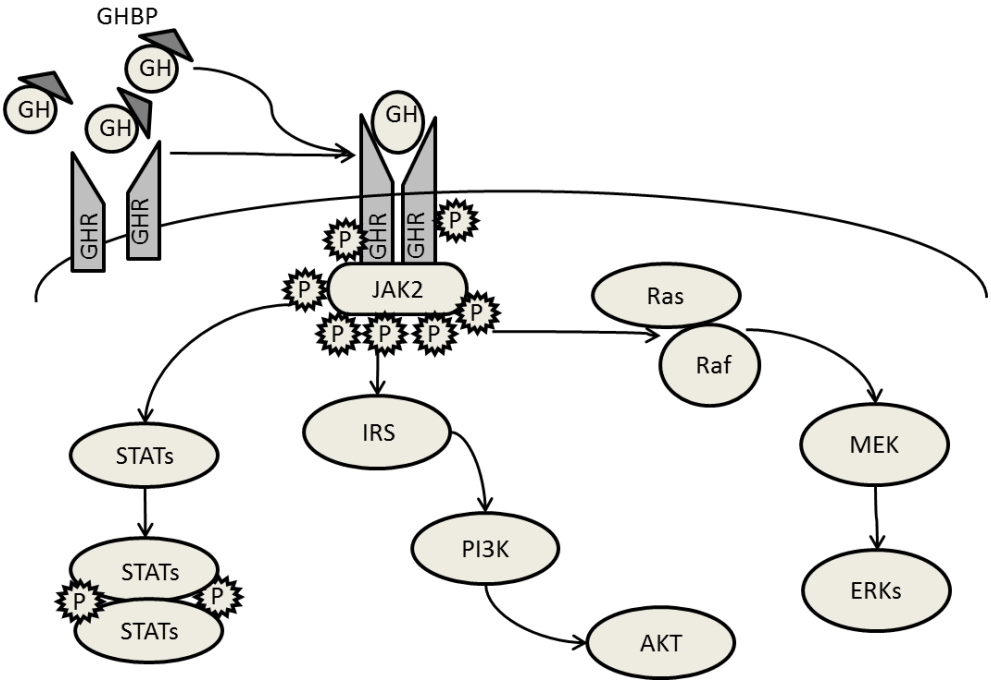


Fig. 1.4. GH intracellular signaling pathways mediated by JAK2 phosphorylation: a) activated JAK2 phosphorylates signal transducers and activators of transcription (STAT) proteins to form dimers to start target gene transcription; b) insulin receptor substrate (IRS) proteins are phosphorylated by JAK2 and serve as docking proteins to activate PI3K/AKT pathway; c) upon JAK2 activation, Ras/Raf phosphorylate MEK/ERK pathway to activate gene transcription.

It is critical to keep the physiological concentration of GH in circulation system. Excessive GH secretion thickens the bones of the jaw, fingers and toes, accompanied with problems of sweating, insulin resistance, muscle weakness, and reduced sexual function. The deficiency of GH leads to growth failure, short stature and sexual immaturity in children. The most common cause of GH deficiency in adults is pituitary adenoma. Adults with GH deficiency only had minor problems including a relative decrease in muscle mass and decreased energy and quality of life (Molitch et al., 2006).

Because of the critical role in growth, GH has long been used in medicine and agriculture. It has been used to treat GH deficiency-caused short stature, such as Turner syndrome, chronic renal failure, Prader-Willi syndrome, intrauterine growth retardation, and severe idiopathic short stature. Higher doses above physiologic level in circulation system are required to produce remarkable improvement of growth in these conditions. In agriculture, there is a long history of using GH to increase livestock production. The potential problems of using GH in medicine and agriculture need to be considered. In medicine, exogenous GH treatment is limited under certain circumstances due to its side effects, such as joint pain, sweating, and diabetes (Molitch et al., 2006). There are also controversies over using GH in animal products. In the US, the only FDA approved use of GH in animal production is the use of bovine somatotropin for milk production in dairy cows.

Insulin-like growth factor-I

IGF-I is a 70-amino-acid single chain containing three intra-molecular disulfide bridges, which shares approximately 50% homology with insulin. It is important for both prenatal development and postnatal growth. Mice null for IGF-I are born smaller and show slower postnatal growth compared to normal litter mates (Baker et al., 1993; Liu et al., 1998; Liu et al., 1993). A major part of IGF-I in the circulation system is secreted by liver under the control of GH. IGF-I exerts endocrine actions on target organs to promote growth. In addition, it stimulates glucose uptake into fat and muscle tissues (Blundell et al., 1983; Rinderknecht and Humbel, 1978). The body size correlates with IGF-I levels in different species. Tall children have elevated plasma IGF-I levels (Gourmelen et al., 1984). Mice with high IGF-I levels have increased body weight (Blair et al., 1988).

Besides liver, many other tissues express IGF-I. IGF-I is expressed in multiple human fetal tissues, including intestine, muscle, kidney, placenta, stomach, heart, skin, pancreas, hypothalamus, brain stem, spleen, and adrenal (Han et al., 1988). Other studies confirmed IGF-I mRNA expression in adult rat tissues such as heart, muscle, fat, spleen, and kidney (Le Roith et al., 2001). The expression of IGF-I in these tissues indicated its autocrine/paracrine effects. GH may or may not participate in regulating the local IGF-I expression in other tissues (D'Ercole et al., 1980; Han et al., 1988; Lowe et al., 1988).

The receptor of IGF-I (IGF-IR) has a heterotetrameric configuration formed with two alpha and two beta subunits. The extracellular region is composed of the two alpha subunits and forms the ligand binding domain. The entire alpha subunit is located extracellularly, and the beta subunit has the transmembrane region of the receptor and contains the cytoplasmic tyrosine kinase domain (Steele-Perkins et al., 1988). Upon IGF-I binding, the alpha subunits mediate the formation of the heterotetrameric configuration and the intracellular domains of beta subunits become phosphorylated to recruit signal proteins (Sasaki et al., 1985).

After binding with IGF-I, the cytoplasmic domain of IGF-IR is subsequently phosphorylated and recruits endogenous substrates that bind to the phosphotyrosine docking sites (Fig. 1.5) (LeRoith et al., 1995). These substrates include IRS family of proteins (IRS-1,2,3,4) and the SHC family of adapter proteins, with SH2 domains. Both docking proteins bind to the IGF-I receptor at the juxtamembrane region and then are able to recruit other substrates that lead to activation of two principal signaling pathways, the MEK/ERK pathway and the PI3K/AKT pathway (Craparo et al., 1995; Tartare-Deckert et al., 1995). The PI3K pathway leads to the activation of protein kinase B (PKB). Protein kinase B is also known as AKT, which has the

mammalian target of rapamycin (mTOR) as one of its important target proteins (D'Mello et al., 1997).

There are six IGF binding proteins (IGFBPs). When IGF-I is being transported to the target tissue in circulation system, IGFBPs bind to IGF-I to prevent proteolytic degradation. In addition to their function in circulation, some tissues also express IGFBPs to control the paracrine/autocrine effects of IGF-I locally (Rechler, 1993). Depending on the conditions, IGFBPs may have positive or negative effects on the responses of IGF-IR to IGF-I stimulation.

Physiological IGF-I concentration is also very critical. Several studies have shown that increased levels of IGF lead to an increased risk of cancer (Smith et al., 2000). Studies with lung cancer cells show that drugs inhibiting such signaling can be of potential interest in cancer therapy (Velcheti and Govindan, 2006).

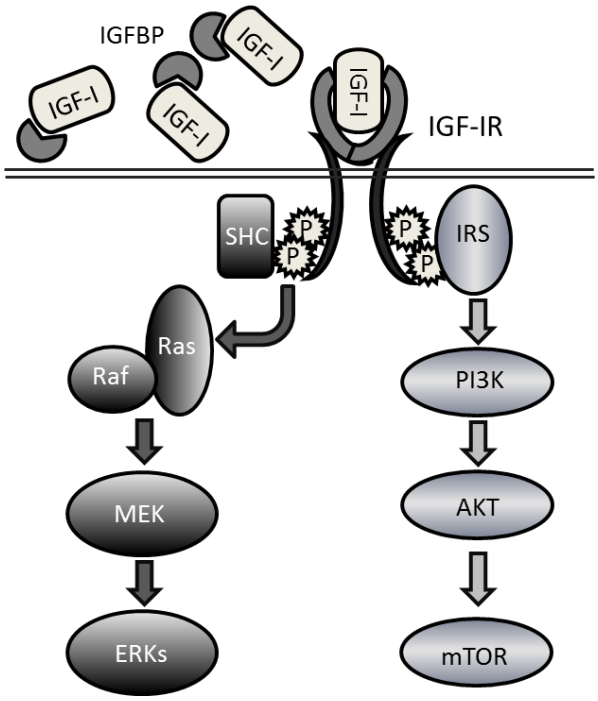


Fig. 1.5. IGF-I intracellular signaling pathways through IGF-IR: autophosphorylation of IGF-I receptor recruits SHC and IRS proteins binding to its intracellular domain. Phosphorylation of SHC leads to the activation of MEK/ERK pathway, and phosphorylation of IRS results in the activation of PI3K/AKT/mTOR pathway.

Somatomedin hypothesis

Both GH and IGF-I play an important role in development and growth during embryonic and postnatal periods. In the original somatomedin hypothesis, GH promotes somatic growth by stimulating synthesis and secretion of IGF-I in liver (Salmon and Daughaday, 1957). Liver-derived IGF-I is delivered to target organs in circulation system and promotes organ growth, such as bone and skeletal muscle, in an endocrine manner. The direct effects of GH and local production of IGF-I were not described at that time. Later studies detected GH receptor expression in multiple tissues, which suggested direct effects by GH on peripheral tissues

(Ballesteros et al., 2000). Expression of IGF-I was also detected in multiple tissues from the fetal mouse (D'Ercole et al., 1980; D'Ercole et al., 1984). In addition, mice with liver-specific IGF-I deletion grew normally (Sjogren et al., 1999; Yakar et al., 1999). These observations suggested that GH had both direct and indirect effects on growth and emphasized the importance of IGF-I paracrine/autocrine effects. Recently, Soloman et al. proposed modifications of the somatomedin hypothesis, because IGF-I counteracts the effects of GH on gluconeogenesis and lipolysis, but enhances the anabolic effects of GH by increasing protein synthesis and decreasing protein degradation (Kaplan and Cohen, 2007). In summary, it appears that whole body growth is controlled by both endocrine and autocrine/paracrine actions of GH and IGF-I.

Role of GH in skeletal muscle growth

Postnatal growth and development are more dependent on GH than prenatal stages. Studies indicate GH-deficient children with congenital absence of the pituitary or GHR deletions are born near normal in size (Gluckman et al., 1981). The effects of GH on skeletal muscle postnatal growth have been well studied in different systems *in vitro* and *in vivo*. Administration of GH to animals with low GH level leads to an increase in muscle mass (Guler et al., 1988). Blocking GH and GHR binding with antiserum to GH substantially reduced the weight, protein and RNA content of hind limb muscles in intact rats (Palmer et al., 1994). Similarly in humans, adults with GH deficiency showed reduction in skeletal muscle mass (Cuneo et al., 1990; Sartorio et al., 1995). Administration of recombinant human GH (rhGH) in GH-deficient patients successfully increased skeletal muscle mass at different ages. Several studies have reported beneficial effects of extended GH treatment on the musculature of GH-deficient humans (Beshyah et al., 1995; Sartorio and Narici, 1994).

The role of IGF gene expression in mediating the effects of GH on skeletal muscle growth is not clear. There is extensive evidence demonstrating that effects of GH on isolated skeletal muscle cells depend on IGF-I. It has been known for more than two decades that cultured myoblasts release IGFs (Hill et al., 1984). Administration of GH in hypophysectomized rats caused an increase in IGF-I mRNA content in the gastrocnemius muscle (Bates et al., 1993; Loughna et al., 1992). Other studies, however, showed that there was a lack of correlation between GH levels and muscle IGF-I mRNA levels (Coleman et al., 1994; Hannon et al., 1991; Pell et al., 1993).

Although there is evidence for IGF-I-independent effects of GH on muscle growth (Wang et al., 1999), most early studies using muscle cells gave negative results (Florini et al., 1996). GH directly stimulated glucose oxidation in cultured BC3HI cells (muscle cells), but GH had no effect on either growth or myogenesis (Adamafio et al., 1991). Direct injection of GH into the forearm muscle leads to increased myosin heavy chain mRNA and protein synthesis in humans (Fong et al., 1989; Fryburg et al., 1991). Still, these reports did not establish a direct effect of GH in stimulating the growth or differentiation of muscle cells. Phosphorylation of GH signal protein JAK2 and STAT5 in skeletal muscle is direct evidence for GH intracellular signal activation. Intravenous injection of GH in rat increased tyrosine phosphorylation of JAK2 and STAT5 (Chow et al., 1996). However, these studies did not exclude the possibility that the effects of GH in muscle is mediated by the induction of IGF-I. The most convincing evidence for direct effect of GH on myogenesis came from the study on mouse myoblasts. Administration of GH increased myogenesis, and this was associated with activation of GH signaling in myoblasts but without upregulating IGF-I expression (Sotiropoulos et al., 2006).

Role of IGF-I in skeletal muscle growth

Many growth factors play a major role in muscle development and growth (Allen et al., 1995; Boonen and Post, 2008; Charge and Rudnicki, 2004; Doumit et al., 1993; Grounds and Yablonka-Reuveni, 1993; Haugk et al., 1995; Hawke et al., 2001; Ratajczak et al., 2003; Robertson et al., 1993). In particular IGF-I is critical for skeletal muscle growth at both fetal and postnatal stages (Menetrey et al., 2000; Sato et al., 2003). This was confirmed by using transgenic mice demonstrating that overexpression of human IGF-I induces muscle hypertrophy (Adams, 1998), while blocking IGF-I signal in mouse skeletal muscle delayed proliferation and differentiation (Fernandez et al., 2002). In addition, IGF-I treatment improves regeneration after muscle injuries or diseases (Kasemkijwattana et al., 1998; Menetrey et al., 2000; Sato et al., 2003). By manipulating functional IGF-I receptors in myoblasts, the mechanisms by which IGF-I regulates myogenesis are revealed. Overexpression of IGF-I receptor in rodent skeletal muscle enhances proliferation and differentiation of myoblasts (Quinn et al., 1994). In vitro, IGF-I regulates the expression of MRFs to promote the proliferation and differentiation of satellite cell-derived myoblasts (Allen and Boxhorn, 1989; Charge and Rudnicki, 2004).

Distinct intracellular signaling pathways mediate the effects of IGF-I on myoblast proliferation and differentiation (Coolican et al., 1997). Some studies suggest that the MAPK pathway leads IGF-I to stimulate proliferation, and that the PI3K pathway mediates differentiation (Coolican et al., 1997; Jones et al., 2001; Singleton and Feldman, 2001; Tamir and Bengal, 2000). Other studies indicated that MAPKs are differentially regulated in proliferation and differentiation, and that p38 is critical for terminal differentiation (Garrington and Johnson, 1999; Gredinger et al., 1998; Wu et al., 2000; Zetser et al., 1999). There is no conclusion about the mechanism by which IGF-I switches myoblasts from proliferation to

differentiation. The proposed mechanism is the crosstalk between the two opposing pathways, depending on the different stages of muscle cells. Taken together, these studies establish IGF-I as an important regulator of mitogenesis and myogenesis of muscle cells.

In addition to proliferation and differentiation, IGF-I also plays a very important role in keeping the balance between anabolic and catabolic signals in muscle cells. Mice overexpressing IGF-I specifically in skeletal muscle develop increased muscle mass (Barton-Davis et al., 1998; Coleman et al., 1995; Musaro et al., 2001). Targeted disruption of IGF-I expression in the mouse muscle leads to a reduction of fiber size and muscle hypoplasia (Coleman et al., 1995; Fournier and Lewis, 2000). Under anabolic conditions, IGF-I leads to muscle hypertrophy by activating the PI3K/Akt pathway at the upstream of mTOR, a key target protein in protein synthesis (DeVol et al., 1990). Phosphorylated mTOR could further activate its target proteins, such as p70S6K. Genetic support for the linear style of Akt/mTOR/p70S6K pathway came from reports which demonstrated that rapamycin can inhibit the activity of mTOR target proteins (Inoki et al., 2002). This finding indicated that mTOR can inhibit p70S6K activity in protein synthesis. Glycogen synthase kinase 3 β (GSK3β) is another target protein at the downstream of Akt that has been shown to be a negative effector of muscle mass. Its activity is inhibited by Akt phosphorylation (Cross et al., 1995). Expression of a dominant negative GSK3β induces dramatic hypertrophy in myotubes (Rommel et al., 2001), as does pharmacologic inhibition of GSK3β (Vyas et al., 2001).

Under catabolic conditions, IGF-I attenuates protein degradation through the PI3K/AKT pathway in skeletal muscle atrophy as well (Svanberg et al., 2000). Overexpressing IGF-I in transgenic mice attenuates muscular atrophy by inhibiting the expression of two E3-ubiquitin ligases, muscle-specific atrogin-1 (MAFbx) and muscle RING-finger protein-1 (MuRF1). Both are involved in muscle atrophy and are related to activity of the ubiquitin-mediated proteasomal

degradation system (Sandri et al., 2004; Song et al., 2005). The mechanism by which IGF-I administration inhibited MAFbx and MuRF1 expression involved the PI3K/AKT activated forkhead transcription factor (FoxO) family in muscle cells (Lee et al., 2004; Sandri et al., 2004; Stitt et al., 2004). The FoxO family includes FOXO1, FOXO3A, and FOXO4. In myotubes, phosphorylated FoxO transcription factors are excluded from the nucleus which down-regulates the expression of target genes, including MAFbx and MuRF1. Inhibitors of PI3K can also block the expression of MAFbs and MuRF1, which confirmed the previous work demonstrating the predominant role for the PI3K/Akt/FOXO pathway in muscle atrophy.

Overall, studies of IGF-I in skeletal muscle showed that IGF-I is the main growth factor in myogenesis and regeneration. It is involved in multiple stages by stimulating distinct intracellular signals depending on muscle cell status. The corresponding IGF-I signals need to be specified depending on cell types, due to the observed discrepancy in different cultured muscle cells. During myogenesis, myoblast proliferation and differentiation are two mutually exclusive procedures both being promoted under IGF-I administration. The mechanisms by which IGF-I manages the switch from one to another still need to be clarified.

STAC Family Genes

There are three family members in the Src homology three and cysteine rich domain (STAC) gene family, including stac, stac2 and stac3. Kawai et al. first mapped the human stac gene to 3p24-p22 using PCR of a radiation hybrid panel (Kawai et al., 1998). The mouse stac gene was mapped by interspecific backcross analysis to the distal portion of chromosome 9, in a region syntenic with human chromosome 3p21. Stac gene is the founding member of this stac family. Computer searches using STAC protein sequence as a query revealed two additional stac proteins: stac2 and stac3. All stac family members are predicted to encode about 40 kDa proteins with sequence similarity close to 60% at amino acid level.

The predicted stac proteins contain two well recognized motifs: a Src homology three (SH3) domain and a cysteine rich (C1) domain. Those two major motifs found in this family are both characteristic of proteins associated with intracellular signal transduction cascades (Suzuki et al., 1996). Although neither of the two domains in stac3 protein has catalytic kinase activity, they are involved in several kinase pathways (Colon-Gonzalez and Kazanietz, 2006; Stahl et al., 1988). These structural motifs support the hypothesis that these proteins might act as adaptor proteins in cell signaling pathways.

The SH3 domain is a prevalent protein-protein interaction motif that was first identified as a conserved sequence in the viral adaptor protein Crk. It is also found in numerous other protein families, such as the Src family of tyrosine kinases, PI3K, phospholipase C and myosins (Geli et al., 2000; Goodson et al., 1996; Mayer and Gupta, 1998; Stahl et al., 1988). The SH3 domain is a 60 amino acid sequence characterized with a β-barrel fold which consists of five or six β-strands arranged as two tightly packed anti-parallel β sheets (Whisstock and Lesk, 1999). It

specifically binds to proline-rich peptide fragment at the nitrogen backbone featured with a consensus sequence as –X-P-p-X-P (Nguyen et al., 2000). The SH3 domains regulate the activity of adaptor proteins and other tyrosine kinases. It can increase the substrate specificity of some tyrosine kinases by binding the non-active domain of the kinase.

The C1 domain contains approximately 50 amino acids and primarily binds to the membrane bound second messenger diacyglcerol (DAG) and phorbol esters (Azzi et al., 1992). Phorbol esters have been implicated in tumor formation (Colon-Gonzalez and Kazanietz, 2006; Kazanietz et al., 1995; Ono et al., 1989; Quest et al., 1994). They are analogues of DAG and potent tumor promoters that cause a variety of physiological changes when administered to both cells and tissues. They can activate protein kinase C (PKC), which is a family of serine/threonine protein kinases (Ono et al., 1989). The N-terminal of PKC has a C1 domain and binds DAG dependent on phospholipid and zinc. The C1 domain found in the stac family is atypical based on amino acid sequence. The atypical C1 domain results from the non-cysteine amino acid changes causing structural modifications. These structural changes prevent the binding of substrates, but not the overall protein folding (Kazanietz et al., 1994). This atypical C1 domain makes it possible that this specific protein family does not directly bind DAG or phorbol ester. However, the atypical C1 domain has been shown to interact with G-proteins (Brtva et al., 1995). Therefore the stac protein family might act in a G-protein coupled receptor pathway.

The first two members, stac and stac2, are expressed predominantly in neurons. They are mutually exclusive markers for nociceptive peptidergicneurons and nonpeptidergic neurons, respectively, in the dorsal root ganglia neurons, suggesting that stac and stac2 might play important roles in signal transduction in neurons (Legha et al., 2010). However, no research has indicated the exact function of stac or stac2 in nervous tissue. The third member, stac3, has an

expression pattern different from stac and stac2. Stac3 is expressed exclusively in skeletal muscle (this dissertation). Still, no data regarding stac3 and its potential function in skeletal muscle has been reported.

Concluding Remarks

Skeletal muscle is the largest tissue in mammals. Its normal function is critical for body posture, movements, producing heat and breathing. The skeletal muscle size is determined by the number and size of myofibers. Myofiber features as a multi-nuclear muscle cell with peripheral nuclei and sarcomere protein structure at the center. The number of myofibers is determined during prenatal myogenesis. The size of myofibers is regulated during both prenatal and postnatal growth.

Satellite cells are the main resource to provide nuclear content during myofiber postnatal growth; they are activated and proliferate as myoblasts. After rounds of proliferation, myoblasts permanently withdraw from cell cycle to differentiate and fuse into existing myofibers to increase nuclear number. Myogenic regulating factors, including Myf5, MRF4, myogenin and MyoD, play important roles during this process. Their expression is up-regulated at different stages in a sequential manner, which guarantees the irreversible progress of myoblast proliferation and differentiation. In addition to nuclear number, the protein mass in myofibers is also dynamically regulated during postnatal growth. The balance between protein synthesis and degradation determines the protein mass.

Growth hormone and IGF-I are major hormonal regulators of postnatal skeletal muscle mass. GH is more important in postnatal growth and IGF-I critical for growth in both prenatal and postnatal stages. Although it has been long believed that liver-derived endocrine IGF-I mediates the growth promoting effect of GH, recent gene targeting studies suggest that GH stimulates muscle growth through either paracrine/autocrine IGF-I or IGF-I-independent mechanisms. IGF-I stimulates skeletal muscle growth by directly acting on muscle cells. The

growth-promoting effects of IGF-I on muscle cells include stimulating proliferation, differentiation, and increasing protein mass. Identifying the signaling pathways mediating the different effects of IGF-I on the same muscle cell has been an active area of research.

Although a lot of advances have been made in understanding the mechanisms by which GH and IGF-I stimulate skeletal muscle growth, many questions remain to be answered. Examples of these questions include whether GH stimulates skeletal muscle growth through locally produced IGF-I or IGF-I-independent mechanisms and how IGF-I exerts different effects on the same muscle cell. Given the unequivocal roles of GH and IGF-I in skeletal muscle growth, hypertrophy, and regeneration, addressing these and other related questions are critical to the development of strategies to enhance muscle regeneration and prevent atrophy in humans and to improve muscle efficiency in agricultural animals.

References

Adamafio, N. A., R. J. Towns, and J. L. Kostyo. 1991. Growth hormone receptors and action in BC3H-1 myocytes. Growth Regul 1: 17-22.

Adams, G. R. 1998. Role of insulin-like growth factor-I in the regulation of skeletal muscle adaptation to increased loading. Exerc Sport Sci Rev 26: 31-60.

Allen, R. E., and L. K. Boxhorn. 1989. Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. J Cell Physiol 138: 311-315.

Allen, R. E., S. M. Sheehan, R. G. Taylor, T. L. Kendall, and G. M. Rice. 1995. Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. J Cell Physiol 165: 307-312.

Andres, V., and K. Walsh. 1996. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J Cell Biol 132: 657-666.

Asakura, A., P. Seale, A. Girgis-Gabardo, and M. A. Rudnicki. 2002. Myogenic specification of side population cells in skeletal muscle. J Cell Biol 159: 123-134.

Azzi, A., D. Boscoboinik, and C. Hensey. 1992. The protein kinase C family. Eur J Biochem 208: 547-557.

Bajard, L. et al. 2006. A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. Genes Dev 20: 2450-2464.

Baker, J., J. P. Liu, E. J. Robertson, and A. Efstratiadis. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75: 73-82.

Ballesteros, M., K. C. Leung, R. J. Ross, T. P. Iismaa, and K. K. Ho. 2000. Distribution and abundance of messenger ribonucleic acid for growth hormone receptor isoforms in human tissues. J Clin Endocrinol Metab 85: 2865-2871.

Barton-Davis, E. R., D. I. Shoturma, A. Musaro, N. Rosenthal, and H. L. Sweeney. 1998. Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. Proc Natl Acad Sci U S A 95: 15603-15607.

Bates, P. C., P. T. Loughna, J. M. Pell, D. Schulster, and D. J. Millward. 1993. Interactions between growth hormone and nutrition in hypophysectomized rats: body composition and production of insulin-like growth factor-I. J Endocrinol 139: 117-126.

Baumann, G. 1995. Growth hormone binding to a circulating receptor fragment--the concept of receptor shedding and receptor splicing. Exp Clin Endocrinol Diabetes 103: 2-6.

Baumann, G., M. W. Stolar, K. Amburn, C. P. Barsano, and B. C. DeVries. 1986. A specific growth hormone-binding protein in human plasma: initial characterization. J Clin Endocrinol Metab 62: 134-141.

Baumbach, W. R., D. L. Horner, and J. S. Logan. 1989. The growth hormone-binding protein in rat serum is an alternatively spliced form of the rat growth hormone receptor. Genes Dev 3: 1199-1205.

Beauchamp, J. R. et al. 2000. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 151: 1221-1234.

Beshyah, S. A., P. Kyd, E. Thomas, A. Fairney, and D. G. Johnston. 1995. The effects of prolonged growth hormone replacement on bone metabolism and bone mineral density in hypopituitary adults. Clin Endocrinol (Oxf) 42: 249-254.

Bischoff, R., and C. Heintz. 1994. Enhancement of skeletal muscle regeneration. Dev Dyn 201: 41-54.

Bittner, R. E. et al. 1999. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. Anat Embryol (Berl) 199: 391-396.
Blair, H. T. et al. 1988. Genetic selection for insulin-like growth factor-1 in growing mice is associated with altered growth. Endocrinology 123: 1690-1692.
Blundell, T. L., S. Bedarkar, and R. E. Humbel. 1983. Tertiary structures, receptor binding, and antigenicity of insulinlike growth factors. Fed Proc 42: 2592-2597.
Bodine, S. C. 2006. mTOR signaling and the molecular adaptation to resistance exercise. Med Sci Sports Exerc 38: 1950-1957.
Bodine, S. C. et al. 2001a. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294: 1704-1708.
Bodine, S. C. et al. 2001b. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 3: 1014-1019.
Boldrin, L., and J. E. Morgan. 2007. Activating muscle stem cells: therapeutic potential in muscle diseases. Curr Opin Neurol 20: 577-582.
Boldrin, L., P. S. Zammit, F. Muntoni, and J. E. Morgan. 2009. Mature adult dystrophic mouse muscle environment does not impede efficient engrafted satellite cell regeneration and self-renewal. Stem Cells 27: 2478-2487.
Boonen, K. J., and M. J. Post. 2008. The muscle stem cell niche: regulation of satellite cells during regeneration. Tissue Eng Part B Rev 14: 419-431.
Brazeau, P. et al. 1973. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science 179: 77-79.
Brtva, T. R. et al. 1995. Two distinct Raf domains mediate interaction with Ras. J Biol Chem 270: 9809-9812.
Cai, D. et al. 2004. IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. Cell 119: 285-298.
Carlson, B. M. 1973. The regeneration of skeletal muscle. A review. Am J Anat 137: 119-149.
Carrera, A. C. 2004. TOR signaling in mammals. J Cell Sci 117: 4615-4616.
Carter-Su, C., J. Schwartz, and L. S. Smit. 1996. Molecular mechanism of growth hormone action. Annu Rev Physiol 58: 187-207.
Cenciarelli, C. et al. 1999. Critical role played by cyclin D3 in the MyoD-mediated arrest of cell cycle during myoblast differentiation. Mol Cell Biol 19: 5203-5217.
Charge, S. B., and M. A. Rudnicki. 2004. Cellular and molecular regulation of muscle regeneration. Physiol Rev 84: 209-238.
Chow, J. C. et al. 1996. Growth hormone stimulates tyrosine phosphorylation of JAK2 and STAT5, but not insulin receptor substrate-1 or SHC proteins in liver and skeletal muscle of normal rats in vivo. Endocrinology 137: 2880-2886.
Ciechanover, A. 2005. Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat Rev Mol Cell Biol 6: 79-87.
Coleman, M. E. et al. 1995. Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. J Biol Chem 270: 12109-12116.
Coleman, M. E., L. Russell, and T. D. Etherton. 1994. Porcine somatotropin (pST) increases IGF-I mRNA abundance in liver and subcutaneous adipose tissue but not in skeletal muscle of growing pigs. J Anim Sci 72: 918-924.
Collins, C. A. et al. 2005. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122: 289-301.

Colon-Gonzalez, F., and M. G. Kazanietz. 2006. C1 domains exposed: from diacylglycerol binding to protein-protein interactions. Biochim Biophys Acta 1761: 827-837.
Conboy, I. M., and T. A. Rando. 2002. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3: 397-409.
Coolican, S. A., D. S. Samuel, D. Z. Ewton, F. J. McWade, and J. R. Florini. 1997. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J Biol Chem 272: 6653-6662.
Cooper, R. N. et al. 1999. In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci 112 (Pt 17): 2895-2901.
Corbel, S. Y. et al. 2003. Contribution of hematopoietic stem cells to skeletal muscle. Nat Med 9: 1528-1532.
Cornelison, D. D., and B. J. Wold. 1997. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol 191: 270-283.
Cossu, G. 1997. Unorthodox myogenesis: possible developmental significance and implications for tissue histogenesis and regeneration. Histol Histopathol 12: 755-760.
Cossu, G. 2004. Fusion of bone marrow-derived stem cells with striated muscle may not be sufficient to activate muscle genes. J Clin Invest 114: 1540-1543.
Craparo, A., T. J. O'Neill, and T. A. Gustafson. 1995. Non-SH2 domains within insulin receptor substrate-1 and SHC mediate their phosphotyrosine-dependent interaction with the NPEY motif of the insulin-like growth factor I receptor. J Biol Chem 270: 15639-15643.
Cross, D. A., D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378: 785-789.
Cuneo, R. C., F. Salomon, C. M. Wiles, and P. H. Sonksen. 1990. Skeletal muscle performance in adults with growth hormone deficiency. Horm Res 33 Suppl 4: 55-60.
D'Ercole, A. J., G. T. Applewhite, and L. E. Underwood. 1980. Evidence that somatomedin is synthesized by multiple tissues in the fetus. Dev Biol 75: 315-328.
D'Ercole, A. J., A. D. Stiles, and L. E. Underwood. 1984. Tissue concentrations of somatomedin C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. Proc Natl Acad Sci U S A 81: 935-939.
D'Mello, S. R., K. Borodezt, and S. P. Soltoff. 1997. Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-1 signaling. J Neurosci 17: 1548-1560.
De Santa, F. et al. 2007. pRb-dependent cyclin D3 protein stabilization is required for myogenic differentiation. Mol Cell Biol 27: 7248-7265.
Dehoux, M. et al. 2007. IGF-I does not prevent myotube atrophy caused by proinflammatory cytokines despite activation of Akt/Foxo and GSK-3beta pathways and inhibition of atrogin-1 mRNA. Am J Physiol Endocrinol Metab 292: E145-150.
Dellavalle, A. et al. 2007. Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. Nat Cell Biol 9: 255-267.
DeVol, D. L., P. Rotwein, J. L. Sadow, J. Novakofski, and P. J. Bechtel. 1990. Activation of insulin-like growth factor gene expression during work-induced skeletal muscle growth. Am J Physiol 259: E89-95.
Doherty, K. R. et al. 2005. Normal myoblast fusion requires myoferlin. Development 132: 5565-5575.

Doumit, M. E., D. R. Cook, and R. A. Merkel. 1993. Fibroblast growth factor, epidermal growth factor, insulin-like growth factors, and platelet-derived growth factor-BB stimulate proliferation of clonally derived porcine myogenic satellite cells. J Cell Physiol 157: 326-332.

Duguez, S., M. Bartoli, and I. Richard. 2006. Calpain 3: a key regulator of the sarcomere? FEBS J 273: 3427-3436.

Emery, A. E. 2002. The muscular dystrophies. Lancet 359: 687-695.

Endo, T., and B. Nadal-Ginard. 1986. Transcriptional and posttranscriptional control of c-myc during myogenesis: its mRNA remains inducible in differentiated cells and does not suppress the differentiated phenotype. Mol Cell Biol 6: 1412-1421.

Fan, Y., M. Maley, M. Beilharz, and M. Grounds. 1996. Rapid death of injected myoblasts in myoblast transfer therapy. Muscle Nerve 19: 853-860.

Fernandez, A. M. et al. 2002. Muscle-specific inactivation of the IGF-I receptor induces compensatory hyperplasia in skeletal muscle. J Clin Invest 109: 347-355.

Ferrari, G. et al. 1998. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279: 1528-1530.

Fisker, S. et al. 2004. Gene expression of the GH receptor in subcutaneous and intraabdominal fat in healthy females: relationship to GH-binding protein. Eur J Endocrinol 150: 773-777.

Florini, J. R., D. Z. Ewton, and S. A. Coolican. 1996. Growth hormone and the insulin-like growth factor system in myogenesis. Endocr Rev 17: 481-517.

Fong, Y. et al. 1989. Recombinant growth hormone enhances muscle myosin heavy-chain mRNA accumulation and amino acid accrual in humans. Proc Natl Acad Sci U S A 86: 3371-3374.

Fournier, M., and M. I. Lewis. 2000. Influences of IGF-I gene disruption on the cellular profile of the diaphragm. Am J Physiol Endocrinol Metab 278: E707-715.

Fryburg, D. A., R. A. Gelfand, and E. J. Barrett. 1991. Growth hormone acutely stimulates forearm muscle protein synthesis in normal humans. Am J Physiol 260: E499-504.

Fuchtbauer, E. M., and H. Westphal. 1992. MyoD and myogenin are coexpressed in regenerating skeletal muscle of the mouse. Dev Dyn 193: 34-39.

Galvez, B. G. et al. 2006. Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. J Cell Biol 174: 231-243.

Garrington, T. P., and G. L. Johnson. 1999. Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr Opin Cell Biol 11: 211-218.

Geli, M. I., R. Lombardi, B. Schmelzl, and H. Riezman. 2000. An intact SH3 domain is required for myosin I-induced actin polymerization. EMBO J 19: 4281-4291.

Gibson, M. C., and E. Schultz. 1983. Age-related differences in absolute numbers of skeletal muscle satellite cells. Muscle Nerve 6: 574-580.

Glass, D. J. 2005a. A signaling role for dystrophin: inhibiting skeletal muscle atrophy pathways. Cancer Cell 8: 351-352.

Glass, D. J. 2005b. Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol 37: 1974-1984.

Glickman, M. H., and A. Ciechanover. 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 82: 373-428.

Gluckman, P. D., M. M. Grumbach, and S. L. Kaplan. 1981. The neuroendocrine regulation and function of growth hormone and prolactin in the mammalian fetus. Endocr Rev 2: 363-395.

Gomes, M. D., S. H. Lecker, R. T. Jagoe, A. Navon, and A. L. Goldberg. 2001. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proc Natl Acad Sci U S A 98: 14440-14445.

Goodson, H. V., B. L. Anderson, H. M. Warrick, L. A. Pon, and J. A. Spudich. 1996. Synthetic lethality screen identifies a novel yeast myosin I gene (MYO5): myosin I proteins are required for polarization of the actin cytoskeleton. J Cell Biol 133: 1277-1291.

Gourmelen, M., Y. Le Bouc, F. Girard, and M. Binoux. 1984. Serum levels of insulin-like growth factor (IGF) and IGF binding protein in constitutionally tall children and adolescents. J Clin Endocrinol Metab 59: 1197-1203.

Gredinger, E., A. N. Gerber, Y. Tamir, S. J. Tapscott, and E. Bengal. 1998. Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells. J Biol Chem 273: 10436-10444.

Gros, J., M. Manceau, V. Thome, and C. Marcelle. 2005. A common somitic origin for embryonic muscle progenitors and satellite cells. Nature 435: 954-958.

Grounds, M. D., K. L. Garrett, M. C. Lai, W. E. Wright, and M. W. Beilharz. 1992. Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. Cell Tissue Res 267: 99-104.

Grounds, M. D., and Z. Yablonka-Reuveni. 1993. Molecular and cell biology of skeletal muscle regeneration. Mol Cell Biol Hum Dis Ser 3: 210-256.

Gu, M. B., P. Todd, and D. S. Kompala. 1993. Foreign gene expression (beta-galactosidase) during the cell cycle phases in recombinant CHO cells. Biotechnol Bioeng 42: 1113-1123.

Guerette, B. et al. 1997. Prevention by anti-LFA-1 of acute myoblast death following transplantation. J Immunol 159: 2522-2531.

Guler, H. P., J. Zapf, E. Scheiwiller, and E. R. Froesch. 1988. Recombinant human insulin-like growth factor I stimulates growth and has distinct effects on organ size in hypophysectomized rats. Proc Natl Acad Sci U S A 85: 4889-4893.

Guo, K., and K. Walsh. 1997. Inhibition of myogenesis by multiple cyclin-Cdk complexes. Coordinate regulation of myogenesis and cell cycle activity at the level of E2F. J Biol Chem 272: 791-797.

Gussoni, E., H. M. Blau, and L. M. Kunkel. 1997. The fate of individual myoblasts after transplantation into muscles of DMD patients. Nat Med 3: 970-977.

Guttridge, D. C. 2004. Signaling pathways weigh in on decisions to make or break skeletal muscle. Curr Opin Clin Nutr Metab Care 7: 443-450.

Halevy, O. et al. 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science 267: 1018-1021.

Han, V. K., P. K. Lund, D. C. Lee, and A. J. D'Ercole. 1988. Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization, and tissue distribution. J Clin Endocrinol Metab 66: 422-429.

Hannon, K., A. Gronowski, and A. Trenkle. 1991. Relationship of liver and skeletal muscle IGF-1 mRNA to plasma GH profile, production of IGF-1 by liver, plasma IGF-1 concentrations, and growth rates of cattle. Proc Soc Exp Biol Med 196: 155-163.

Hasty, P. et al. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364: 501-506.

Haugk, K. L., R. A. Roeder, M. J. Garber, and G. T. Schelling. 1995. Regulation of muscle cell proliferation by extracts from crushed muscle. J Anim Sci 73: 1972-1981.

Hawke, T. J., S. Lessard, L. Vickery, S. L. Lipskie, and M. I. Lindinger. 2001. Ouabain stimulates unidirectional and net potassium efflux in resting mammalian skeletal muscle. Can J Physiol Pharmacol 79: 932-941.

He, K. et al. 2003. Janus kinase 2 determinants for growth hormone receptor association, surface assembly, and signaling. Mol Endocrinol 17: 2211-2227.

Hill, D. J., C. J. Crace, L. Fowler, A. T. Holder, and R. D. Milner. 1984. Cultured fetal rat myoblasts release peptide growth factors which are immunologically and biologically similar to somatomedin. J Cell Physiol 119: 349-358.

Horsley, V. et al. 2001. Regulation of the growth of multinucleated muscle cells by an NFATC2-dependent pathway. J Cell Biol 153: 329-338.

Horsley, V., K. M. Jansen, S. T. Mills, and G. K. Pavlath. 2003. IL-4 acts as a myoblast recruitment factor during mammalian muscle growth. Cell 113: 483-494.

Horsley, V., and G. K. Pavlath. 2004. Forming a multinucleated cell: molecules that regulate myoblast fusion. Cells Tissues Organs 176: 67-78.

Huard, J., L. P. Fortier, G. Dansereau, C. Labrecque, and J. P. Tremblay. 1992. A light and electron microscopic study of dystrophin localization at the mouse neuromuscular junction. Synapse 10: 83-93.

Inoki, K., Y. Li, T. Zhu, J. Wu, and K. L. Guan. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 4: 648-657.

Jones, N. C., Y. V. Fedorov, R. S. Rosenthal, and B. B. Olwin. 2001. ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. J Cell Physiol 186: 104-115.

Kaplan, S. A., and P. Cohen. 2007. The somatomedin hypothesis 2007: 50 years later. J Clin Endocrinol Metab 92: 4529-4535.

Kasemkijwattana, C. et al. 1998. Development of approaches to improve the healing following muscle contusion. Cell Transplant 7: 585-598.

Kassar-Duchossoy, L. et al. 2004. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature 431: 466-471.

Kawai, J., H. Suzuki, A. Hara, K. Hirose, and S. Watanabe. 1998. Human and mouse chromosomal mapping of Stac, a neuron-specific protein with an SH3 domain. Genomics 47: 140-142.

Kazanietz, M. G. et al. 1994. Zinc finger domains and phorbol ester pharmacophore. Analysis of binding to mutated form of protein kinase C zeta and the vav and c-raf proto-oncogene products. J Biol Chem 269: 11590-11594.

Kazanietz, M. G. et al. 1995. Residues in the second cysteine-rich region of protein kinase C delta relevant to phorbol ester binding as revealed by site-directed mutagenesis. J Biol Chem 270: 21852-21859.

Kiess, M., R. M. Gill, and P. A. Hamel. 1995. Expression of the positive regulator of cell cycle progression, cyclin D3, is induced during differentiation of myoblasts into quiescent myotubes. Oncogene 10: 159-166.

Kitzmann, M. et al. 1999. cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity. Mol Cell Biol 19: 3167-3176.

Kramerova, I., E. Kudryashova, G. Venkatraman, and M. J. Spencer. 2005. Calpain 3 participates in sarcomere remodeling by acting upstream of the ubiquitin-proteasome pathway. Hum Mol Genet 14: 2125-2134.

Kramerova, I., E. Kudryashova, B. Wu, and M. J. Spencer. 2006. Regulation of the M-cadherin-beta-catenin complex by calpain 3 during terminal stages of myogenic differentiation. Mol Cell Biol 26: 8437-8447.

LaBarge, M. A., and H. M. Blau. 2002. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell 111: 589-601.

Le Roith, D., C. Bondy, S. Yakar, J. L. Liu, and A. Butler. 2001. The somatomedin hypothesis: 2001. Endocr Rev 22: 53-74.

Lee, S. W. et al. 2004. Regulation of muscle protein degradation: coordinated control of apoptotic and ubiquitin-proteasome systems by phosphatidylinositol 3 kinase. J Am Soc Nephrol 15: 1537-1545.

Legha, W. et al. 2010. stac1 and stac2 genes define discrete and distinct subsets of dorsal root ganglia neurons. Gene Expr Patterns 10: 368-375.

LeRoith, D., H. Werner, D. Beitner-Johnson, and C. T. Roberts, Jr. 1995. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 16: 143-163.

Leung, D. W. et al. 1987. Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature 330: 537-543.

Leung, K. C., N. Doyle, M. Ballesteros, M. J. Waters, and K. K. Ho. 2000. Insulin regulation of human hepatic growth hormone receptors: divergent effects on biosynthesis and surface translocation. J Clin Endocrinol Metab 85: 4712-4720.

Li, L., and E. N. Olson. 1992. Regulation of muscle cell growth and differentiation by the MyoD family of helix-loop-helix proteins. Adv Cancer Res 58: 95-119.

Ling, N. et al. 1984. Isolation, primary structure, and synthesis of human hypothalamic somatocrinin: growth hormone-releasing factor. Proc Natl Acad Sci U S A 81: 4302-4306.

Liu, J. L. et al. 1998. Insulin-like growth factor-I affects perinatal lethality and postnatal development in a gene dosage-dependent manner: manipulation using the Cre/loxP system in transgenic mice. Mol Endocrinol 12: 1452-1462.

Liu, J. P., J. Baker, A. S. Perkins, E. J. Robertson, and A. Efstratiadis. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75: 59-72.

Loughna, P. T., P. Mason, and P. C. Bates. 1992. Regulation of insulin-like growth factor 1 gene expression in skeletal muscle. Symp Soc Exp Biol 46: 319-330.

Lowe, W. L., Jr., S. R. Lasky, D. LeRoith, and C. T. Roberts, Jr. 1988. Distribution and regulation of rat insulin-like growth factor I messenger ribonucleic acids encoding alternative carboxyterminal E-peptides: evidence for differential processing and regulation in liver. Mol Endocrinol 2: 528-535.

Macconnachie, H. F., M. Enesco, and C. P. Leblond. 1964. The Mode of Increase in the Number of Skeletal Muscle Nuclei in the Postnatal Rat. Am J Anat 114: 245-253.

Macharia, R., A. Otto, P. Valasek, and K. Patel. 2010. Neuromuscular junction morphology, fiber-type proportions, and satellite-cell proliferation rates are altered in MyoD(-/-) mice. Muscle Nerve 42: 38-52.

Mammucari, C. et al. 2007. FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab 6: 458-471.

Maroto, M. et al. 1997. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell 89: 139-148.

Mathews, L. S. et al. 1988. Growth enhancement of transgenic mice expressing human insulin-like growth factor I. Endocrinology 123: 2827-2833.
Mayer, B. J., and R. Gupta. 1998. Functions of SH2 and SH3 domains. Curr Top Microbiol Immunol 228: 1-22.
Megeney, L. A., B. Kablar, K. Garrett, J. E. Anderson, and M. A. Rudnicki. 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev 10: 1173-1183.
Menetrey, J. et al. 2000. Growth factors improve muscle healing in vivo. J Bone Joint Surg Br 82: 131-137.
Molitch, M. E. et al. 2006. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab 91: 1621-1634.
Montarras, D. et al. 2005. Direct isolation of satellite cells for skeletal muscle regeneration. Science 309: 2064-2067.
Mourkioti, F. et al. 2006. Targeted ablation of IKK2 improves skeletal muscle strength, maintains mass, and promotes regeneration. J Clin Invest 116: 2945-2954.
Mourkioti, F., and N. Rosenthal. 2005. IGF-1, inflammation and stem cells: interactions during muscle regeneration. Trends Immunol 26: 535-542.
Musaro, A. et al. 2001. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 27: 195-200.
Negroni, E. et al. 2009. In vivo myogenic potential of human CD133+ muscle-derived stem cells: a quantitative study. Mol Ther 17: 1771-1778.
Nguyen, J. T. et al. 2000. Improving SH3 domain ligand selectivity using a non-natural scaffold. Chem Biol 7: 463-473.
Nigg, E. A. 1995. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. Bioessays 17: 471-480.
Olguin, H. C., and B. B. Olwin. 2004. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol 275: 375-388.
Ono, Y. et al. 1989. Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. Proc Natl Acad Sci U S A 86: 4868-4871.
Palmer, R. M. et al. 1994. Effects of a polyclonal antiserum to rat growth hormone on circulating insulin-like growth factor (IGF)-I and IGF-binding protein concentrations and the growth of muscle and bone. J Endocrinol 142: 85-91.
Parrish, E. P. et al. 1996. Targeting widespread sites of damage in dystrophic muscle: engrafted macrophages as potential shuttles. Gene Ther 3: 13-20.
Partridge, T. A., J. E. Morgan, G. R. Coulton, E. P. Hoffman, and L. M. Kunkel. 1989. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. Nature 337: 176-179.
Patel, K., B. Christ, and F. E. Stockdale. 2002. Control of muscle size during embryonic, fetal, and adult life. Results Probl Cell Differ 38: 163-186.
Peault, B. et al. 2007. Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Mol Ther 15: 867-877.
Pell, J. M., J. C. Saunders, and R. S. Gilmour. 1993. Differential regulation of transcription initiation from insulin-like growth factor-I (IGF-I) leader exons and of tissue IGF-I expression in response to changed growth hormone and nutritional status in sheep. Endocrinology 132: 1797-1807.

Pownall, M. E., M. K. Gustafsson, and C. P. Emerson, Jr. 2002. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu Rev Cell Dev Biol 18: 747-783.

Price, H. M., E. L. Howes, Jr., and J. M. Blumberg. 1964. Ultrastructural Alterations in Skeletal Muscle Fibers Injured by Cold. Ii. Cells on the Sarcolemmal Tube: Observations on "Discontinuous" Regeneration and Myofibril Formation. Lab Invest 13: 1279-1302.

Qu-Petersen, Z. et al. 2002. Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. J Cell Biol 157: 851-864.

Quest, A. F., E. S. Bardes, and R. M. Bell. 1994. A phorbol ester binding domain of protein kinase C gamma. Deletion analysis of the Cys2 domain defines a minimal 43-amino acid peptide. J Biol Chem 269: 2961-2970.

Quinn, L. S., B. Steinmetz, A. Maas, L. Ong, and M. Kaleko. 1994. Type-1 insulin-like growth factor receptor overexpression produces dual effects on myoblast proliferation and differentiation. J Cell Physiol 159: 387-398.

Rao, S. S., C. Chu, and D. S. Kohtz. 1994. Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators. Mol Cell Biol 14: 5259-5267.

Ratajczak, M. Z. et al. 2003. Expression of functional CXCR4 by muscle satellite cells and secretion of SDF-1 by muscle-derived fibroblasts is associated with the presence of both muscle progenitors in bone marrow and hematopoietic stem/progenitor cells in muscles. Stem Cells 21: 363-371.

Rechler, M. M. 1993. Insulin-like growth factor binding proteins. Vitam Horm 47: 1-114.

Reinstein, E., and A. Ciechanover. 2006. Narrative review: protein degradation and human diseases: the ubiquitin connection. Ann Intern Med 145: 676-684.

Rinderknecht, E., and R. E. Humbel. 1978. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 253: 2769-2776.

Robertson, T. A., J. M. Papadimitriou, and M. D. Grounds. 1993. Fusion of myogenic cells to the newly sealed region of damaged myofibres in skeletal muscle regeneration. Neuropathol Appl Neurobiol 19: 350-358.

Rommel, C. et al. 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 3: 1009-1013.

Rudnicki, M. A. et al. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75: 1351-1359.

Sacco, A., R. Doyonnas, P. Kraft, S. Vitorovic, and H. M. Blau. 2008. Self-renewal and expansion of single transplanted muscle stem cells. Nature 456: 502-506.

Sakamoto, K., D. E. Arnolds, I. Ekberg, A. Thorell, and L. J. Goodyear. 2004. Exercise regulates Akt and glycogen synthase kinase-3 activities in human skeletal muscle. Biochem Biophys Res Commun 319: 419-425.

Salisbury, E. et al. 2008. Ectopic expression of cyclin D3 corrects differentiation of DM1 myoblasts through activation of RNA CUG-binding protein, CUGBP1. Exp Cell Res 314: 2266-2278.

Salleo, A., G. La Spada, G. Falzea, M. G. Denaro, and R. Cicciarello. 1983. Response of satellite cells and muscle fibers to long-term compensatory hypertrophy. J Submicrosc Cytol 15: 929-940.

Salmon, W. D., Jr., and W. H. Daughaday. 1957. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J Lab Clin Med 49: 825-836.

Sampaolesi, M. et al. 2006. Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. Nature 444: 574-579.
Sandhu, C., and J. Slingerland. 2000. Deregulation of the cell cycle in cancer. Cancer Detect Prev 24: 107-118.
Sandri, M. 2008. Signaling in muscle atrophy and hypertrophy. Physiology (Bethesda) 23: 160-170.
Sandri, M. et al. 2004. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 117: 399-412.
Sartorio, A., A. Conti, S. Ferrero, A. Spada, and G. Faglia. 1995. GH responsiveness to repeated GHRH or hexarelin administration in normal adults. J Endocrinol Invest 18: 718-722.
Sartorio, A., and M. V. Narici. 1994. Growth hormone (GH) treatment in GH-deficient adults: effects on muscle size, strength and neural activation. Clin Physiol 14: 527-537.
Sasaki, N., R. W. Rees-Jones, Y. Zick, S. P. Nissley, and M. M. Rechler. 1985. Characterization of insulin-like growth factor I-stimulated tyrosine kinase activity associated with the beta-subunit of type I insulin-like growth factor receptors of rat liver cells. J Biol Chem 260: 9793-9804.
Sato, K. et al. 2003. Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis. Muscle Nerve 28: 365-372.
Sato, T., D. Rocancourt, L. Marques, S. Thorsteinsdottir, and M. Buckingham. 2010. A Pax3/Dmrt2/Myf5 regulatory cascade functions at the onset of myogenesis. PLoS Genet 6: e1000897.
Schiaffino, S., S. P. Bormioli, and M. Aloisi. 1972. Cell proliferation in rat skeletal muscle during early stages of compensatory hypertrophy. Virchows Arch B Cell Pathol 11: 268-273.
Shafiq, S. A., and M. A. Gorycki. 1965. Regeneration in skeletal muscle of mouse: some electron-microscope observations. J Pathol Bacteriol 90: 123-127.
Shafiq, S. A., M. A. Gorycki, and A. Mauro. 1968. Mitosis during postnatal growth in skeletal and cardiac muscle of the rat. J Anat 103: 135-141.
Sherr, C. J. 1995. Mammalian G1 cyclins and cell cycle progression. Proc Assoc Am Physicians 107: 181-186.
Singleton, J. R., and E. L. Feldman. 2001. Insulin-like growth factor-I in muscle metabolism and myotherapies. Neurobiol Dis 8: 541-554.
Sjogren, K. et al. 1999. Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. Proc Natl Acad Sci U S A 96: 7088-7092.
Skapek, S. X., J. Rhee, D. B. Spicer, and A. B. Lassar. 1995. Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. Science 267: 1022-1024.
Smith, C. K., 2nd, M. J. Janney, and R. E. Allen. 1994. Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. J Cell Physiol 159: 379-385.
Smith, G. D., D. Gunnell, and J. Holly. 2000. Cancer and insulin-like growth factor-I. A potential mechanism linking the environment with cancer risk. BMJ 321: 847-848.
Song, A., Q. Wang, M. G. Goebl, and M. A. Harrington. 1998. Phosphorylation of nuclear MyoD is required for its rapid degradation. Mol Cell Biol 18: 4994-4999.

Song, B. L., N. Sever, and R. A. DeBose-Boyd. 2005. Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. Mol Cell 19: 829-840.

Sotiropoulos, A. et al. 2006. Growth hormone promotes skeletal muscle cell fusion independent of insulin-like growth factor 1 up-regulation. Proc Natl Acad Sci U S A 103: 7315-7320.

Spate, U., and P. C. Schulze. 2004. Proinflammatory cytokines and skeletal muscle. Curr Opin Clin Nutr Metab Care 7: 265-269.

Spiess, J., J. Rivier, and W. Vale. 1983. Characterization of rat hypothalamic growth hormone-releasing factor. Nature 303: 532-535.

Stahl, M. L., C. R. Ferenz, K. L. Kelleher, R. W. Kriz, and J. L. Knopf. 1988. Sequence similarity of phospholipase C with the non-catalytic region of src. Nature 332: 269-272.

Steele-Perkins, G. et al. 1988. Expression and characterization of a functional human insulin-like growth factor I receptor. J Biol Chem 263: 11486-11492.

Stitt, T. N. et al. 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 14: 395-403.

Suzuki, H. et al. 1996. Stac, a novel neuron-specific protein with cysteine-rich and SH3 domains. Biochem Biophys Res Commun 229: 902-909.

Svanberg, E. et al. 2000. IGF-I/IGFBP-3 binary complex modulates sepsis-induced inhibition of protein synthesis in skeletal muscle. Am J Physiol Endocrinol Metab 279: E1145-1158.

Takeda, K., and S. Akira. 2000. STAT family of transcription factors in cytokine-mediated biological responses. Cytokine Growth Factor Rev 11: 199-207.

Tamir, Y., and E. Bengal. 2000. Phosphoinositide 3-kinase induces the transcriptional activity of MEF2 proteins during muscle differentiation. J Biol Chem 275: 34424-34432.

Tartare-Deckert, S., D. Sawka-Verhelle, J. Murdaca, and E. Van Obberghen. 1995. Evidence for a differential interaction of SHC and the insulin receptor substrate-1 (IRS-1) with the insulin-like growth factor-I (IGF-I) receptor in the yeast two-hybrid system. J Biol Chem 270: 23456-23460.

Torrente, Y. et al. 2004. Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. J Clin Invest 114: 182-195.

Uezumi, A. et al. 2006. Functional heterogeneity of side population cells in skeletal muscle. Biochem Biophys Res Commun 341: 864-873.

Velcheti, V., and R. Govindan. 2006. Insulin-like growth factor and lung cancer. J Thorac Oncol 1: 607-610.

Vyas, V. K., S. Kuchin, and M. Carlson. 2001. Interaction of the repressors Nrg1 and Nrg2 with the Snf1 protein kinase in Saccharomyces cerevisiae. Genetics 158: 563-572.

Walsh, K., and H. Perlman. 1997. Cell cycle exit upon myogenic differentiation. Curr Opin Genet Dev 7: 597-602.

Wang, J., J. Zhou, and C. A. Bondy. 1999. Igf1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. FASEB J 13: 1985-1990.

Wang, Z., R. W. Hipkin, and M. Ascoli. 1996. Progressive cytoplasmic tail truncations of the lutropin-choriogonadotropin receptor prevent agonist- or phorbol ester-induced phosphorylation, impair agonist- or phorbol ester-induced desensitization, and enhance agonist-induced receptor down-regulation. Mol Endocrinol 10: 748-759.

Waters, M. J. et al. 1999. Growth hormone as a cytokine. Clin Exp Pharmacol Physiol 26: 760-764.

Wei, Y., Z. Rhani, and C. G. Goodyer. 2006. Characterization of growth hormone receptor messenger ribonucleic acid variants in human adipocytes. J Clin Endocrinol Metab 91: 1901-1908.

Weintraub, H. et al. 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. Science 251: 761-766.

Whisstock, J. C., and A. M. Lesk. 1999. SH3 domains in prokaryotes. Trends Biochem Sci 24: 132-133.

White, J. D. et al. 2000. Myotube formation is delayed but not prevented in MyoD-deficient skeletal muscle: studies in regenerating whole muscle grafts of adult mice. J Histochem Cytochem 48: 1531-1544.

Winter, B., T. Braun, and H. H. Arnold. 1993. cAMP-dependent protein kinase represses myogenic differentiation and the activity of the muscle-specific helix-loop-helix transcription factors Myf-5 and MyoD. J Biol Chem 268: 9869-9878.

Wong, E. A., S. M. Ohlsen, J. A. Godfredson, D. M. Dean, and J. E. Wheaton. 1989. Cloning of ovine insulin-like growth factor-I cDNAs: heterogeneity in the mRNA population. DNA 8: 649-657.

Woods, K. A., C. Camacho-Hubner, M. O. Savage, and A. J. Clark. 1996. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. N Engl J Med 335: 1363-1367.

Wu, X. et al. 2006. Growth hormone receptor overexpression predicts response of rectal cancers to pre-operative radiotherapy. Eur J Cancer 42: 888-894.

Wu, Z. et al. 2000. p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. Mol Cell Biol 20: 3951-3964.

Yablonka-Reuveni, Z., and A. J. Rivera. 1994. Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. Dev Biol 164: 588-603.

Yakar, S. et al. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci U S A 96: 7324-7329.

Zammit, P. S. et al. 2004. Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol 166: 347-357.

Zetser, A., E. Gredinger, and E. Bengal. 1999. p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the Mef2c transcription factor. J Biol Chem 274: 5193-5200.

Zhang, J. M., Q. Wei, X. Zhao, and B. M. Paterson. 1999. Coupling of the cell cycle and myogenesis through the cyclin D1-dependent interaction of MyoD with cdk4. EMBO J 18: 926-933.

Zhu, T., E. L. Goh, R. Graichen, L. Ling, and P. E. Lobie. 2001. Signal transduction via the growth hormone receptor. Cell Signal 13: 599-616.

Chapter II

Growth hormone stimulates protein synthesis in bovine skeletal muscle cells without altering insulin-like growth factor-I mRNA expression

ABSTRACT

Growth hormone is a major stimulator of skeletal muscle growth in animals, including cattle. In this study, we determined whether GH stimulates skeletal muscle growth in cattle by direct stimulation of proliferation or fusion of myoblasts, by direct stimulation of protein synthesis, or by direct inhibition of protein degradation in myotubes. We also determined whether these direct effects of GH are mediated by IGF-I produced by myoblasts or myotubes. Satellite cells were isolated from cattle skeletal muscle and were allowed to proliferate as myoblasts or induced to fuse into myotubes in culture. Growth hormone at 10 and 100 ng/mL increased protein synthesis in myotubes ($P < 0.05$), but had no effect on protein degradation in myotubes or proliferation of myoblasts ($P > 0.05$). Insulin like growth factor I at 50 and 500 ng/mL stimulated protein synthesis ($P < 0.01$), and this effect of IGF-I was much greater than that of GH ($P < 0.05$). Besides stimulating protein synthesis, IGF-I at 50 and 500 ng/mL also inhibited protein degradation in myotubes ($P < 0.01$), and IGF-I at 500 ng/mL stimulated proliferation of myoblasts ($P < 0.05$). Neither GH nor IGF-I had effects on fusion of myoblasts into myotubes ($P > 0.1$). These data indicate that GH and IGF-I have largely different direct effects on bovine muscle cells. Growth hormone at 10 and 100 ng/mL had no effect on *IGF-I* mRNA expression in either myoblasts or myotubes ($P > 0.1$). This lack of effect was not because the cultured myoblasts or myotubes were not responsive to GH, as *GHR* mRNA was detectable in them and the expression of the cytokine-inducible SH2-containing protein (*CISH*) gene, a

well-established GH target gene, was increased by GH in bovine myoblasts ($P < 0.05$). Overall, the data suggest that GH stimulates skeletal muscle growth in cattle in part through stimulation of protein synthesis in the muscle and that this stimulation is not mediated through increased *IGF-I* mRNA expression in the muscle.

Keywords: cattle, growth hormone, insulin-like growth factor I, muscle

INTRODUCTION

Growth hormone is a major regulator of animal growth and metabolism (Etherton and Bauman, 1998). At the cellular level, the action of GH starts with binding to the GH receptors (**GHR**), and binding of GH to GHR leads to the activation of multiple intracellular signaling pathways (Herrington et al., 2000; Piwien-Pilipuk et al., 2002), of which the Janus kinase 2 (**JAK2**)-signal transducer and the activator of transcription 5 (**STAT5**) pathway is responsible for GH regulation of many genes, including *IGF-I* (Eleswarapu et al., 2008; Wang and Jiang, 2005), *GHR* (Jiang et al., 2007), and *cytokine-inducible SH2-containing protein* gene (*CISH*; (Sadowski et al., 2001; Tollet-Egnell et al., 1999).

Muscle growth in animals can result from an increased number of nuclei in the multinucleated myofiber, increased cytoplasmic volume of the myofiber, or both. In postnatal animals, new nuclei for myofibers are provided by satellite cells, the muscle stem cells (Rhoads et al., 2009; Yablonka-Reuveni et al., 2008; Zammit et al., 2006). The cytoplasmic volume of a myofiber is determined by protein synthesis and protein degradation. The mechanism by which GH stimulates skeletal muscle growth in postnatal animals is not fully understood. There has been much controversy about the relative contribution of circulating IGF-I, locally produced IGF-I, and IGF-I-independent factors to the effect of GH on muscle growth in the last 10 yr (Klover and Hennighausen, 2007; Ohlsson et al., 2009; Stratikopoulos et al., 2008; Wu et al., 2009; Yakar et al., 1999).

The objective of this study was to determine whether GH stimulates skeletal muscle growth in cattle by stimulating myoblast proliferation, myoblast fusion, or protein synthesis in myotubes or by inhibiting protein degradation in myotubes. We also determined the effect of GH

on *IGF-I* mRNA expression in bovine myoblasts and myotubes.

MATERIALS AND METHODS

Isolation and Culturing of Bovine Satellite Cells

Bovine satellite cells were isolated from 5 cattle according to the published procedures (Burton et al., 2000; Kamanga-Sollo et al., 2004; Kamanga-Sollo et al., 2008). Briefly, extensor carpi radialis muscle, which was easy to collect, was removed from adult cattle (mostly crossbred Angus steers) at a local abattoir and was transported to the laboratory in sterile PBS on ice within 30 min of collection. Following removal of the adipose and connective tissues, the muscle was minced into small pieces and ground with a meat grinder. The muscle was then digested in PBS containing 1 mg/mL of Pronase (Calbiochem, San Diego, CA) at 37 ℃ for 40 min. The digested muscle was centrifuged for 4 min at 1,500 × *g* at room temperature. The pellet was washed with PBS followed by centrifugation for 6 min at 1,500 × *g* at room temperature. This washing procedure was repeated 3 times to enrich satellite cells.

The isolated satellite cells were allowed to proliferate as myoblasts in growth medium composed of Dulbecco's Modified Eagle Medium (**DMEM**; Mediatech, Manassas, VA), 10% fetal bovine serum (**FBS**; Atlanta Biologicals, Lawrenceville, GA), and 1% antibiotics-antimycotics (**ABAM**; Mediatech, Manassas, VA) in plates pre-coated with reduced growth factor basement membrane Matrigel (BD, Franklin Lakes, NJ). We verified the purity of the isolated satellite cells by determining the percentage of MyoD-positive cells, i.e., myogenic cells, using immunocytochemistry (described below). Most of our isolations of satellite cells had a percentage of MyoD-positive cells of at least 80%. The isolations used in subsequent experiments had at least 80% MyoD-positive cells.

To generate myotubes, the medium of myoblasts at confluence was replaced by

differentiation medium composed of DMEM, 2% horse serum (**HS**; Atlanta Biologicals), and 1% ABAM. Based on immunocytochemistry (described below), approximately 70% of the nuclei were in multinucleated (≥ 3 nuclei) myotubes by day 3 of differentiation.

Immunocytochemistry

Myoblasts or myotubes were fixed with 4% paraformaldehyde (**PFA**) in PBS at room temperature for 10 min, followed by washing twice with ice-cold PBS for 5 min. The cell membrane was permeated with 0.25% Triton–X 100 at room temperature for 10 min. After washing 3 times with PBS for 5 min, the cells were blocked with 0.05% Tween and 1% BSA in PBS at room temperature for 30 min. Myoblasts were incubated with 1:100 diluted anti-MyoD antibody (sc-760, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, followed by 1:200 diluted anti-rabbit IgG FITC antibody (sc-2012, Santa Cruz Biotechnology) in dark at room temperature for 1 h. Myotubes were incubated with 1:100 diluted anti-myosin heavy chain (**MHC**) antibody (NA4, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at 4 ^{o}C overnight and 1:200 diluted anti-mouse IgG fluorescein isothiocyanate (**FITC**) antibody (F0382, Sigma, St. Louis, MO) in a dark at room temperature for 1 h. After washing with PBS, nuclei of the cells were stained with 4',6-diamidino-2-phenylindole (**DAPI**; Thermo Scientific, Rockford, IL). The stained cells were visualized with a fluorescence microscope. Representative immunocytochemical images of myoblasts and myotubes are shown in Figure 2.1.

Myoblast Proliferation and Fusion Assays

Myoblasts were seeded in 96-well plates at the density of 5×10^3 cells/well and cultured in medium composed of DMEM, 10% FBS, 1% ABAM, and 10 ng/mL or 100 ng/mL of

recombinant bovine GH (National Hormone and Peptide Program, Torrance, CA), or 50 ng/mL or 500 ng/mL of recombinant human IGF-I (Shenandoah Biotechnology, Inc., Warwick, PA), or an equal volume of PBS for 24 h, 48 h, and 72 h. The viable cells were counted using the nonradioactive CellTiter 96 assay kit (Promega, Madison, WI), as previously reported (Zhou et al., 2008). For fusion assays, myoblasts were seeded in 6-well plates at the density of 1×10^5 cells/well and were initially cultured in growth medium. Approximately 24 h later, the medium was replaced with differentiation medium composed of DMEM, 2% HS, and 1% ABAM supplemented with GH, IGF-I, or PBS as described above for 48 h. At the end of this 48-h period, the cells were fixed with 4% PFA and stained with DAPI. The numbers of nuclei located in mononucleated, as well as multinucleated, cells were counted. At least 2,000 total nuclei were counted for each treatment. The fusion index was defined as the percentage of total nuclei being in the cells containing at least 3 nuclei. The myoblast proliferation experiment was repeated 5 times, and the fusion experiment 4 times, each time using satellite cells isolated from a different animal.

Protein Synthesis and Degradation Assays

Myoblasts were induced to fuse into myotubes by switching medium that contained 10% FBS to medium that contained 2% HS. When 70% of myoblasts were fused into myotubes, they were treated with GH, IGF-I, or PBS in serum-free medium for 24 h. Myotubes were then labeled with 0.5 μCi L-[2,3,4,5,6-^{3}H] phenylalanine (GE Healthcare, Pittsburgh, PA) for 8 h. After being washed 3 times with PBS, myotubes were lysed in 0.5 *M* NaOH and 0.1% Triton X-100 for 2 h at 37^{o}C. Total protein was precipitated with 10% Thichloroacetic acid (**TCA**) at room temperature for 30 min. The precipitated protein was captured by filtering through a glass-fiber

filter (Millipore, Billerica, MA). Free [^{3}H] phenylalanine was removed by washing the filter 5 times with 5 mL of 10% TCA. Radioactivity of protein-incorporated [^{3}H] phenylalanine on the filter was counted with a liquid scintillation counter (LS-6000LL, Beckman, Brea, CA). The DNA concentration of the same cellular lysate was measured using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA). The radioactivity from the cells in a well was divided by the DNA concentration from the same well to normalize the potential well-to-well variation in cell number.

To quantify protein degradation, myotubes fused from myoblasts were cultured in the presence of 0.5 μCi L-[2,3,4,5,6-^{3}H] phenylalanine for 24 h. Myotubes were washed 3 times with PBS to remove the extracellular L-[2,3,4,5,6-^{3}H] phenylalanine and then cultured in fresh medium containing GH or IGF-I as described above for 8 h. At the end of this 8-h period, medium samples were taken and the cells were lysed in 0.5 *M* NaOH and 0.1% Triton X-100. Medium activity of ^{3}H and DNA concentration of the cell lysate were determined as described earlier. These protein synthesis and degradation assays were repeated 4 times, each time using satellite cells isolated from a different animal

RNA Extraction and Real-time Reverse Transcription-Polymerase Chain Reaction

Total RNA from cultured bovine muscle cells was extracted using TRI Reagent according to the manufacturer's instructions (MRC, Cincinnati, OH). The extracted RNA was dissolved in diethypyrocarbonate (**DEPC**)-treated water. Concentrations of total RNA were determined using a NanoDrop1000 (Thermo Scientific, Wilmington, DE). Integrity of total RNA was validated by formaldehyde gel electrophoresis. Total RNA (0.1 μg) was reverse-transcribed into cDNA in a total volume of 20 μL using the ImProm-II reverse transcriptase (Promega) according to the

manufacturer's instructions. Real-time PCR was performed on 1 μL of cDNA product in a total volume of 25 μL containing 12.5 μL of SyberGreen PCR Master Mix (Applied Biosystems Inc., Foster City, CA) and 0.2 μ*M* of gene-specific forward and reverse primers (Table 1) under 40 cycles of 15 s at 95°C and 1 min at 60°C. The specificity of all primers was verified by gel electrophoresis and DNA sequencing of their PCR products. The efficiency of all primers was verified by standard curve analysis of serially diluted cDNA. The amplification efficiency of the primers used in this study was between 90% and 110%. The relative abundance of an mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with *18S rRNA* being the internal control. Based on the Ct values, abundance of *18S rRNA* was not different between groups in this study ($P > 0.1$).

Statistical Analyses

Data were analyzed by ANOVA followed by the Tukey test using the General Linear Model of SAS (SAS Institute, Inc., Cary, NC). A difference was considered significant if the associated *P* value was < 0.05, and not significant if the *P* value was > 0.1. All data were expressed as mean ± SEM. In the proliferation assay, main effects of treatment, time and treatment-time interaction were tested. In other assays, the main effect of treatment was tested. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Growth Hormone and Insulin-like Growth Factor-I on Proliferation and Fusion of Bovine Myoblasts

To determine the effects of GH and IGF-I on proliferation of bovine myoblasts, bovine satellite cells were isolated and cultured in medium supplemented with 10 or 100 ng/mL of GH, or 50 or 500 ng/mL of IGF-I for 24, 48, and 72 h. Addition of 10 ng/mL or 100 ng/mL of GH or 50 ng/mL of IGF-I did not alter the proliferation of these cells ($P > 0.1$, Figure 2.2). Addition of 500 ng/mL of IGF-I increased the proliferation of bovine myoblasts by 20%, compared to addition of PBS ($P < 0.05$, Figure 2.2). Reducing percentage of FBS in the medium from 10% to 5% did not change the proliferation response to the cells to GH or IGF-I ($P > 0.1$, data not shown).

To determine whether GH and IGF-I affect the ability of bovine myoblasts to fuse into myotubes, the myoblasts were induced to fuse in the presence of 10 or 100 ng/mL of GH, or 50 or 500 ng/mL of IGF-I. As shown in Figure 2.3, in the absence of added GH or IGF-I, approximately 40% of the myoblasts fused into the multinucleated myotubes by 48 h of differentiation. This percentage was not altered by addition of GH or IGF-I at both high and low concentrations ($P > 0.1$).

Effects of Growth Hormone and Insulin-like Growth Factor-I on Protein Synthesis and Degradation in Bovine Myotubes

We next determined the effects of GH and IGF-I on protein synthesis and degradation in bovine myotubes. As shown in Figure 2.4A, the myotubes treated with GH for 24 h accumulated 15% more protein than those not treated with GH within the subsequent 8-h period ($P < 0.05$).

This effect of GH was not different between the 2 concentrations of GH tested ($P > 0.1$, Figure 2.4A). The myotubes treated with IGF-I for 24 h accumulated approximately 70% more protein than PBS controls and 50% more protein than those treated with GH ($P < 0.01$, Figure 2.4A). This effect of IGF-I was also not different between the 2 concentrations tested ($P > 0.1$, Figure 2.4A).

To determine whether the stimulatory effects of GH and IGF-I on protein accumulation in bovine myotubes is the result of increased protein synthesis or that of decreased protein degradation, we labeled the newly synthesized protein in myotubes with ^{3}H-phenylalanine and determined the effects of GH and IGF-I on the amount of ^{3}H-phenylalanine released to the medium over a period of 8 h. As shown in Figure 2.4B, GH at either 10 ng/mL or 100 ng/mL had no effect on medium amount of ^{3}H-phenylalanine, compared to PBS ($P > 0.1$). However, the medium of myotubes treated with 50 ng/mL or 500 ng/mL of IGF-I had 30% less ^{3}H-phenylalanine than that of untreated myotubes ($P < 0.01$, Figure 2.4B). This data indicates that GH stimulated protein accumulation in bovine myotubes by stimulating protein synthesis, whereas IGF-I stimulated protein accumulation in myotubes by both stimulating protein synthesis and inhibiting protein degradation.

Effect of Growth Hormone on Insulin-like Growth Factor-I mRNA Expression in Bovine Myoblasts and Myotubes

To determine the possibility that the stimulatory effect of GH on protein synthesis in bovine myotubes is mediated by paracrine/autocrine action of the locally produced IGF-I, we determined whether GH stimulates *IGF-I* mRNA expression in bovine myotubes and myoblasts. As shown in Figure 2.5A, GH at 10 ng/mL or 100 ng/mL did not alter *IGF-I* mRNA expression

in either myotube or myoblast culture ($P > 0.1$). The *GHR* mRNA was detectable in both bovine myoblasts and myotubes (Figure 2.5B). The *CISH* gene is known to be regulated by GH action (Sadowski et al., 2001), and its mRNA expression in bovine myoblasts was increased by GH ($P < 0.05$, Figure 2.5C). These latter observations indicated that the lack of GH effect on *IGF-I* mRNA expression in bovine myoblasts and myotubes was not because these cells were not responsive to GH.

DISCUSSION

The role of GH as a major stimulator of growth in animals, including cattle, has been known for decades (Etherton and Bauman, 1998), but the underlying cellular and molecular mechanisms are not fully understood. We have conducted this study to determine whether GH may stimulate skeletal muscle growth in cattle through direct action on myoblasts or myofibers and whether the direct effect of GH is mediated by IGF-I produced in these muscle cells.

In the study we first tested the possibility that GH stimulates skeletal muscle growth by directly stimulating proliferation of myoblasts. Our data showed that GH at 10 ng/mL, a physiological concentration, or 100 ng/mL, a supraphysiological concentration (Wu et al., 2010), had no effect on proliferation of bovine myoblasts. This result is consistent with the majority of the studies that have examined the effects of GH on proliferation of primary myoblasts or myoblast cell lines (Allen et al., 1986; Florini et al., 1996; Gospodarowicz et al., 1976; Kim et al., 2005). Conflicting results have been reported as to the effect of GH on proliferation of bovine myoblasts in vivo. In one report, GH administration did not influence the number of satellite cells (Vann et al., 1998), but in another report GH appeared to stimulate proliferation of bovine satellite cells because GH administration was associated with an in increase in the DNA content of skeletal muscle (Maltin et al., 1990). Apparently, whether GH stimulates proliferation of satellite cells or myoblasts in cattle remains to be clarified. Our result that GH had no direct effect on proliferation of bovine myoblasts in culture suggests that even if GH stimulates proliferation of satellite cells or myoblasts in cattle, this effect is probably not the result of direct action of GH on these cells.

Skeletal muscle growth could also result from increased fusion of myoblasts into

myotubes. We observed that GH had no effect on fusion of bovine myoblasts into myotubes. This in vitro observation is in line with the in vivo finding that GH administration does not alter the number of nuclei per myofiber in cattle (Vann et al., 1998; Vann et al., 2001). Therefore, it is likely that GH does not stimulate skeletal muscle growth in cattle by increasing myoblast fusion. However, GH has been shown to stimulate murine myoblasts to fuse to myotubes (Florini et al., 1991; Heron-Milhavet et al., 2010; Hsu et al., 1997; Mavalli et al., 2010; Sotiropoulos et al., 2006). Thus, GH may have species-dependent effects on myoblast fusion.

In this study, we also investigated the possibility that GH stimulates skeletal muscle growth in cattle by directly stimulating protein synthesis or by directly inhibiting protein degradation in muscle. Our data show that GH at a physiological concentration increased protein accumulation in bovine myotubes by 15%. We also observed that the same concentration of GH had no effect on protein degradation, indicating that GH-increased accumulation of protein in myotubes resulted from increased protein synthesis. Growth hormone administration to cattle stimulates muscle protein synthesis and muscle hypertrophy without affecting the number of nuclei per myofiber or the number of myofibers in muscle (Vann et al., 1998; Vann et al., 2001). So our result that GH can directly stimulate protein synthesis in bovine myotubes suggests that the stimulatory effect of GH on muscle protein synthesis in cattle is at least partly due to direct action of GH on myofibers.

The second major question we intended to address in this study was whether the action of GH on skeletal muscle growth is mediated by IGF-I produced in myoblasts or myofibers. Our data show that GH at physiological and supraphysiological concentrations did not stimulate *IGF-I* mRNA expression in either bovine myoblasts or bovine myotubes, and that this lack of effect was not because the cultured bovine myoblasts and myotubes were not responsive to GH. Our

observation that GH had no direct effect on *IGF-I* mRNA expression in bovine muscle cells is consistent with the in vivo observations that GH administration did not affect skeletal muscle *IGF-I* mRNA expression in cattle (Pell et al., 1993), and that skeletal muscle *IGF-I* mRNA was not correlated with serum GH concentrations or growth rate in bulls, growing steers, and heifers (Hannon et al., 1991). We also observed that whereas GH at either physiological concentration or supraphysiological concentration had no effect on proliferation of bovine myoblasts, IGF-I at physiological concentrations stimulated proliferation of these cells, that IGF-I had a much greater effect on protein accumulation in bovine myotubes than GH, and that IGF-I not only inhibited protein degradation but also stimulated protein synthesis, whereas GH only affected protein synthesis in bovine myotubes. These differences further suggest that GH does not stimulate protein synthesis in bovine skeletal muscle through increased *IGF-I* mRNA expression.

In summary, this study shows that GH has a small stimulatory effect on protein synthesis in bovine myotubes, has no effect on proliferation or fusion of bovine myoblasts, and has no effect on protein degradation in bovine myotubes. These results suggest that the direct growth-stimulating effect of GH on skeletal muscle in cattle may be limited to a modest effect on protein synthesis. This study also shows that GH has no effect on *IGF-I* mRNA expression in bovine muscle cells. This result suggests that the direct effect of GH on protein synthesis in cattle skeletal muscle is unlikely mediated through increased expression of *IGF-I* mRNA in muscle. Interestingly, the mechanisms of GH action on skeletal muscle implied by this study seem to be supported by the result of a recent knockout mouse study, which showed that skeletal muscle-specific inactivation of the *GHR* gene caused only a small decrease in BW, and did not alter muscle *IGF-I* mRNA expression (Vijayakumar et al., 2010).

References

Allen, R. E., M. V. Dodson, L. K. Boxhorn, S. L. Davis, and K. L. Hossner. 1986. Satellite cell proliferation in response to pituitary hormones. J Anim Sci 62: 1596-1601.

Burton, N. M., J. Vierck, L. Krabbenhoft, K. Bryne, and M. V. Dodson. 2000. Methods for animal satellite cell culture under a variety of conditions. Methods Cell Sci 22: 51-61.

Eleswarapu, S., Z. Gu, and H. Jiang. 2008. Growth hormone regulation of insulin-like growth factor-I gene expression may be mediated by multiple distal signal transducer and activator of transcription 5 binding sites. Endocrinology 149: 2230-2240.

Etherton, T. D., and D. E. Bauman. 1998. Biology of somatotropin in growth and lactation of domestic animals. Physiol Rev 78: 745-761.

Florini, J. R., D. Z. Ewton, and S. A. Coolican. 1996. Growth hormone and the insulin-like growth factor system in myogenesis. Endocr Rev 17: 481-517.

Florini, J. R., D. Z. Ewton, and S. L. Roof. 1991. Insulin-like growth factor-I stimulates terminal myogenic differentiation by induction of myogenin gene expression. Mol Endocrinol 5: 718-724.

Gospodarowicz, D., J. Weseman, J. S. Moran, and J. Lindstrom. 1976. Effect of fibroblast growth factor on the division and fusion of bovine myoblasts. J Cell Biol 70: 395-405.

Hannon, K., A. Gronowski, and A. Trenkle. 1991. Relationship of liver and skeletal muscle IGF-1 mRNA to plasma GH profile, production of IGF-1 by liver, plasma IGF-1 concentrations, and growth rates of cattle. Proc Soc Exp Biol Med 196: 155-163.

Heron-Milhavet, L., D. Mamaeva, D. LeRoith, N. J. Lamb, and A. Fernandez. 2010. Impaired muscle regeneration and myoblast differentiation in mice with a muscle-specific KO of IGF-IR. J Cell Physiol 225: 1-6.

Herrington, J., L. S. Smit, J. Schwartz, and C. Carter-Su. 2000. The role of STAT proteins in growth hormone signaling. Oncogene 19: 2585-2597.

Hsu, H. H., M. M. Zdanowicz, V. R. Agarwal, and P. W. Speiser. 1997. Expression of myogenic regulatory factors in normal and dystrophic mice: effects of IGF-1 treatment. Biochem Mol Med 60: 142-148.

Jiang, H. et al. 2007. Growth hormone stimulates hepatic expression of bovine growth hormone receptor messenger ribonucleic acid through signal transducer and activator of transcription 5 activation of a major growth hormone receptor gene promoter. Endocrinology 148: 3307-3315.

Kamanga-Sollo, E. et al. 2004. IGF-I mRNA levels in bovine satellite cell cultures: effects of fusion and anabolic steroid treatment. J Cell Physiol 201: 181-189.

Kamanga-Sollo, E. et al. 2008. Roles of IGF-I and the estrogen, androgen and IGF-I receptors in estradiol-17beta- and trenbolone acetate-stimulated proliferation of cultured bovine satellite cells. Domest Anim Endocrinol 35: 88-97.

Kim, H. et al. 2005. Intact insulin and insulin-like growth factor-I receptor signaling is required for growth hormone effects on skeletal muscle growth and function in vivo. Endocrinology 146: 1772-1779.

Klover, P., and L. Hennighausen. 2007. Postnatal body growth is dependent on the transcription factors signal transducers and activators of transcription 5a/b in muscle: a role for autocrine/paracrine insulin-like growth factor I. Endocrinology 148: 1489-1497.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

Maltin, C. A., M. I. Delday, S. M. Hay, G. M. Innes, and P. E. Williams. 1990. Effects of bovine pituitary growth hormone alone or in combination with the beta-agonist clenbuterol on muscle growth and composition in veal calves. Br J Nutr 63: 535-545.

Mavalli, M. D. et al. 2010. Distinct growth hormone receptor signaling modes regulate skeletal muscle development and insulin sensitivity in mice. J Clin Invest 120: 4007-4020.

Ohlsson, C. et al. 2009. The role of liver-derived insulin-like growth factor-I. Endocr Rev 30: 494-535.

Pell, J. M., J. C. Saunders, and R. S. Gilmour. 1993. Differential regulation of transcription initiation from insulin-like growth factor-I (IGF-I) leader exons and of tissue IGF-I expression in response to changed growth hormone and nutritional status in sheep. Endocrinology 132: 1797-1807.

Piwien-Pilipuk, G., J. S. Huo, and J. Schwartz. 2002. Growth hormone signal transduction. J Pediatr Endocrinol Metab 15: 771-786.

Rhoads, R. P. et al. 2009. Extrinsic regulation of domestic animal-derived myogenic satellite cells II. Domest Anim Endocrinol 36: 111-126.

Sadowski, C. L., T. T. Wheeler, L. H. Wang, and H. B. Sadowski. 2001. GH regulation of IGF-I and suppressor of cytokine signaling gene expression in C2C12 skeletal muscle cells. Endocrinology 142: 3890-3900.

Sotiropoulos, A. et al. 2006. Growth hormone promotes skeletal muscle cell fusion independent of insulin-like growth factor 1 up-regulation. Proc Natl Acad Sci U S A 103: 7315-7320.

Stratikopoulos, E., M. Szabolcs, I. Dragatsis, A. Klinakis, and A. Efstratiadis. 2008. The hormonal action of IGF1 in postnatal mouse growth. Proc Natl Acad Sci U S A 105: 19378-19383.

Tollet-Egnell, P., A. Flores-Morales, A. Stavreus-Evers, L. Sahlin, and G. Norstedt. 1999. Growth hormone regulation of SOCS-2, SOCS-3, and CIS messenger ribonucleic acid expression in the rat. Endocrinology 140: 3693-3704.

Vann, R. C., T. G. Althen, W. K. Smith, J. J. Veenhuizen, and S. B. Smith. 1998. Recombinant bovine somatotropin (rbST) administration to creep-fed beef calves increases muscle mass but does not affect satellite cell number or concentration of myosin light chain-1f mRNA. J Anim Sci 76: 1371-1379.

Vann, R. C. et al. 2001. Recombinant bovine somatotropin (rbST) increases size and proportion of fast-glycolytic muscle fibers in semitendinosus muscle of creep-fed steers. J Anim Sci 79: 108-114.

Vijayakumar, A., R. Novosyadlyy, Y. Wu, S. Yakar, and D. LeRoith. 2010. Biological effects of growth hormone on carbohydrate and lipid metabolism. Growth Horm IGF Res 20: 1-7.

Wang, Y., and H. Jiang. 2005. Identification of a distal STAT5-binding DNA region that may mediate growth hormone regulation of insulin-like growth factor-I gene expression. J Biol Chem 280: 10955-10963.

Wu, M. H. et al. 2010. Relationships between critical period of estrogen exposure and circulating levels of insulin-like growth factor-I (IGF-I) in breast cancer: evidence from a case-control study. Int J Cancer 126: 508-514.

Wu, Y., H. Sun, S. Yakar, and D. LeRoith. 2009. Elevated levels of insulin-like growth factor (IGF)-I in serum rescue the severe growth retardation of IGF-I null mice. Endocrinology 150: 4395-4403.

Yablonka-Reuveni, Z., K. Day, A. Vine, and G. Shefer. 2008. Defining the transcriptional signature of skeletal muscle stem cells. J Anim Sci 86: E207-216.

Yakar, S. et al. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci U S A 96: 7324-7329.
Zammit, P. S., T. A. Partridge, and Z. Yablonka-Reuveni. 2006. The skeletal muscle satellite cell: the stem cell that came in from the cold. J Histochem Cytochem 54: 1177-1191.

Table 2.1. Primer sequences for RT-PCR analysis of bovine muscle cells

Target name[1]	Primer sequence (5'to 3')[2]	GenBank #	Amplicon size (bp)
IGF-I mRNA	GTTGGTGGATGCTCTCCAGT	BC126802	148
	CTCCAGCCTCCTCAGATCAC		
CISH mRNA	TTCCTGGAGGAGGCAGTAGA	BC113307	113
	TCCCGAAGGTAGGAGAAGGT		
GHR mRNA	CGTCTCTGCTGGTGAAAACA	AY748827	148
	AACGGGTGGATCTGGTTGTA		
18S rRNA mRNA	GTAACCCGTTGAACCCCATT	DQ222453	150
	CCATCCAATCGGTAGTAGCG		

[1]*IGF-I* = insulin-like growth factor I; *CISH* = cytokine inducible SH2-containing protein; *GHR* = growth hormone receptor.
[2]The upper sequence for a target is the forward primer, and the lower is the reverse primer.

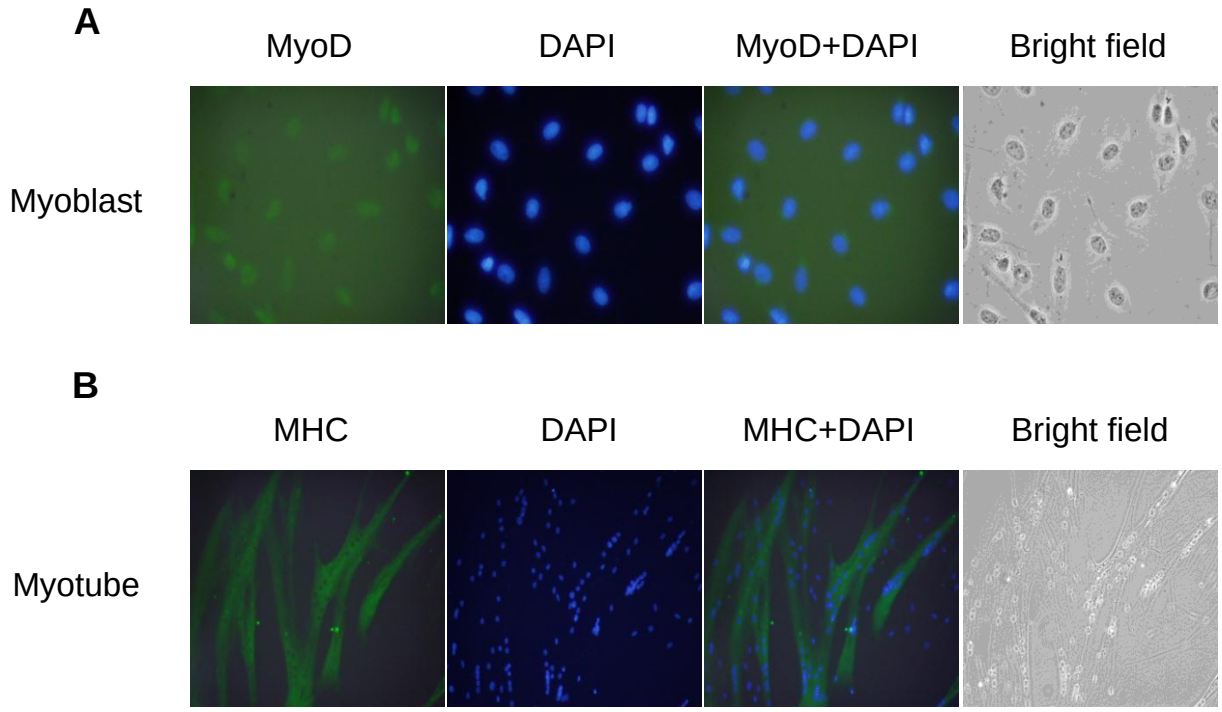


Fig. 2.1. Representative images of cultured bovine myoblasts and myotubes. (A) Newly cultured bovine satellite cells stained with an anti-MyoD antibody and 4',6-diamidino-2-phenylindole (DAPI; 40× magnification). (B) Bovine myotubes stained with an anti-myosin heavy chain antibody and DAPI (20× magnification).

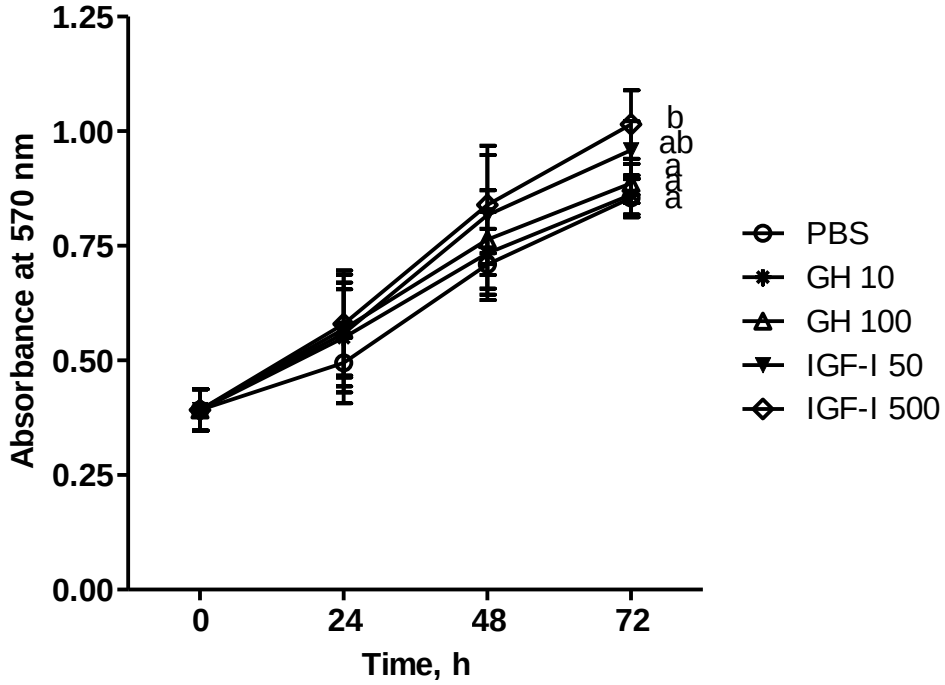


Fig. 2.2. Effects of GH and IGF-I on proliferation of bovine myoblasts. Equal numbers of bovine myoblasts were cultured in the presence of 10 ng/mL or 100 ng/mL of GH, or 50 ng/mL or 500 ng/mL of IGF-I, or PBS for 24, 48, and 72 h before the numbers of viable cells were determined. The absorbance at 570 nm on the y-axis represents the number of viable cells. The lines labeled with different letters differ in the effect of treatment ($P < 0.05$, n = 5). There is also a difference in the effect of time ($P < 0.05$), but not in that of time × treatment interaction ($P > 0.1$).

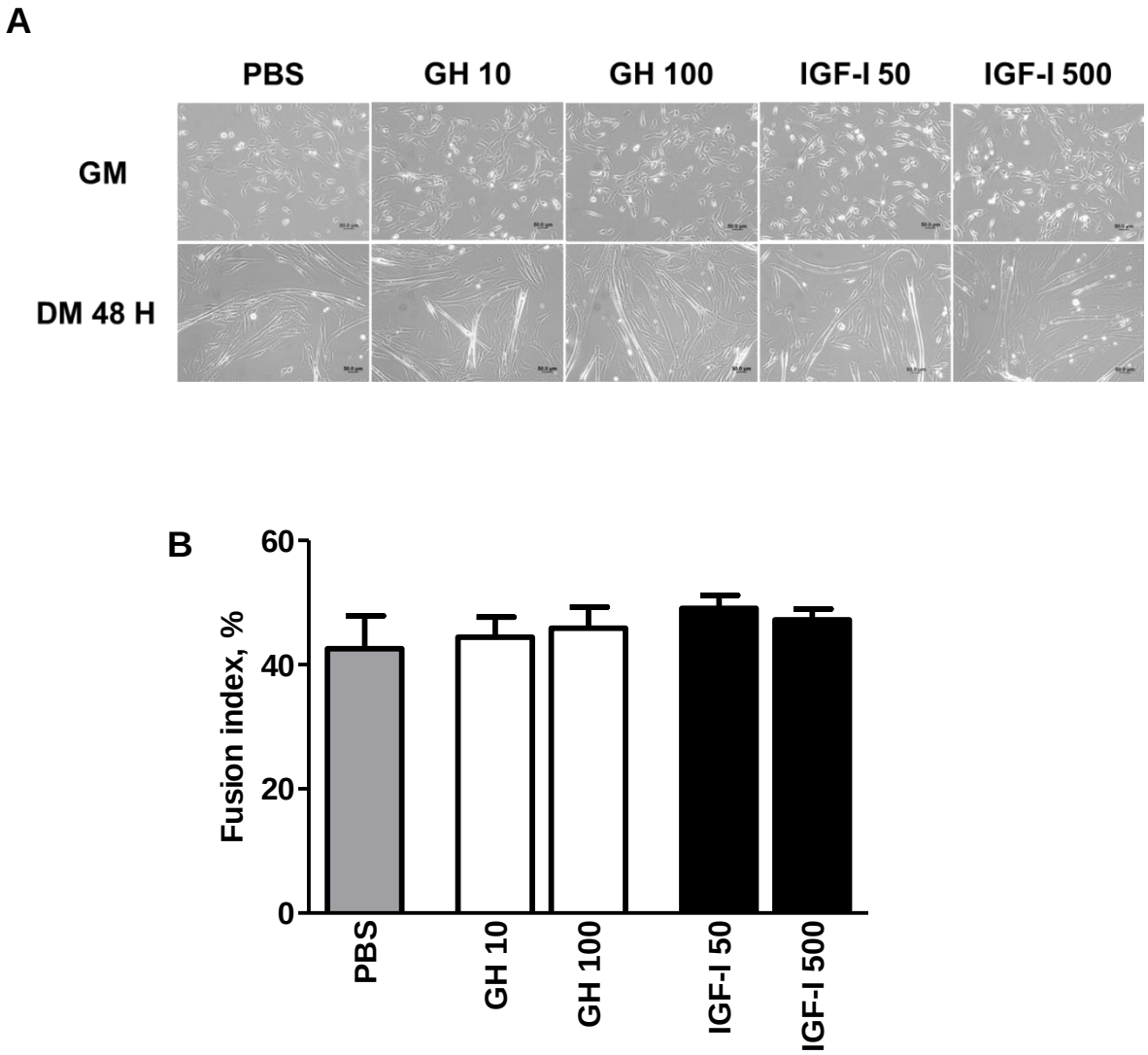


Fig. 2.3. Effects of GH and IGF-I on fusion of bovine myoblasts into myotubes. Bovine satellite cells were cultured initially in growth medium (GM) and then in differentiation medium (DM) containing 10 or 100 ng/mL of GH, or 50 or 100 ng/mL of IGF-I, or PBS, for 48 h. (A) Representative images of the formed myotubes. (B) Percentages of myoblasts forming myotubes (i.e., fusion indexes). Fusion indexes were not different between the treatments ($P > 0.1$, n = 4).

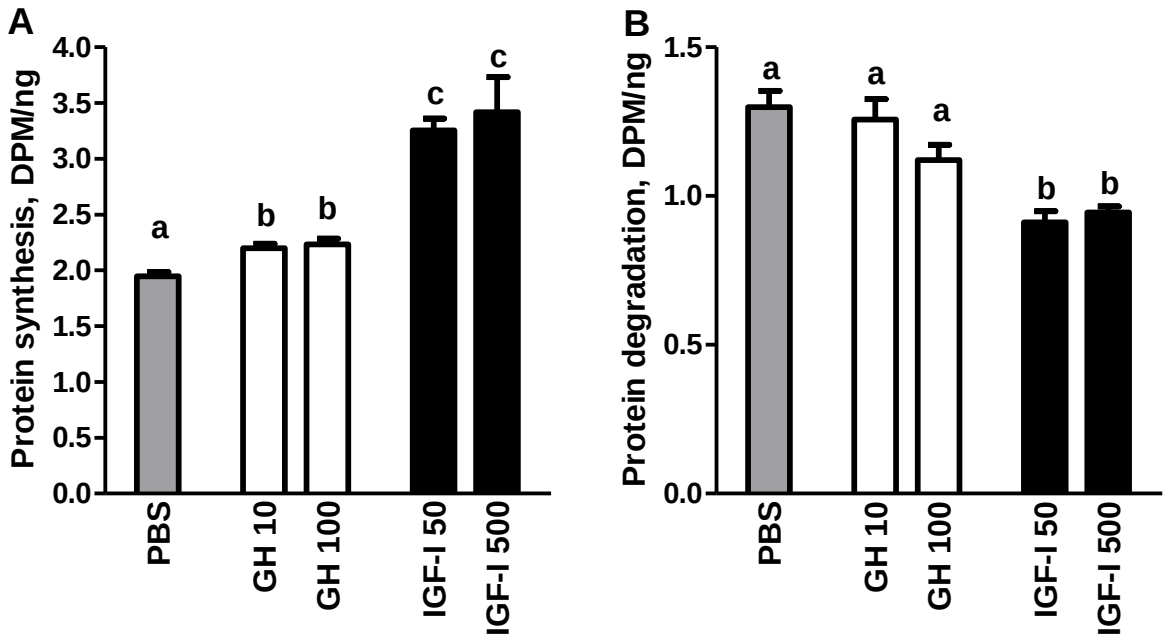


Fig. 2.4. Effects of GH and IGF-I on protein synthesis and degradation in bovine myotubes. (A) Effects of GH and IGF-I on protein accumulation. Bovine myotubes were treated with 10 ng/mL or 100 ng/mL of GH, or 50 ng/mL or 500 ng/mL of IGF-I, or PBS for 24 h, and incorporation of [^{3}H]-phenylalanine into protein was measured in the subsequent 8-h period. The y-axis represents the ^{3}H activity in protein normalized to the amount of DNA in the same cells. Bars labeled with different superscript letters differ in the y-axis value ($P < 0.05$, n = 5). (B) Effects of GH and IGF-I on protein degradation. Myotubes pre-labeled with [^{3}H]-phenylalanine were treated with 10 ng/mL or 100 ng/mL of GH, or 50 ng/mL or 500 ng/mL of IGF-I, or PBS for 8 h, and medium ^{3}H activity was used to represent the rate of protein degradation. Bars without a common superscript are different ($P < 0.05$, n = 4).

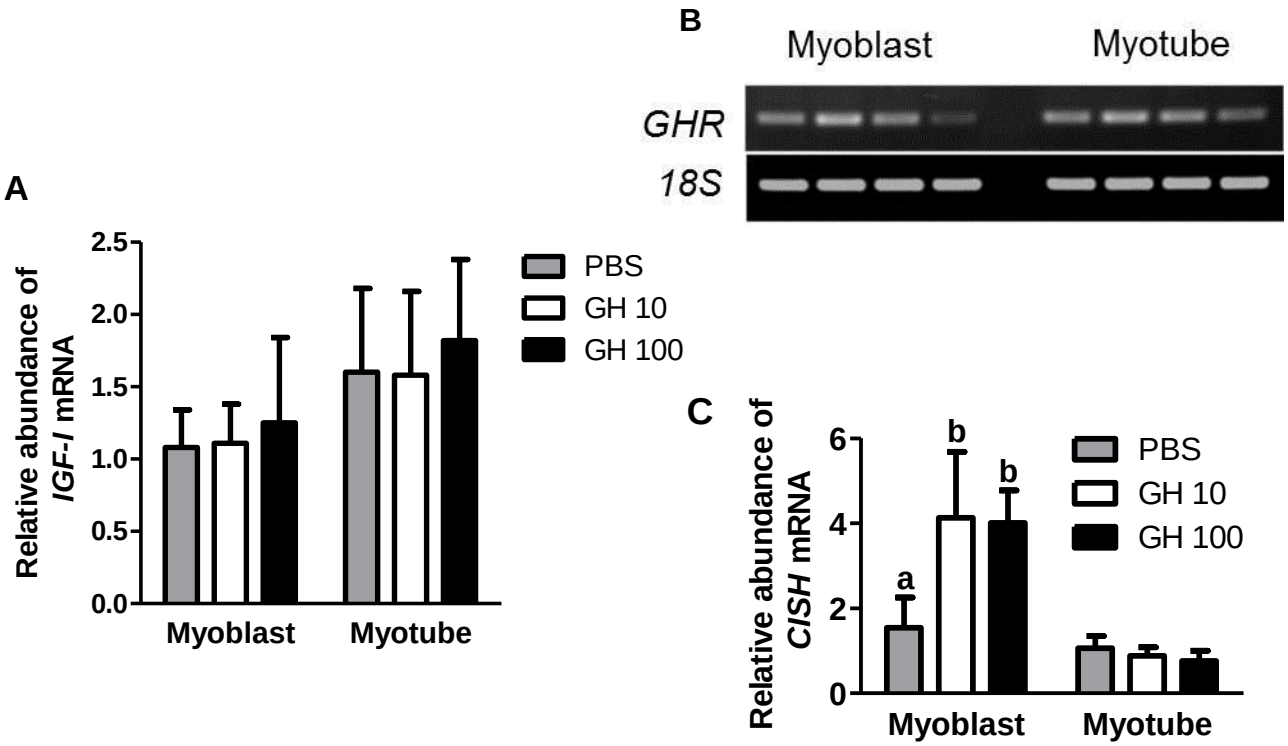


Fig. 2.5. Effects of GH on *IGF-I* and *cytokine-inducible SH2-containing protein* gene. (*CISH*) mRNA expression in bovine myoblasts and myotubes. Myoblasts or myotubes derived from bovine satellite cells were treated with 10 ng/mL or 100 ng/mL of GH or PBS for 12 h prior to RNA extraction. Abundance of mRNA was quantified by real-time reverse-transcription-PCR using *18S rRNA* as the internal control. (A) Effect of GH on *IGF-I* mRNA expression. Expression of *IGF-I* mRNA was not different between the 3 groups ($P > 0.1$, n = 4). (B) Reverse transcription-PCR (RT-PCR) of *GHR* mRNA in myoblasts and myotubes. Shown is a representative gel image of the RT-PCR. (C) Effect of GH on *CISH* mRNA expression. Growth hormone increased *CISH* mRNA expression in myoblasts ($P < 0.05$, n = 4) but not in myotubes ($P > 0.1$, n = 4).

Chapter III

Signaling pathways mediating the effects of insulin-like growth factor-I on proliferation, protein synthesis, and protein degradation in bovine muscle cells

ABSTRACT

The objective of this work was to identify the signaling pathways mediating the effects of IGF-I on proliferation, fusion, protein synthesis, and protein degradation in bovine muscle cells. Satellite cells were isolated from adult cattle skeletal muscle and were allowed to activate and proliferate or were induced to form myotubes following standard protocols. Cell proliferation was determined by measuring the numbers of viable cells at different times. Protein synthesis and degradation were determined by measuring the accumulation of ^{3}H-phenylalanine in cellular protein and the release of ^{3}H-phenylalanine to the medium, respectively. The signaling pathway involved was identified by including in the medium rapamycin, LY294002, or PD98059, which are specific inhibitors of the IGF-I receptor signaling molecules mTOR, AKT (PKB), and ERK (MAPK), respectively. Western blotting confirmed that IGF-I action caused phosphorylations of p70S6K (a signaling molecule immediately downstream of mTOR), AKT, and ERK, and that these phosphorylations were completely or near completely blocked by their corresponding inhibitors. Proliferation of bovine myoblasts was stimulated by 500 ng/mL IGF-I ($P < 0.01$), and this stimulation was partially blocked by PD98059 ($P < 0.05$), and was completely blocked by rapamycin or LY294002 ($P < 0.01$). Protein degradation in myotubes was inhibited by approximately 20% by 500 ng/mL IGF-I ($P < 0.05$), and this inhibition was completely relieved by LY294002 ($P < 0.01$), but not at all by rapamycin or PD98059. Protein synthesis in myotubes was increased by 30% by 500 ng/mL IGF-I ($P < 0.01$), and this increase was completely blocked

by rapamycin, LY294002, or PD98059 ($P < 0.01$). Addition of IGF-I to the culture medium had no effect on fusion of myoblasts into myotubes. These data suggest that IGF-I stimulates proliferation of bovine myoblasts and protein synthesis in bovine myotubes through both the PI3K/AKT and the MAPK signaling pathways, and that IGF-I inhibits protein degradation in bovine myotubes through the PI3K/AKT pathway only from the IGF-I receptor.

Keywords: Insulin-like growth factor I, muscle cells

INTRODUCTION

Insulin-like growth factor I (IGF-I) is an essential growth factor for normal development and growth of skeletal muscle (Baker et al., 1993; Liu et al., 1993). Deletion of IGF-I or its receptor gene, IGF-IR, causes severe growth retardation and abnormalities in skeletal muscle (Blair et al., 1988; Gourmelen et al., 1984; Liu et al., 1993; Wong et al., 1989). Although IGF-I is present at high concentrations in the bloodstream, recent tissue-specific gene targeting studies suggest that locally produced IGF-I is more important to skeletal muscle growth. These studies indicate that whereas liver-specific deletion of the IGF-I gene causes no growth retardation (Sjogren et al., 1999; Yakar et al., 1999), muscle-specific overexpression of IGF-I results in skeletal muscle hypertrophy and prevents atrophy (Coleman et al., 1995; Musaro et al., 2001) and muscle-specific inactivation of IGF-I reduces skeletal muscle and body growth (Klover and Hennighausen, 2007). Besides regulating normal development and growth, IGF-I is also believed to mediate exercise-induced skeletal muscle hypertrophy and injury-triggered skeletal muscle regeneration (Adams, 1998; Mourkioti and Rosenthal, 2005).

Skeletal muscle mass is determined by activation of satellite cells, proliferation and differentiation of myoblasts, and protein synthesis and degradation in myotubes or myofibers (Gibson and Schultz, 1983; Glass, 2005b; Macconnachie et al., 1964; Schiaffino et al., 1972; Shafiq et al., 1968). IGF-I plays a role in almost all of these processes (Adams and McCue, 1998; Fernandez et al., 2002; Menetrey et al., 2000; Sato et al., 2003). IGF-I stimulates proliferation of myoblasts, and this effect was suggested to be mediated by the MEK/MAPK pathway from the IGF-IR (Coolican et al., 1997; Jones et al., 2001). IGF-I stimulates protein synthesis and inhibits protein degradation in myotubes, and both of these effects appeared to be mediated through the PI3K/AKT pathway (Bassel-Duby and Olson, 2006; Glass, 2005b;

Rommel et al., 2001). More recent research indicates that the PI3K/AKT pathway mediates the effects of IGF-I on protein synthesis by inducing phosphorylation of p70S6K and 4EBP1 and that on protein degradation by phosphorylation of the FOXO transcription factors. Phosphorylated p70S6K stimulates protein synthesis by targeting S6 ribosomal protein (Glass, 2005b). Unphosphorylated 4EBP1 inhibits translation of mRNA, and phosphorylation causes it to lose this inhibition (Glass, 2005b). FOXO transcription factors regulate the transcription of E3 ubiquitin ligases MuRF-1 and atrogin-1, which target protein for degradation by proteasomes, and phosphorylation prevents FOXO proteins from translocating to the nucleus and thus from binding to the MuRF-1 and atrogin 1 gene (Sandri et al., 2004; Stitt et al., 2004).

Most of the aforementioned understanding of IGF-I signaling in skeletal muscle has come from studying the myoblast cell lines, such as the L6E9, C2C12, and L6A1 myoblasts (Coleman et al., 1995; Coolican et al., 1997; Engert et al., 1996; Rommel et al., 2001), which might not always have the same physiology as myoblasts or satellite cells in vivo. In this study, we determined the signaling pathways that mediate the effects of IGF-I on cell proliferation, protein synthesis, and protein degradation in myoblasts and myotubes derived from bovine satellite cells, which are readily available (Ge et al., 2012). Our results indicate that while the PI3K/AKT pathway is solely responsible for IGF-I stimulation of protein synthesis and IGF-I inhibition of protein breakdown, both this and the MEK/MAPK pathways are involved in the effect of IGF-I on myoblast proliferation. Our results also suggest that the effect of IGF-I-activated MEK/MAPK signaling on bovine myoblast proliferation is mediated by cyclin D2 and that phosphorylation of p70S6K can be induced by both the PI3K/AKT and the MEK/MAPK pathways in bovine myotubes.

MATERIALS AND METHODS

Isolation and culture of bovine satellite cells

Bovine satellite cells were isolated as described previously (Ge et al., 2012). Briefly, the extensor carpi radialis muscle was collected from crossbred Angus steers and dissected to remove adipose and connective tissues. The animal protocol was approved by the Virginia Tech Animal Care Committee. Extensor carpi radialis was chosen because of its easy accessibility. The muscle was ground and satellite cells were released by incubating the muscle in 1 mg/mL Pronase in phosphate-buffered saline (PBS) at 37 ℃ for 40 min. The satellite cells were collected and purified by four rounds of centrifugation at 1,500 × g at 4 ℃. At least 80% of the isolated cells were myogenic cells (Ge et al., 2012). The satellite cells were allowed to proliferate as myoblasts in growth medium (GM) composed of Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Manassas, VA), 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), and 1% antibotics-antimycotics (ABAM) (Mediatech). To induce differentiation, myoblasts were allowed to reach confluence in GM and then cultured in differentiation medium consisting of DMEM, 2% horse serum (HS), and 1% ABAM.

Proliferation assay

The bovine myoblasts were split into 96-well plates at 5×10^3 cells/well and cultured in DMEM and 10% FBS. The cells in 5 replicates were treated with 500 ng/mL recombinant human IGF-I, or IGF-I and 20 nM rapamycin (inhibitor of mTOR), 50 μM LY294002 (inhibitor of PI3K), 50 μM PD98059 (inhibitor of MEK1), or a combination of 50 μM LY294002 and 50 μM PD98059, for 0, 24, 48, and 72 h. Both IGF-I and its signaling inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). The concentrations of IGF-I signaling inhibitors were based on

previous studies (Cuenda and Alessi, 2000; Davies et al., 2000; Price et al., 1992) and their effectiveness in inhibiting their targets in this study were validated by Western blot analysis (see below). The number of viable cells were quantified using the nonradioactive CellTiter 96 assay kit following the manufacturer's instructions (Promega, Madison, WI). This cell proliferation experiment was repeated 3 times, each time with satellite cells freshly isolated from a different animal.

Protein accumulation and degradation assay

Myoblasts were cultured in GM for 72 h to form myotubes. The myotubes were treated with 500 ng/mL IGF-I, or IGF-I and 20 nM rapamycin, 50 μM LY294002, 50 μM PD98059, or a combination of LY294002 and PD98059, in serum-free DMEM for 24 h. The myotubes were then labeled with 0.5 μCi/mL L-[2,3,4,5,6-^{3}H]phenylalanine (GE Healthcare, Pittsburgh, PA) in DMEM for 8 h. Subsequently, the myotubes were washed twice with PBS to remove extracellular L-[2,3,4,5,6-^{3}H]phenylalanine. The myotubes were lysed in 0.5 M NaOH and 0.1% Triton X-100 for 2 h at 37 ℃. 50 μl of the cell lysate were taken for DNA concentration assay. The protein in the remaining lysate was precipitated by an equal volume of 10% trichloroacetic acid (TCA) and incubation at room temperature for 30 min. The precipitated protein was subsequently applied to a glass fiber filter (Millipore, Billerica, MA). Free L-[2,3,4,5,6-^{3}H]phenylalanine was washed by 10% TCA. The radioactivity remaining on the filter was counted in 5 mL of scintillation fluid on a liquid scintillation counter (Beckman, Brea, CA). DNA concentration of the lysate was determined using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA). The radioactivity in protein was normalized to DNA concentration in the same cell lysate to control for potential variation in cell numbers across different wells.

To determine protein degradation, the myotubes were cultured in the presence of 0.5 μCi/mL L-[2,3,4,5,6-^{3}H]phenylalanine for 24 h. The myotubes were subsequently washed twice with PBS, followed by the addition of fresh DMEM and IGF-I, or IGF-I and rapamycin, LY294002, PD98059, or a combination of LY294002 and PD98059, for 8 h. At the end of this 8 h treatment, the medium was collected and the cells were lysed as described above. The radioactivity in the medium and the DNA concentration in the cell lysate were quantified as described above. The former was normalized to the latter, and the normalized radioactivity value was used to represent the rate of protein degradation.

RNA extraction and real-time RT-PCR

Total RNA extraction and real-time RT-PCR were performed as described previously (Ge et al., 2012). Sequences of the primers for PCR are shown in Table 1. The efficiency of primers was tested in previous experiments (Wang and Jiang, 2010). The relative abundance of mRNA was calculated using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001), using 18S rRNA as the internal control. Based on the Ct values, abundance of 18S rRNA was not different between treatments ($P > 0.1$).

Western blot analyses

Myoblasts or myotubes cultured in 6-well plates were lysed in 200 μL RIPA buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitors and phosphatase inhibitors (Roche Diagnostics Corporation, Indianapolis, IN), according to the mnufacturer's instructions. The cell lysates were centrifuged at 14,000 × g for 15 min at 4 °C, and the supernatants were collected. Protein

concentrations of the supernatants were determined with a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). For western blot analyses, 30 µg of total cellular protein was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% nonfat dried milk in TBST buffer (20 mM Tris-HCl, 500 mM NaCl, and 0.05% Tween-20) and then incubated with phospho-p70S6K, phospho-AKT, phospho-ERK, or phospho-FoxO3a antibody (Cell Signaling Technology, Inc., Danvers, MA) at 1:1000 dilution in TBST and 5% BSA overnight at 4 °C. These antibodies were detected using a horseradish peroxidase-conjugated goat IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific, Rockford, IL). Following detection of a phosphor-protein, the membrane was stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and re-probed with the antibody for total p70S6K, AKT, ERK, or FoxO3a (Cell Signaling Technology, Inc., Danvers, MA). Signal intensities were measured using the ImageJ software (http://rsb.info.nih.gov/ij/index.html).

Statistical analyses

All data are expressed as mean ± S.E.M (standard error of the mean). Each experiment used cells isolated from three or more individual animals. Analysis was performed using General Linear Model ANOVA followed by the Tukey test of SAS (SAS Inst., Inc., Cary, NC) to compare means of multiple groups. In the proliferation assay, main effects of treatment, time and treatment-time interaction were tested. In other assays, the main effect of treatment was tested. A value of $P < 0.05$ was considered statistically significant.

RESULTS

IGF-I stimulation of bovine myoblast proliferation involves both PI3K/AKT and MEK/ERK pathways

The bovine myoblasts maintained proliferation in the control medium (DMEM and 10% FBS), as their number doubled by 72 h of culture (Fig. 3.1A). Addition of IGF-I to the medium increased the proliferation rate of bovine myoblasts by nearly 150% ($P < 0.05$); the number of bovine myoblasts at the end of 72 h of culture was more than 3 times that at the beginning of the culture (Fig. 3.1A).

Addition of PD98059, the MEK1 inhibitor, to the medium completely blocked the stimulation of IGF-I on proliferation of bovine myoblasts ($P < 0.01$, Fig. 3.1A). Indeed, in the presence of PD98059, the myoblasts proliferated at a slower rate than those in medium without IGF-I supplementation ($P < 0.05$, Fig. 3.1A). Addition of the mTOR inhibitor, rapamycin, also completely blocked the stimulatory effect of IGF-I on proliferation of bovine myoblasts ($P < 0.01$, Fig. 3.1A). The cells in the presence of rapamycin tended to proliferate at a slower rate than those in the control medium ($P = 0.074$, Fig. 3.1A). Addition of the PI3K inhibitor, LY294002, completely blocked not only the IGF-I-stimulated proliferation ($P < 0.01$, Fig. 3.1A). Indeed, in the presence of LY294002, the number of myoblasts was even less at 72 h than at the beginning of the culture ($P < 0.05$, Fig. 3.1A). Addition of LY294002 and PD98059 together had a similar effect on IGF-I-induced proliferation, basal proliferation, and death of bovine myoblasts compared to addition of LY294002 only to the medium (Fig. 3.1A).

To validate the effectiveness of PD98059, rapamycin, and LY294002 in inhibiting signal transduction from the IGF-I receptor in bovine myoblasts, the phosphorylation levels of ERK1/2,

p70S6K, and AKT in those cells were analyzed by Western blot analysis. There was no detectable phosphorylation of AKT or p70S6K, and there was some phosphorylation of ERK1/2 in untreated myoblasts (Fig. 3.1B). IGF-I induced remarkable phosphorylation in AKT and p70S6K, and increased phosphorylation in ERK1/2 (Fig. 3.1B). Rapamycin and LY294002 completely prevented IGF-I induced phosphorylation in p70S6K and AKT, respectively (Fig. 3.1B). PD98059 blocked both IGF-I induced and basal phosphorylation of ERK1/2 (Fig. 3.1B).

IGF-I stimulated Cyclin D2 expression in bovine myoblasts

Cell proliferation is controlled primarily by cell cycle regulators (King and Cidlowski, 1998). To determine whether IGF-I stimulates proliferation of bovine myoblast through cell cycle regulators, we determined the effects of IGF-I on mRNA expression of major cell cycle regulators, including cyclin D1, D2, and D3 (CCND1, CCND2, and CCND3), cyclin E1 and E2 (CCNE1 and CCNE2), cyclin-dependent kinase inhibitors 1A, 1B, and 2B (CDKN1A, CDKN1B, CDKN2B) (Nigg, 1995; Sandhu and Slingerland, 2000; Sherr, 1995). Compared to the control (i.e., PBS), IGF-I increased cyclin D2 mRNA expression by more than 2-fold at all three time points ($P < 0.05$, Fig. 2A). IGF-I, however, had no effect on mRNA expression of any of the remaining cell cycle regulators at any of the three time points (Fig. 3.2A).

To identify the signaling pathway that mediates IGF-I-stimulated expression of cyclin D2 mRNA, we compared CCND2 mRNA levels in bovine myoblasts treated with IGF-I or IGF-I and its signaling inhibitor at different times. LY294002 completely blocked the IGF-I-induced increases in CCND2 mRNA expression at 2, 6, and 24 h of the treatment ($P < 0.01$, Fig. 3.2B). PD98059 partially blocked IGF-I-induced increases in CCND2 mRNA expression ($P < 0.05$, Fig. 3.2B). Rapamycin had no effect on IGF-I-induced increases in CCND2 mRNA expression at any

of the three times (Fig. 3.2B).

IGF-I activated PI3K/AKT and MEK/ERK pathways to stimulate protein accumulation, and PI3K/AKT pathway to inhibit protein degradation in bovine myotubes.

To identify the signaling pathways that mediate the effects of IGF-I on protein synthesis and degradation in myotubes, we induced myoblasts to form myotubes and treated the latter with IGF-I, or IGF-I and its signaling inhibitor for 24 h, followed by protein accumulation and degradation assays. Incorporation of ^{3}H-labeled phenylalanine into protein during a period of 8 h was used to estimate protein accumulation in myotubes. Myotubes pre-treated with IGF-I accumulated 50% more protein than myotubes without IGF-I treatment ($P < 0.05$, Fig. 3.3A). This effect of IGF-I was completely blocked by rapamycin or PD98059 ($P < 0.05$, Fig. 3.3A). Myotubes co-treated with IGF-I and LY294002 accumulated 70% less protein than IGF-I-treated myotubes and 50% less than myotubes without IGF-I treatment ($P < 0.01$, Fig. 3.3A). Myotubes co-treated with IGF-I, PD98059, and LY294002 accumulated the same amount of protein as myotubes co-treated with IGF-I and LY294002 (Fig. 3.3A) but 25% less than myotubes co-treated with IGF-I and PD98905 ($P < 0.05$, Fig. 3.3A).

In the protein degradation assay, we labeled the newly synthesized proteins in myotubes with ^{3}H-phenylalanine and determined the effects of IGF-I or its signaling inhibitor on the release of ^{3}H-phenylalanine into the medium over a period of 8 h. Myotubes treated with IGF-I released 15% less ^{3}H-phenylalanine to the medium than myotubes without IGF-I treatment ($P < 0.05$, Fig. 3.3B). This difference was not affected by co-treating the myotubes with rapamycin or PD98059 ($P > 0.1$, Fig. 3.3B), but was reversed by co-treating the myotubes with LY294002 ($P < 0.01$, Fig. 3.3B).

The effectiveness of PD98059, rapamycin, and LY294002 in inhibiting IGF-I signaling in bovine myotubes was validated by their effects on IGF-I-induced phosphorylation of ERK1/2, p70S6K, and AKT, respectively (Fig. 3.3C).

IGF-I-induced phosphorylation of FoxO3a in bovine myotubes is mediated solely by the PI3K-AKT pathway but that of p70S6K by both the PI3K/AKT and MEK/ERK pathways

The effects of IGF-I on protein synthesis and degradation are thought to be mediated respectively through phosphorylation of p70S6K and FoxO3a (Glass, 2005b; Sandri et al., 2004; Stitt et al., 2004). Upon phosphorylation, p70S6K stimulates mRNA translation on the ribosome (Glass, 2005b), and FoxO3a loses the ability to translocate to the nucleus, thereby being unable to activate the transcription of the MuRF-1 and MAF box genes, which encode E3 ubiquitin ligases that target proteins for degradation (Gomes et al., 2001; Jeno et al., 1988; Sandri et al., 2004). To determine the roles of these two proteins in IGF-I-stimulated protein synthesis and inhibited protein degradation in bovine myotubes, we determined if they were phosphorylated by IGF-I in bovine myotubes and identified the signaling pathways involved. As shown in Fig. 3.4A and 3.4C, IGF-I stimulated phosphorylation of p70S6K in bovine myotubes. The IGF-I-induced phosphorylation of p70S6K was completely blocked by rapamycin, LY294002, or PD98059 ($P < 0.05$). This indicated that IGF-I stimulates phosphorylation of p70S6 in bovine myotubes not only through the PI3K/AKT/mTOR pathway, but also through the MER/ERK pathway. IGF-I also stimulated phosphorylation of FoxO3a in bovine myotubes ($P < 0.05$, Fig. 3.4B and 3.4D), and this stimulation was completely blocked by LY294002, but was not affected by rapamycin or PD98059 (Fig. 3.4B and 3.4D). This indicated that IGF-I stimulated phosphorylation of FoxO3a in bovine myotubes through the PI3K/AKT pathway.

DISCUSSION

In this study, we demonstrated that IGF-I stimulated proliferation of bovine myoblasts and that this stimulation was accompanied by increased mRNA expression of CCND2 which is the cell cycle regulator responsible for progression through the G1 phase (King and Cidlowski, 1998). We also showed that IGF-I did not affect mRNA expression of other major cell cycle regulators. These results support the hypothesis that IGF-I stimulates proliferation of bovine myoblasts through upregulation of CCND2 expression.

Inhibitor of PI3K or MEK completely blocked IGF-I induced proliferation of bovine myoblasts. This suggests that both the PI3K/AKT and MEK/ERK signaling pathways are essential to the effect of IGF-I on bovine myoblast proliferation. These inhibitors also blunted the IGF-I stimulated CCND2 mRNA expression, further supporting the possibility that IGF-I stimulates proliferation of bovine myoblasts through CCND2. In other muscle cell lines, IGF-I leads to increased mRNA expression of CCND1, CCND2 and p27 (Engert et al., 1996; Machida et al., 2003). Although CCND2 was the only cell cycle regulator with increased mRNA expression in this study, other regulators could potentially be affected at protein level. The IGF-I signaling pathways control the expression of CCND1, CCNE, p21 and p27 at protein level in smooth muscle cells and satellite cells (Braun-Dullaeus et al., 2001; Chakravarthy et al., 2000). Here we found that IGF-I coupled PI3K/AKT and MEK/ERK pathways to increase CCND2 mRNA expression to promote bovine myoblast proliferation.

In addition to providing more DNA content by stimulating proliferation, IGF-I also increased protein synthesis and inhibited protein degradation to increase muscle mass (Fryburg, 1994). In this study, we confirmed that IGF-I increased protein accumulation in bovine myotubes

which is the balance between protein synthesis and degradation. This effect was blocked by administration of inhibitors of mTOR, PI3K, or MEK in bovine myotubes, as a result of inhibiting the phosphorylation of p70S6K. These result together suggested that both PI3K/AKT and MEK/ERK pathways increased protein synthesis in bovine myotubes. Similarly, overexpression of activated Akt induced hypertrophy of C2C12 myotubes, and MEK/ERK pathway mediated IGF-I hypertrophic effect in rat skeletal muscle (Haddad and Adams, 2004; Rommel et al., 2001). The effect of LY294002 on reducing protein accumulation was more promising than Rapamycin in bovine myotubes due to the increased protein degradation. In addition to increase protein synthesis, IGF-I also decreased protein degradation via FoxO3a, which was blocked by LY294002. These results were consistent with earlier findings that the activity of IGF-I on myotubes requires the blockade of FOXO by PI3K/AKT (Lee et al., 2004; Sandri et al., 2004; Stitt et al., 2004).

To summarize, as shown in Fig. 3.5, we found that IGF-I triggers multiple signaling pathways that collaborate to control both the proliferation and growth of bovine muscle cells. At the myoblast stage, IGF-I-dependent signals branch into two pathways controlling cell growth via CCND2 that directly influence the cell cycle. At the myotube stage, IGF-I activated both PI3K/AKT and the MEK/ERK to increase protein synthesis and inhibit protein degradation coordinately. By controlling both DNA and protein content, IGF-I optimized the efficiency of bovine muscle growth.

References

Adams, G. R. 1998. Role of insulin-like growth factor-I in the regulation of skeletal muscle adaptation to increased loading. Exerc Sport Sci Rev 26: 31-60.

Adams, G. R., and S. A. McCue. 1998. Localized infusion of IGF-I results in skeletal muscle hypertrophy in rats. J Appl Physiol 84: 1716-1722.

Baker, J., J. P. Liu, E. J. Robertson, and A. Efstratiadis. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75: 73-82.

Bassel-Duby, R., and E. N. Olson. 2006. Signaling pathways in skeletal muscle remodeling. Annu Rev Biochem 75: 19-37.

Blair, H. T. et al. 1988. Genetic selection for insulin-like growth factor-1 in growing mice is associated with altered growth. Endocrinology 123: 1690-1692.

Braun-Dullaeus, R. C. et al. 2001. Cell cycle protein expression in vascular smooth muscle cells in vitro and in vivo is regulated through phosphatidylinositol 3-kinase and mammalian target of rapamycin. Arterioscler Thromb Vasc Biol 21: 1152-1158.

Chakravarthy, M. V., T. W. Abraha, R. J. Schwartz, M. L. Fiorotto, and F. W. Booth. 2000. Insulin-like growth factor-I extends in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3'-kinase/Akt signaling pathway. J Biol Chem 275: 35942-35952.

Coleman, M. E. et al. 1995. Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. J Biol Chem 270: 12109-12116.

Coolican, S. A., D. S. Samuel, D. Z. Ewton, F. J. McWade, and J. R. Florini. 1997. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J Biol Chem 272: 6653-6662.

Cuenda, A., and D. R. Alessi. 2000. Use of kinase inhibitors to dissect signaling pathways. Methods Mol Biol 99: 161-175.

Davies, S. P., H. Reddy, M. Caivano, and P. Cohen. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351: 95-105.

Engert, J. C., E. B. Berglund, and N. Rosenthal. 1996. Proliferation precedes differentiation in IGF-I-stimulated myogenesis. J Cell Biol 135: 431-440.

Fernandez, A. M. et al. 2002. Muscle-specific inactivation of the IGF-I receptor induces compensatory hyperplasia in skeletal muscle. J Clin Invest 109: 347-355.

Fryburg, D. A. 1994. Insulin-like growth factor I exerts growth hormone- and insulin-like actions on human muscle protein metabolism. Am J Physiol 267: E331-336.

Ge, X., J. Yu, and H. Jiang. 2012. Growth hormone stimulates protein synthesis in bovine skeletal muscle cells without altering insulin-like growth factor-I mRNA expression. J Anim Sci.

Gibson, M. C., and E. Schultz. 1983. Age-related differences in absolute numbers of skeletal muscle satellite cells. Muscle Nerve 6: 574-580.

Glass, D. J. 2005. Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol 37: 1974-1984.

Gomes, M. D., S. H. Lecker, R. T. Jagoe, A. Navon, and A. L. Goldberg. 2001. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proc Natl Acad Sci U S A 98: 14440-14445.

Gourmelen, M., Y. Le Bouc, F. Girard, and M. Binoux. 1984. Serum levels of insulin-like growth

factor (IGF) and IGF binding protein in constitutionally tall children and adolescents. J Clin Endocrinol Metab 59: 1197-1203.
Haddad, F., and G. R. Adams. 2004. Inhibition of MAP/ERK kinase prevents IGF-I-induced hypertrophy in rat muscles. J Appl Physiol 96: 203-210.
Jeno, P., L. M. Ballou, I. Novak-Hofer, and G. Thomas. 1988. Identification and characterization of a mitogen-activated S6 kinase. Proc Natl Acad Sci U S A 85: 406-410.
Jones, N. C., Y. V. Fedorov, R. S. Rosenthal, and B. B. Olwin. 2001. ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. J Cell Physiol 186: 104-115.
King, K. L., and J. A. Cidlowski. 1998. Cell cycle regulation and apoptosis. Annu Rev Physiol 60: 601-617.
Klover, P., and L. Hennighausen. 2007. Postnatal body growth is dependent on the transcription factors signal transducers and activators of transcription 5a/b in muscle: a role for autocrine/paracrine insulin-like growth factor I. Endocrinology 148: 1489-1497.
Lee, S. W. et al. 2004. Regulation of muscle protein degradation: coordinated control of apoptotic and ubiquitin-proteasome systems by phosphatidylinositol 3 kinase. J Am Soc Nephrol 15: 1537-1545.
Liu, J. P., J. Baker, A. S. Perkins, E. J. Robertson, and A. Efstratiadis. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75: 59-72.
Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
Macconnachie, H. F., M. Enesco, and C. P. Leblond. 1964. The Mode of Increase in the Number of Skeletal Muscle Nuclei in the Postnatal Rat. Am J Anat 114: 245-253.
Machida, S., E. E. Spangenburg, and F. W. Booth. 2003. Forkhead transcription factor FoxO1 transduces insulin-like growth factor's signal to p27Kip1 in primary skeletal muscle satellite cells. J Cell Physiol 196: 523-531.
Menetrey, J. et al. 2000. Growth factors improve muscle healing in vivo. J Bone Joint Surg Br 82: 131-137.
Mourkioti, F., and N. Rosenthal. 2005. IGF-1, inflammation and stem cells: interactions during muscle regeneration. Trends Immunol 26: 535-542.
Musaro, A. et al. 2001. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 27: 195-200.
Nigg, E. A. 1995. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. Bioessays 17: 471-480.
Price, D. J., J. R. Grove, V. Calvo, J. Avruch, and B. E. Bierer. 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. Science 257: 973-977.
Rommel, C. et al. 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 3: 1009-1013.
Sandhu, C., and J. Slingerland. 2000. Deregulation of the cell cycle in cancer. Cancer Detect Prev 24: 107-118.
Sandri, M. et al. 2004. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 117: 399-412.
Sato, K. et al. 2003. Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis. Muscle Nerve 28: 365-372.
Schiaffino, S., S. P. Bormioli, and M. Aloisi. 1972. Cell proliferation in rat skeletal muscle

during early stages of compensatory hypertrophy. Virchows Arch B Cell Pathol 11: 268-273.
Shafiq, S. A., M. A. Gorycki, and A. Mauro. 1968. Mitosis during postnatal growth in skeletal and cardiac muscle of the rat. J Anat 103: 135-141.
Sherr, C. J. 1995. Mammalian G1 cyclins and cell cycle progression. Proc Assoc Am Physicians 107: 181-186.
Sjogren, K. et al. 1999. Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. Proc Natl Acad Sci U S A 96: 7088-7092.
Stitt, T. N. et al. 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 14: 395-403.
Wang, A., and H. Jiang. 2010. Rumen fluid inhibits proliferation and stimulates expression of cyclin-dependent kinase inhibitors 1A and 2A in bovine rumen epithelial cells. J Anim Sci 88: 3226-3232.
Wong, E. A., S. M. Ohlsen, J. A. Godfredson, D. M. Dean, and J. E. Wheaton. 1989. Cloning of ovine insulin-like growth factor-I cDNAs: heterogeneity in the mRNA population. DNA 8: 649-657.
Yakar, S. et al. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci U S A 96: 7324-7329.

Table 3.1. Real-time PCR primers used in this study

Gene name	Sequence*	GenBank #	Amplicon size
bCCND1	GCACTTCCTCTCCAAGATGC GTCAGGCGGTGATAGGAGAG	NM_001046273	204 bp
bCCND2	CCAGACCTTCATCGCTCTGT GATCTTTGCCAGGAGATCCA	NM_001076372	163 bp
bCCND3	TCCAAGCTGCGCGAGACTAC GAGAGAGCCGGTGCAGAATC	NM_001034709	178 bp
bCCNE1	TTGACAGGACTGTGAGAAGC TTCAGTACAGGCAGTGGCGA	XM_612960	187 bp
bCCNE2	CTGCATTCTGAGTTGGAACC CTTGGAGCTTAGGAGCGTAG	NM_001015665	229 bp
bCDKN1A	GCAGACCAGCATGACAGATT GTATGTACAAGAGGAGGCGT	NM_001098958	205 bp
bCDKN1B	GACCTGCCGCAGATGATTCC CCATTCTTGGAGTCAGCGAT	NM_001100346	249 bp
bCDKN2B	GCGGTGGATTATCCTGGACA CATCATCATCACCTGGATCG	NM_001075894	210 bp
b18S	GTAACCCGTTGAACCCCATT CCATCCAATCGGTAGTAGCG	DQ222453	150 bp

*All sequences are written from 5’ to 3’.

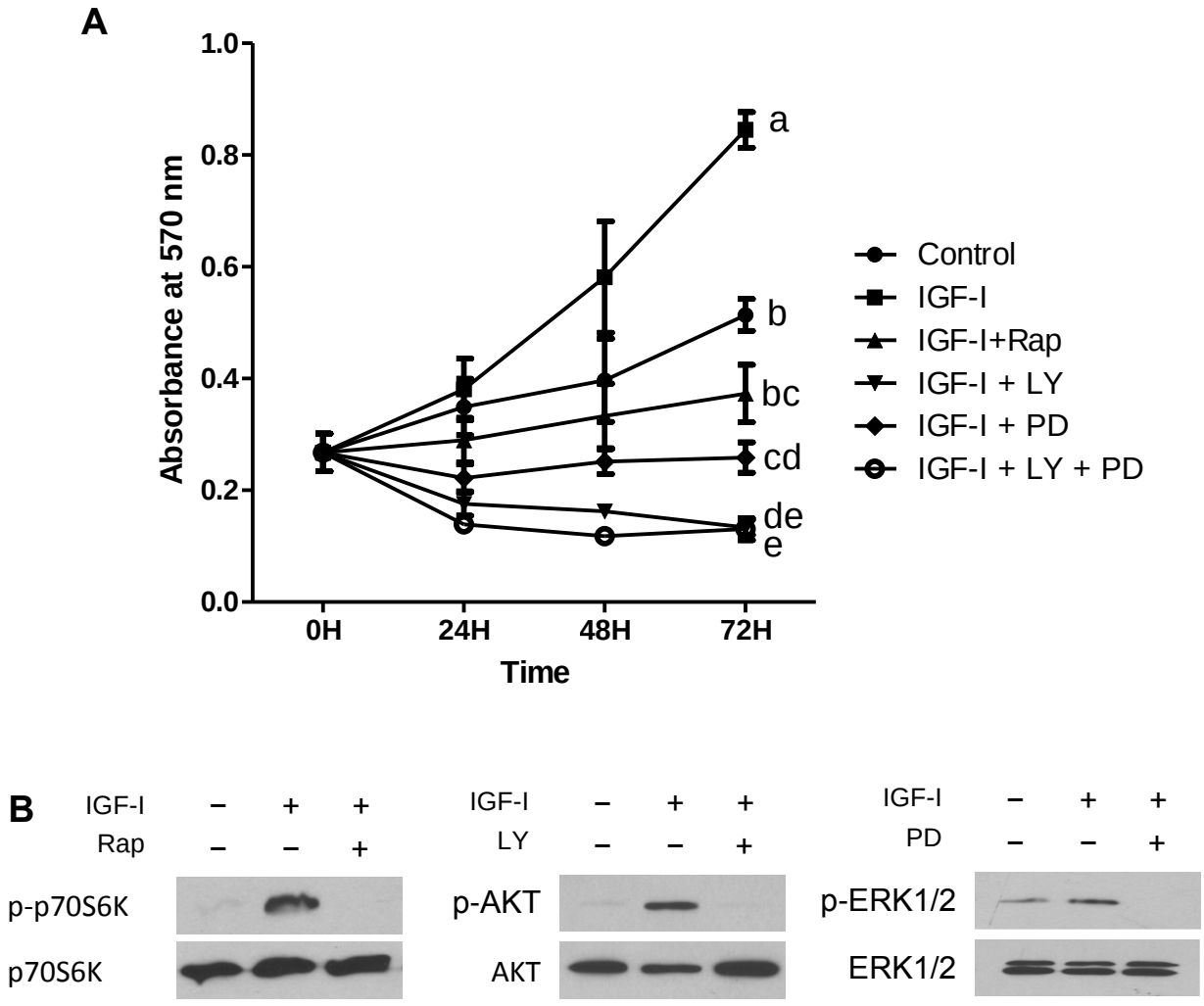
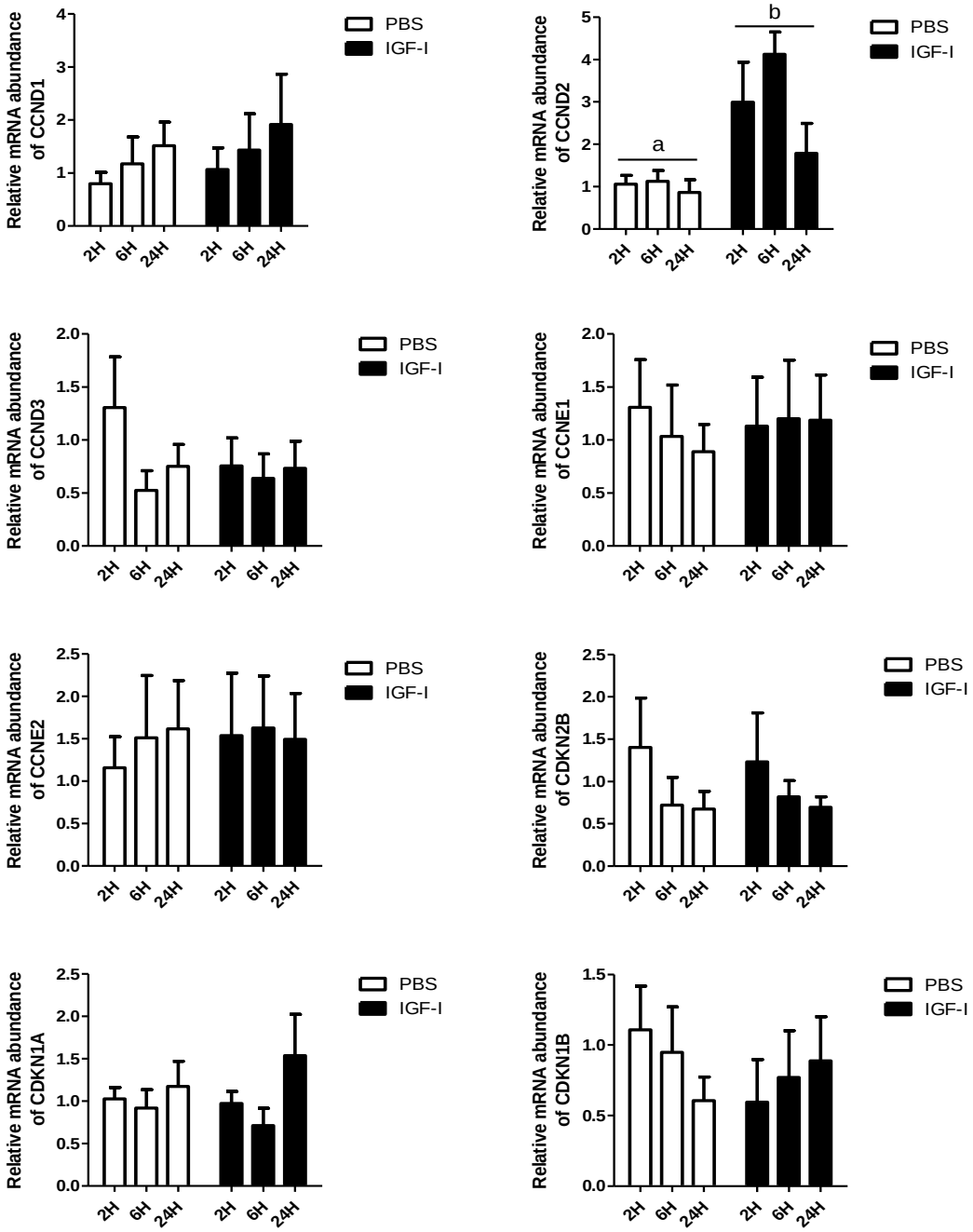


Fig. 3.1. Effects of IGF-I activated signaling pathways on myoblast proliferation. (A) Effects of IGF-I and signal inhibitors on proliferation of bovine myoblasts. Control myoblasts were treated with vehicles. The absorbance at 570 nm on the y-axis represents the number of viable cells. Treatments labeled with different letters had different effects on proliferation ($P < 0.05$, n = 3). (B) Western blot analyses of phosphorylated and total signal proteins targeted by the inhibitors. Shown is a representative image of the western blot using total p70S6K and AKT as loading control.

A


Relative mRNA abundance of CCND1
Relative mRNA abundance of CCND2
Relative mRNA abundance of CCND3
Relative mRNA abundance of CCNE1
Relative mRNA abundance of CCNE2
Relative mRNA abundance of CDKN2B
Relative mRNA abundance of CDKN1A
Relative mRNA abundance of CDKN1B
PBS
IGF-I
2H
6H
24H
a
b

B

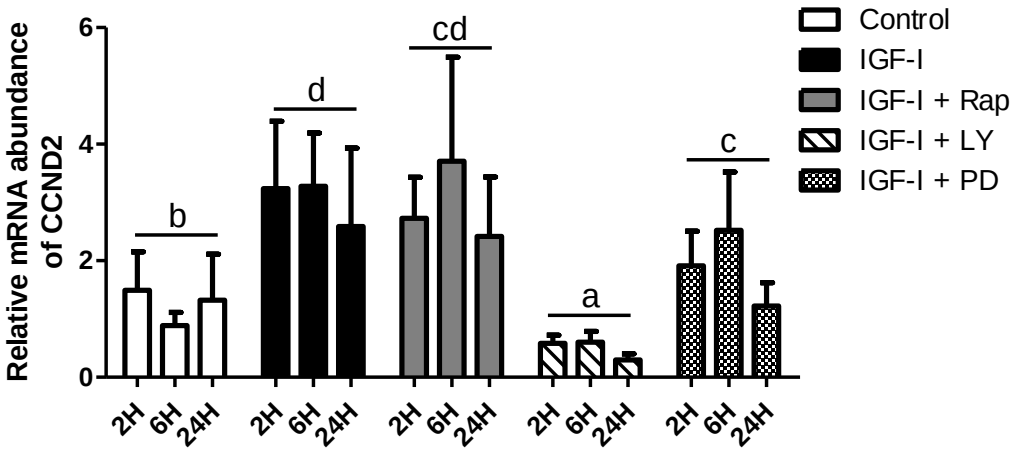


Fig. 3.2. Effects of IGF-I activated signaling pathways on mRNA expression of cell cycle regulators. (A) Effects of IGF-I on mRNA expression of major cell cycle regulators in bovine myoblasts. mRNA abundance labeled with different letters had different effects ($P < 0.05$, n = 4). (B) Identification of the signaling pathway mediating the effect of IGF-I on CCND2 mRNA expression. Treatments labeled with different letters had different effects on mRNA abundance ($P < 0.05$, n = 3).

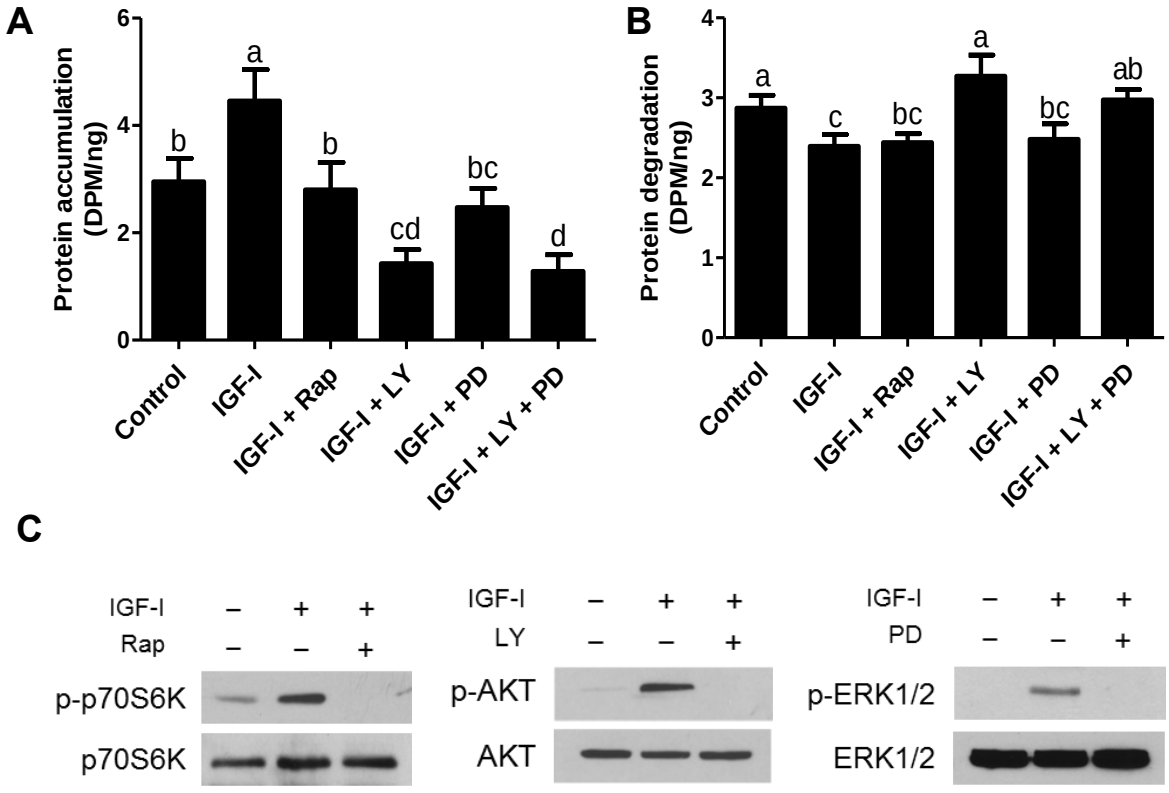


Fig. 3.3. Effects of IGF-I activated signaling pathways on protein accumulation and degradation in bovine myotubes. (A) Effects of IGF-I and signal inhibitors on protein accumulation in bovine myotubes. Protein-incorporated ^{3}H activity normalized with total cellular DNA was used to represent protein accumulation. Treatments labeled with different letters differed in effects ($P < 0.05$, n = 3). (B) Effects of IGF-I and signal inhibitors on protein degradation in bovine myotubes. Medium ^{3}H activity normalized with total cellular DNA was used to represent protein degradation. Treatments labeled with different letters differed in effects ($P < 0.05$, n = 3). (C) Western blot analyses of phosphorylated and total proteins targeted directly or indirectly by the inhibitors.

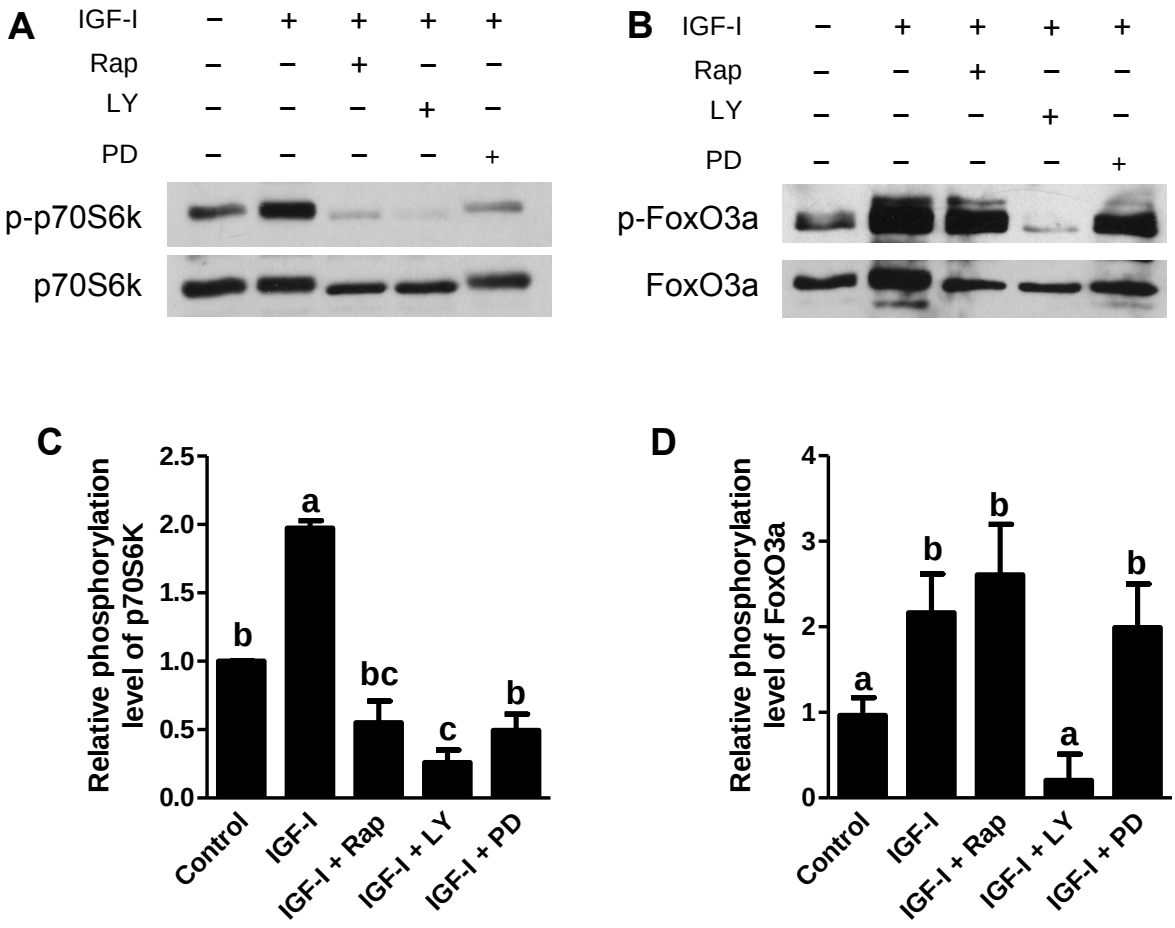


Fig. 3.4. Effects of IGF-I activated signaling pathways on phosphorylation of p70S6K and FoxO3a. (A, B) Western blot analyses of phosphorylated and total proteins of p70S6K and FoxO3a with administration of IGF-I and signal inhibitors. (C, D) Densitometric quantification of the protein bands in panel A and B. Y-axis indicates the ratio of the abundance of phosphorylated p70S6K (p-p70S6K) or FoxO3a (p-FoxO3a) to that of total p70S6K or FoxO3a protein respectively. Ratios labeled with different letters are different ($P < 0.01$, n = 3).

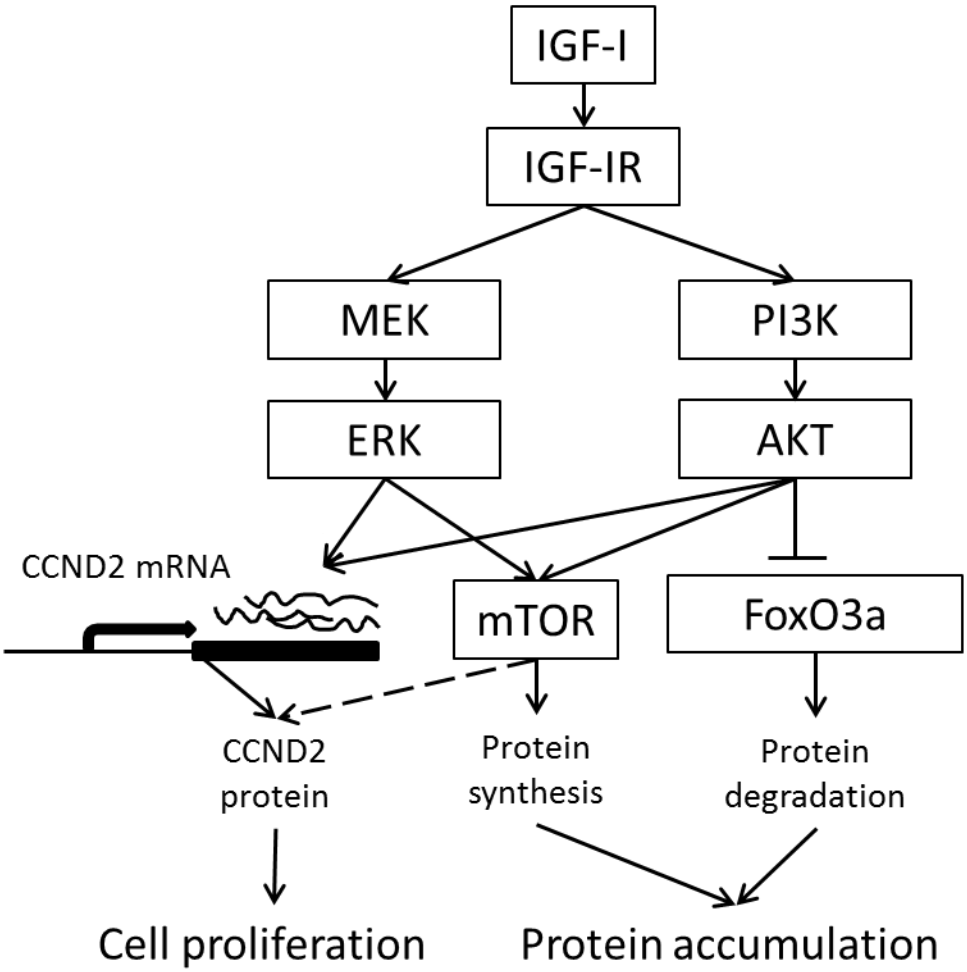


Fig. 3.5. Signal transduction pathways mediating the effects of IGF-I on proliferation of bovine myoblasts and on protein synthesis and degradation in bovine myotubes.

Chapter IV

Identification of the SH3 and cysteine rich domain 3 (STAC3) gene as a novel player in skeletal myogenesis

ABSTRACT

The SH3 and cysteine rich domain 3 gene is the third member of the STAC gene family. The first two members, STAC and STAC2, are exclusively expressed in neural tissues. Here we demonstrated that STAC3 is almost exclusively expressed in skeletal muscle in adult mice. By transiently overexpressing or knocking down STAC3 in C2C12 myoblasts, we showed that STAC3 knockdown promoted fusion of C2C12 cells whereas STAC3 overexpression inhibited their fusion. C2C12 proliferation was not changed in either case. Consistently, STAC3 overexpression inhibited whereas STAC3 knockdown stimulated mRNA expression for several myogenic regulatory factors (MRFs) in C2C12 cells. STAC3 overexpression decreased myosin heavy chain 3 (MyHC3) mRNA expression, while STAC3 knockdown had no effect. The role of STAC3 in myogenesis was also studied using embryonic mouse myoblasts lacking STAC3. Myoblasts derived from STAC3-deficient mouse embryos showed increased fusion ability compared to those from control embryos. The former cells also expressed more MyoD, Myogenin, and MyHC1mRNAs than the latter when differentiated. These data together suggest that STAC3 inhibits differentiation and fusion of both C2C12 and embryonic mouse myoblasts.

Keywords: SH3 and cysteine rich domain 3 (STAC3), myoblast, differentiation and fusion.

INTRODUCTION

During myogenesis, a fine balance among proliferation, differentiation, and fusion of the muscle progenitor cells, myoblasts, is required for the formation of the functional multinucleated myofibers. Many positive and negative regulatory factors are involved in this process. The most studied are the myogenic regulatory factors (MRFs), including Myf5, MyoD, myogenin, and MRF4. These transcription factors act in a cascade format through different stages of myogenesis (Fuchtbauer and Westphal, 1992; Grounds et al., 1992; Weintraub et al., 1991; Yablonka-Reuveni and Rivera, 1994). First, quiescent satellite cells up-regulate MyoD and Myf5 and then enter the cell cycle as myoblasts to proliferate (Beauchamp et al., 2000; Cooper et al., 1999; Cornelison and Wold, 1997; Smith et al., 1994b; Yablonka-Reuveni and Rivera, 1994). Subsequently, myoblasts up-regulate MRF4 and Myogenin to exit the cell cycle to differentiate (Cornelison and Wold, 1997; Smith et al., 1994b; Yablonka-Reuveni and Rivera, 1994). Differentiating myoblasts fuse with each other to form new myotubes or into existing myofibers to increase fiber size. At this stage, they start to express the contractile proteins for myofiber structures, such as myosin heavy chain (MyHC) (Sabourin et al., 1999). MyHC is encoded by a family of six genes, whose expression is developmentally regulated during skeletal muscle formation (Mahdavi et al., 1987; Wydro et al., 1983). The MyHC3 gene is the major MyHC expressed in embryos; therefore, it is also called the embryonic MyHC gene. The MyHC8 gene is expressed mainly in the perinatal stage. The MyHC7, MyHC2, MyHC4, and MyHC1 genes encode type I, type IIa, IIb, and IIx muscle fibers in adults, respectively. Type I fiber is also called a slow fiber, and type IIa, IIb, and IIx are fast fibers (Pette and Staron, 2000; Staron and Johnson, 1993).

In an effort to identify novel regulators of myogenesis, we searched the gene expression profiles at NCBI for genes that are highly expressed in the skeletal muscle. This search led to the identification of the STAC3 gene. The STAC3 gene belongs to a gene family consisting of three members. The other two members are STAC (also called STAC1) and STAC2. The STAC proteins encoded by this family of genes contain a Src homology three (SH3) domain and a cysteine rich (C1) domain. These two protein motifs are associated with cytoplasmic signal transduction (Colon-Gonzalez and Kazanietz, 2006; Stahl et al., 1988), indicating that these proteins might act as part of cellular signaling pathways (Suzuki et al., 1996). Unlike STAC3, both STAC and STAC2 are specifically expressed in the nervous system, and they are mutually exclusive markers for nociceptive peptidergicneurons and nonpeptidergic neurons in the dorsal root ganglia neurons (Legha et al., 2010). In this study, we investigated the potential function of STAC3 in myoblast proliferation, differentiation, and fusion using the C2C12 myoblast cell line and mouse embryonic myoblasts. Our data suggest that STAC3 is a novel inhibitor of myoblast differentiation and fusion.

MATERIALS AND METHODS

Animal experiments

The male C57BL/6 mice were housed at Virginia Tech animal care facility on a timed 12-h light/dark schedule with free access to standard diet and water. The animal related procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

STAC3 knockdown and overexpression in C2C12 cells

The C2C12 myoblasts were plated in 24-well plates, and cultured in DMEM supplemented with 10% FBS at 37 ℃ under 5% CO_2. At 50 ~ 60% confluence, the myoblasts were transfected with 30 pmol siRNAs targeting STAC3 mRNA or 0.5 μg of a pcDNA3.1-based FLAG-tagged STAC3 expression plasmid, using Lipofectamine 2000 as the transfection reagent (Invitrogen). Transfection of an equal amount of a scrambled siRNA (Invitrogen) or the empty pcDNA3.1 plasmid served as a control. Efficiency of STAC3 knockdown or overexpression was confirmed by real-time PCR and western blot analysis of STAC3 mRNA and STAC3-Flag fusion protein in the transfected cells, respectively.

Establishment of C2C12 cell lines stably expressing STAC3

The C2C12 cells were cultured in a 10 cm dish with DMEM supplemented with 10% FBS. At 50% confluence, they were transfected with 5 μg of FLAG-tagged STAC3 expression plasmid pcDNA3.1/hygro/STAC3-FLAG or 5 μg LacZ expression plasmid pcDNA3.1/hygro/LacZ as a control, using Lipofectamine 2000 (Invitrogen). The transfected cells were cultured under 1 mg/mL of hygromycin B (H1012DI, A.G. Scientific) for a week to select the colonies stably expressing STAC3-FLAG or LacZ. The colonies that were formed

under hygromycin selection were picked and expanded in the same culture medium supplemented with 0.5 mg/mL of hygromycin B.

Knockout mouse establishment

STAC3 chimeric mice were generated from microinjection of two STAC3-trapped embryonic stem clones from the KOMP Repository (Stanford et al., 2001). Heterozygous STAC3 mutants were bred to generate homozygous STAC3 mutants. Embryonic day 17.5 STAC3 homozygous embryos and their heterozygous and wild type littermates were used in the present study. Generation and characterization of STAC3 knockout mice are described in detail in the M.S. thesis by Brad Reinholt.

Mouse embryonic myoblast isolation and culture

Mouse embryonic myoblasts were isolated as described before (Springer and Blau, 1997) with minor modifications. Briefly, limb muscles were dissected from embryos at embryonic day 17.5 (E17.5). The muscles were digested in a solution consisting of 1.5 U/mL collagenase (Roche), 2.4 U/ml dispase (Roche), and 2.5 mM $CaCl_2$ at 37 ℃ for 30 min. The released cells were collected by centrifuging at 3000 rpm for 3 min and re-suspended with F-10 primary myoblast growth medium containing 20% FBS (SH30070.02, Hyclone), followed by 2 h preplating. The supernatant was transferred to Collagen I coated dishes (08-774-7, BD Biosciences) and cultured for 48 h before the medium was changed. When cells reached 50% confluence, the procedure of pre-plating for 20 min and transferring the supernatant into Collagen I coated dishes was repeated. The step of pre-plating was repeated until the purity of myoblasts reached ~80%.

Cell proliferation assay

The C2C12 cells transiently transfected with STAC3-targetng siRNA or STAC3 expression plasmid or stably transfected with STAC3 or LacZ expression plasmid were seeded in 96-well plates at a density of 2,000 cells/well and cultured in DMEM supplemented with 10% FBS and 1% ABAM for 0 h, 24 h, 48 h, and 72 h. The viable cells were counted using the nonradioactive CellTiter 96 assay kit (Promega, Madison, WI), according to the manufacturer's instructions.

Cell staining and myotube quantification

In differentiation and fusion assay, C2C12 cells were seeded at 10,000 cells / cm^2 before transfection. Cells were transfected at 70% confluence for 24 h, and grown in the growth medium for another 24 h before changing into DMEM with 2% Horse serum (Differentiation Medium) to induce myoblast differentiation for 72 h. To visualize myotube and nuclei, cells were washed in PBS before fixing for 10 min with MeOH. Cells were then stained with Giemsa (Invitrogen, Carlsbad, CA) for 1 h. C2C12 constantly overexpressing STAC3 were plated into 24-well plate at 20,000 / cm^2 24 h before starting differentiation. Primary embryonic muscle cells were plated at the density of 20,000 cells / cm^2 into a 24-well plate. The plate was pre-coated with type I Collagen overnight at room temperature before use. Cells were grown in F-10 growth medium for 24 h before switching to differentiation medium. By the end of differentiation, nuclei were stained with DAPI.

To quantify the differentiation and fusion rate, total nuclei and nuclei inside myotube were counted using NIH ImageJ software. Fusion index was calculated as nuclei in myotubes

divided by total nuclei. The nuclear number per myotube was determined by dividing the number of nuclei within multinucleated myotubes by the total number of myotubes.

Total RNA isolation and real-time RT-PCR

Total RNA from tissue or cells was isolated using TRI Reagent (MRC, Cincinnati, OH), according to the manufacturer's instructions. Total RNA (0.1 μg) was reverse-transcribed into cDNA in a total volume of 20 μL using the ImProm-II reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. Real-time PCR was performed on 1 μL of cDNA product in a total volume of 25 μl containing 12.5 μL of SybrGreen PCR Master Mix (Applied Biosystems Inc., Foster City, CA) and 0.2 μM of gene-specific forward and reverse primers (Table 1), under conditions suggested by the manufacturer. The relative abundance of an mRNA was calculated using 18S rRNA as the internal control. Based on the Ct values, abundance of 18S rRNA was not different between treatments ($P > 0.1$).

Total cellular protein isolation and Western blotting analysis

Cells were lysed with ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitors and phosphatase inhibitors (Roche Diagnostics Corporation, Indianapolis, IN). The lysates were centrifuged at 14,000 × g for 15 min at 4 ℃. Protein concentrations in the supernatants were measured using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). For Western blotting analyses, 30 μg of total cellular protein was resolved by 8% SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% nonfat dried milk in TBST buffer (20 mM Tris-HCl, 500 mM NaCl, and 0.05% Tween-20) and then

incubated with anti-Flag antibody (Sigma) or anti-STAC3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution at 4 ℃ overnight. Primary antibody was detected using a horseradish peroxidase-conjugated goat IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific, Rockford, IL). Following detection of Flag-STAC3 or STAC3 protein, the membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and reprobed with the antibody for tubulin (Santa Cruz Biotechnology). Signal intensities were measured using the ImageJ software (http://rsb.info.nih.gov/ij/index.html).

Immunocytochemistry

Cells were fixed with 4% PFA in PBS for 15 min and then permeated with 0.25% Triton X-100 in PBS for 10 min at room temperature. Thereafter, cells were blocked with 1% BSA in PBST (PBS + 0.05% Tween-20) and incubated with anti-MyHC antibody (NA4, Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa) at 1:200 dilution in PBST supplemented with 1% BSA at 4 ℃ overnight. Primary antibody was detected by incubating the cells with anti-mouse IgG FITC antibody (F9137, Sigma) at 1:200 dilution in 1% BSA at room temperature for 1 h. Nuclei were detected by staining with 1 μg/mL DAPI (46190, Pierce) in PBS for 1 min at room temperature. Fluorescence from the anti-mouse IgG FITC antibody or DAPI was detected with a fluorescence microscope (Nikon).

Statistical analyses

Student's *t* test was used to determine the statistical significance of the difference between two groups. ANOVA followed by the Tukey test was used to analyze differences

among multiple groups. A difference was considered significant if the associated *P* value was less than 0.05, and not significant if the *P* value was greater than 0.1. All data were expressed as means ± S.E.M (standard error of the mean).

RESULTS

STAC3 is preferentially expressed in skeletal muscle

To evaluate the expression profile of STAC3, total mRNA or protein was isolated from multiple tissues of 4-week old male mice, and subjected to RT-PCR or western blot analysis. As shown in Fig. 4.1, STAC3 mRNA was detected in soleus, extensor digitorum longus, tibialis anterior and gastrocnemius skeletal muscles, but not in other tissues (Fig. 4.1A). At the protein level, STAC3 was detected in all four types of skeletal muscles at high levels (Fig. 4.1B). STAC3 protein was not detected in non-skeletal muscle tissues except in the heart at a very low level (Fig. 4.1B). This nearly skeletal muscle exclusive expression pattern of STAC3 suggested that it has important functions in skeletal muscle.

Transient overexpression or knockdown of STAC3 affected fusion but not proliferation of C2C12 cells

To determine the function of STAC3 in skeletal muscle, we overexpressed or knocked down STAC3 in the C2C12 cells by transiently transfecting them with a STAC3-Flag fusion protein expression plasmid or a pool of three STAC3 specific siRNAs, respectively, and examined the effects of changed STAC3 expression on proliferation and fusion of C2C12 cells.

At day 3 of differentiation, 56% of the control C2C12 cells, i.e., those transfected with an empty pcDNA3.1 plasmid or scrambled siRNA, formed myotubes; 15% of those over expressing STAC3 formed myotubes; and 77% of those transfected with STAC3 siRNAs formed myotubes ($P < 0.05$, Fig. 4.2A and 4.2B; Sungkwon Park). These data indicated that STAC3 knockdown promoted whereas STAC3 overexpression inhibited fusion of C2C12 cells. Consistently, myotubes formed from STAC3 overexpressing C2C12 cells had more nuclei than those formed

from control cells, whereas myotubes derived from STAC3 knockdown C2C12 had more nuclei compared with scramble transfected cells ($P < 0.05$, Fig. 4.2C). Neither STAC3 overexpression nor STAC3 knockdown affect proliferation of C2C12 cells ($P > 0.1$, Fig, 4.2D).

We analyzed STAC3 mRNA levels in C2C12 cells by real-time RT-PCR. As shown in Fig. 4.2E, STAC3 overexpression caused an 8-fold increase whereas STAC3 siRNA knockdown caused a 50% reduction in STAC3 mRNA expression compared to their respective controls (Fig. 4.2E, $P < 0.05$). This data confirmed the efficiency of STAC3 overexpression or knockdown.

Transient overexpression or knockdown of STAC3 affected C2C12 differentation

To investigate the effect of STAC3 on differentiation of C2C12 cells, we measured the mRNA expression levels of Myf5, MyoD, MRF4, myogenin and embryonic myosin heavy chain (MyHC3) in C2C12 cells with STAC3 overexpression or knockdown at 48 h of differentiation. As shown in Fig. 4.3A, STAC3 overexpression decreased Myf5, MyoD and myogenin mRNA expression compared to transfection with pcDNA3.1 plasmids ($P < 0.05$, Fig. 4.3A), whereas STAC3 siRNA knockdown increased Myf5, MyoD and myogenin mRNA expression compared to transfection with scrambled siRNA ($P < 0.05$, Fig. 4.3B). MRF4 expression was not affected by STAC3 overexpression ($P > 0.1$, Fig. 4.3A), but tended to be increased by STAC3 knockdown ($P = 0.07$, Fig. 4.3B). Expression of MyHC3 mRNA was decreased by STAC3 overexpression ($P < 0.05$, Fig. 4.3A), but was not affected by STAC3 siRNA knockdown ($P > 0.1$, Fig. 4.3B). This was also confirmed at the protein level (Fig. 4.3C). Taken together, these data in general indicated that STAC3 overexpression inhibited whereas STAC3 knockdown stimulated mRNA expression for several myogenic markers in C2C12 cells.

Stable STAC3overexpression inhibited differentiation and fusion without affecting proliferation of C2C12 cells

To overcome the limited efficiency of transient transfection, we generated C2C12 cell lines constantly overexpressing STAC3. We also generated C2C12 cell lines constantly overexpressing LacZ as controls. Western blot analysis confirmed STAC3-Flag fusion protein expression in selected C2C12 cells (Fig. 4.4A). The morphology of cells differentiated for 72 h was shown in Fig. 4.4B. There was no difference in the proliferation rate between STAC3 and LacZ overexpressing C2C12 cells ($P > 0.1$, Fig. 4.4C). At 72 h of differentiation, 13.9% of the STAC3-overexpressing C2C12 cells formed myotubes, and this percentage was much smaller than that (41.5%) of the LacZ overexpressing C2C12 cells ($P < 0.05$, Fig. 4.4D). Myotubes formed from STAC3 overexpressing C2C12 cells on average had less nuclei than those formed from LacZ overexpressing C2C12 cells ($P < 0.05$, Fig. 4.4E). Overall, these data indicated that stable overexpression of STAC3 inhibited fusion of C2C12 myoblasts into myotubes while having no effect on proliferation of these cells.

STAC3 deletion promoted differentiation of mouse embryonic myoblasts

To determine the role of STAC3 in myogenesis in vivo, we generated STAC3 knockout mice. All homozygous STAC3 knockout mice died perinatally. Characterization of these mice will be described in detail in a separate report. In this study, we isolated myoblasts from E17.5 STAC3 null mouse embryos and their littermate controls, and compared their ability to differentiate and fuse into myotubes in cell culture. Determined by real-time RT-PCR, STAC3 mRNA levels in myoblasts from STAC3 knockout embryos were barely detectable at only 1% that in control myoblasts l (Fig. 4.5C). When cultured in differentiation medium, STAC3-

deficient myoblasts showed typical morphological changes as control myoblasts, including cell elongation, alignment, and myotube formation (Fig. 4.5A). This indicated that STAC3 is not essential to myoblast proliferation, differentiation, or fusion. However, by 72 h of differentiation (Fig. 4.5B), 53% of STAC3 knockout myoblasts had fused into myotubes compared to 18% of control myoblasts ($P < 0.01$, Fig. 4.5D). Similarly, the myotubes formed from STAC3 knockout myoblasts had more nuclei than control myoblasts (6 vs. 4 nuclei/myotube) ($P < 0.05$, Fig. 4.5E). These data together suggested that STAC3 deficiency promoted myoblast fusion.

STAC3 deletion led to dysregulation of MRFs and MyHC transcription

Expression levels of 4 MRF and 6 MyHC mRNAs were compared between STAC3 knockout and control myoblasts at 48 h of differentiation. Expression of Myf5 mRNA was lower in STAC3 knockout than in control myoblasts ($P < 0.01$, Fig. 4.6A). Both MyoD and MyoG were expressed at higher levels in STAC3 knockout than in control myoblasts ($P < 0.05$, Fig. 4.6A). Expression of MRF4 mRNA was not different between STAC3 knockout and control myoblasts ($P > 0.1$, Fig. 4.6A). The levels of MyHC3 mRNA were lower in STAC3 knockout than in control myoblasts, but those of MyHC1 mRNA were higher in STAC3 knockout than in control myoblasts ($P < 0.01$, Fig. 4.6B). The expression levels of the remaining 4 MHC mRNAs were not different between STAC3 knockout and control myoblasts ($P > 0.1$, Fig. 4.6B). These data indicated that myoblasts from STAC3 knockout embryos had a greater ability to differentiate and to fuse into myotubes than myoblasts from control embryos.

DISCUSSION

STAC3 is the third member of the STAC gene family. Both STAC1 and STAC2 are predominantly expressed in the brain and neurons (Legha et al., 2010; Suzuki et al., 1996). Gene expression profiles at NCBI suggested that STAC3 is mainly expressed in skeletal muscle. Through RT-PCR and Western blotting analyses of mouse tissues, we confirmed that STAC3 mRNA and protein are nearly exclusively expressed in skeletal muscle. Being expressed specifically in skeletal muscle suggested that STAC3 might play an important role in this tissue. In this study, we carefully examined the role of STAC3 in myogenesis using three different cellular systems: 1) C2C12 cells with transient overexpression or knockdown of STAC3; 2) C2C12 cells with constant overexpression of STAC3; and 3) STAC3 deficient mouse embryonic myoblasts. These experiments consistently showed that STAC3 overexpression inhibited fusion of C2C12 myoblasts into myotubes whereas STAC3 knockdown or complete deletion promoted fusion of C2C12 myblasts or embryonic myoblasts, respectively. These results suggest that endogenous STAC3 is an inhibitor of myoblast fusion.

From proliferation to fusion of myoblasts into multinucleated myotubes, myogenesis is under the tight regulation of MRFs (Relaix and Marcelle, 2009; Tajbakhsh, 2009). This process is accompanied with the upregulation of genes encoding structural and contractile proteins (Sabourin et al., 1999). MyoD and Myf-5 are expressed in the proliferating myoblasts whereas myogenin increases as myoblasts commit to differentiate (Sabourin and Rudnicki, 2000). After differentiation, MRF4 transcripts become detectable for a short period (Bober et al., 1991; Tajbakhsh and Buckingham, 2000). In this study, STAC3 overexpression-induced decreases in fusion rate were accompanied by decreased expression of Myf5, MyoD, and MyoG in C2C12 myoblasts ($P < 0.05$), whereas STAC3 knockdown-induced increases in fusion rate were

associated with increased expression in these MRFs. These results suggest that STAC3 may have a role in regulating the expression of these MRFs and thereby regulate myoblast differentiation and fusion. The MyHC3 expression level was inhibited by STAC3 overexpression, and this was consistent with the observation that STAC3-overexpressing myoblasts formed smaller myotubes. Surprisingly, the MyHC3 expression level was not lower in myotubes formed from STAC3 knockdown myoblasts, perhaps because of compensation from other factors that control MyHC3 expression.

Despite being widely used in studies of myogenesis in vitro, the C2C12 myoblasts might not have the same physiology as myoblasts in vivo. To study the role of STAC3 in myogenesis in vivo, we examined STAC3 function and MRFs and MyHC expression in primary myoblasts isolated from STAC3 knockout mouse embryos. This experiment indicated that STAC3 deficient myoblasts formed more and larger myotubes than control myoblasts and that this difference was associated with greater expression of MyoD and MyoG mRNAs. These results were similar to those from studying C2C12 cells. However, one discrepancy between the two models was that Myf5 expression was decreased in STAC3-deficient myoblasts but not in STAC3-knocked down C2C12 cells compared to their respective controls. This discrepancy may be due to the difference of STAC3 expression levels between STAC3 knockdown C2C12 cells and STAC3 knockout myoblasts, or other more fundamental differences between C2C12 cell line and primary myoblasts. Myf5 has been reported to be important for myogenic determination of quiescent satellite cells. As the C2C12 cells are a myogenic predetermined mouse cell line, like other cell lines, the do not always represent the same biological functions as satellite cells in vivo. Another noteworthy finding from STAC3 deficient mouse myoblasts was that MyHC1, but not MyHC3, was upregulated in those cells. During muscle development, there

are successive generations of new myofibers that give rise to more developed muscle fibers. The majority of the primary generation of myofibers predominantly expresses the MyHC7 gene (Harris et al., 1989; Ontell et al., 1993). During the second generation, MyHC7 remains in part of those muscle fibers. In some of the other myofibers, MyHC7 is replaced by MyHC1, MyHC2 or MyHC4 and becomes mature myofiber in adult skeletal muscle (Lefaucheur, 2010). Higher level of MyHC1 and lower level of MyHC3 in myotubes derived from STAC3 deficient myoblasts indicated a possibly precocious status of STAC3 deficient myotubes. This might be the result of the upregulated MyoD, which has been documented to accumulate in fast muscles (Allen et al., 2001; Harrison et al., 2011).

In conclusion, this is the first report about the expression profile and biological function of the STAC3 gene. By manipulating STAC3 expression in C2C12 cells and mouse embryonic myoblasts, we have demonstrated that STAC3 is an inhibitor of myoblast differentiation and fusion. Further studies are needed to understand the underlying molecular mechanism.

References

Allen, D. L., C. A. Sartorius, L. K. Sycuro, and L. A. Leinwand. 2001. Different pathways regulate expression of the skeletal myosin heavy chain genes. J Biol Chem 276: 43524-43533.

Beauchamp, J. R. et al. 2000. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 151: 1221-1234.

Bober, E. et al. 1991. The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. J Cell Biol 113: 1255-1265.

Brown, D. M., T. Parr, and J. M. Brameld. 2011. Myosin heavy chain mRNA isoforms are expressed in two distinct cohorts during C2C12 myogenesis. J Muscle Res Cell Motil.

Buckingham, M. et al. 2003. The formation of skeletal muscle: from somite to limb. J Anat 202: 59-68.

Colon-Gonzalez, F., and M. G. Kazanietz. 2006. C1 domains exposed: from diacylglycerol binding to protein-protein interactions. Biochim Biophys Acta 1761: 827-837.

Cooper, R. N. et al. 1999. In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. J Cell Sci 112 (Pt 17): 2895-2901.

Cornelison, D. D., and B. J. Wold. 1997. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol 191: 270-283.

Fuchtbauer, E. M., and H. Westphal. 1992. MyoD and myogenin are coexpressed in regenerating skeletal muscle of the mouse. Dev Dyn 193: 34-39.

Glass, D. J. 2005. Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol 37: 1974-1984.

Grounds, M. D., K. L. Garrett, M. C. Lai, W. E. Wright, and M. W. Beilharz. 1992. Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. Cell Tissue Res 267: 99-104.

Guttridge, D. C. 2004. Signaling pathways weigh in on decisions to make or break skeletal muscle. Curr Opin Clin Nutr Metab Care 7: 443-450.

Harris, A. J., R. B. Fitzsimons, and J. C. McEwan. 1989. Neural control of the sequence of expression of myosin heavy chain isoforms in foetal mammalian muscles. Development 107: 751-769.

Harrison, B. C., D. L. Allen, and L. A. Leinwand. 2011. IIb or not IIb? Regulation of myosin heavy chain gene expression in mice and men. Skelet Muscle 1: 5.

Lefaucheur, L. 2010. A second look into fibre typing--relation to meat quality. Meat Sci 84: 257-270.

Legha, W. et al. 2010. stac1 and stac2 genes define discrete and distinct subsets of dorsal root ganglia neurons. Gene Expr Patterns 10: 368-375.

Macconnachie, H. F., M. Enesco, and C. P. Leblond. 1964. The Mode of Increase in the Number of Skeletal Muscle Nuclei in the Postnatal Rat. Am J Anat 114: 245-253.

Mahdavi, V., S. Izumo, and B. Nadal-Ginard. 1987. Developmental and hormonal regulation of sarcomeric myosin heavy chain gene family. Circ Res 60: 804-814.

Ontell, M. et al. 1993. Contractile protein gene expression in primary myotubes of embryonic mouse hindlimb muscles. Development 117: 1435-1444.

Pette, D., and R. S. Staron. 2000. Myosin isoforms, muscle fiber types, and transitions. Microsc Res Tech 50: 500-509.

Price, H. M., E. L. Howes, Jr., and J. M. Blumberg. 1964. Ultrastructural Alterations in Skeletal Muscle Fibers Injured by Cold. Ii. Cells on the Sarcolemmal Tube: Observations on "Discontinuous" Regeneration and Myofibril Formation. Lab Invest 13: 1279-1302.
Relaix, F., and C. Marcelle. 2009. Muscle stem cells. Curr Opin Cell Biol 21: 748-753.
Sabourin, L. A., A. Girgis-Gabardo, P. Seale, A. Asakura, and M. A. Rudnicki. 1999. Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J Cell Biol 144: 631-643.
Sabourin, L. A., and M. A. Rudnicki. 2000. The molecular regulation of myogenesis. Clin Genet 57: 16-25.
Shafiq, S. A., and M. A. Gorycki. 1965. Regeneration in skeletal muscle of mouse: some electron-microscope observations. J Pathol Bacteriol 90: 123-127.
Smith, T. H., A. M. Kachinsky, and J. B. Miller. 1994. Somite subdomains, muscle cell origins, and the four muscle regulatory factor proteins. J Cell Biol 127: 95-105.
Springer, M. L., and H. M. Blau. 1997. High-efficiency retroviral infection of primary myoblasts. Somat Cell Mol Genet 23: 203-209.
Stahl, M. L., C. R. Ferenz, K. L. Kelleher, R. W. Kriz, and J. L. Knopf. 1988. Sequence similarity of phospholipase C with the non-catalytic region of src. Nature 332: 269-272.
Staron, R. S., and P. Johnson. 1993. Myosin polymorphism and differential expression in adult human skeletal muscle. Comp Biochem Physiol B 106: 463-475.
Suzuki, H. et al. 1996. Stac, a novel neuron-specific protein with cysteine-rich and SH3 domains. Biochem Biophys Res Commun 229: 902-909.
Tajbakhsh, S. 2009. Skeletal muscle stem cells in developmental versus regenerative myogenesis. J Intern Med 266: 372-389.
Tajbakhsh, S., and M. Buckingham. 2000. The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. Curr Top Dev Biol 48: 225-268.
Weintraub, H. et al. 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. Science 251: 761-766.
Wydro, R. M., H. T. Nguyen, R. M. Gubits, and B. Nadal-Ginard. 1983. Characterization of sarcomeric myosin heavy chain genes. J Biol Chem 258: 670-678.
Yablonka-Reuveni, Z., and A. J. Rivera. 1994. Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. Dev Biol 164: 588-603.

Table 4.1. Nucleotide sequences of the primers used for real-time PCR

gene	direction	Primer sequence	Accession #
STAC3	Forward	5’-TAC AGC GAC CAA CAG TAC GC-3’	NM_177707
	Reverse	5’-TCT GCA TTG TTT CCA TCC TG-3’	
GAPDH	Forward	5-‘ACC CAG AAG ACT GTG GAT GG-3’	NM_008084
	Reverse	5’-GGA TGC AGG GAT GAT GTT CT-3’	
18S	Forward	5’-TTAAGAGGGACGGCCGGGGG-3’	NR_003278
	Reverse	5’-CTCTGGTCCGTCTTGCGCCG-3’	
Myogenin	Forward	5’-CGGCTGCCTAAAGTGGAGAT-3’	NM_031189
	Reverse	5’-AGGCCTGTAGGCGCTCAA-3’	
MyoD	Forward	5’-CCACTCCGGGACATAGACTTG-3’	NM_010866
	Reverse	5’-AAAAGCGCAGGTCTGGTGAG-3’	
Myf5	Forward	5’-ATCCAGGTATTCCCACCTGCT-3’	NM_011566
	Reverse	5’-ACTGGTCCCCAAACTCATCCT-3’	
MRF4	Forward	5’-AGTCTTCAGCGCCTTTCTTCC-3’	NM_008657
	Reverse	5’-CTGCTGGGTGAAGAATGTTCC-3’	
MyHC 1	Forward	5’-AGTCCCAGGTCAACAAGCTG-3’	NM_030679
	Reverse	5’-CACATTTTGCTCATCTTTGG-3’	
MyHC 2	Forward	5’-AGTCCCAGGTCAACAAGCTG-3’	NM_001039545
	Reverse	5’-GCATGACCAAAGGTTTCACA-3’	
MyHC 3	Forward	5’-CGCAGAATCGCAAGTCAATA-3’	NM_001099635
	Reverse	5’-ATATCTTCTGCCCTGCACCA-3’	
MyHC 4	Forward	5’-AGTCCCAGGTCAACAAGCTG-3’	NM_010855
	Reverse	5’-TTTCTCCTGTCACCTCTCAACA-3’	
MyHC 7	Forward	5’-AGTCCCAGGTCAACAAGCTG-3’	NM_080728
	Reverse	5’-TTCCACCTAAAGGGCTGTTC-3’	
MyHC 8	Forward	5’-AGTCCCAGGTCAACAAGCTG-3’	NM_177369
	Reverse	5’-CCTCCTGTGCTTTCCTTCAG-3’	

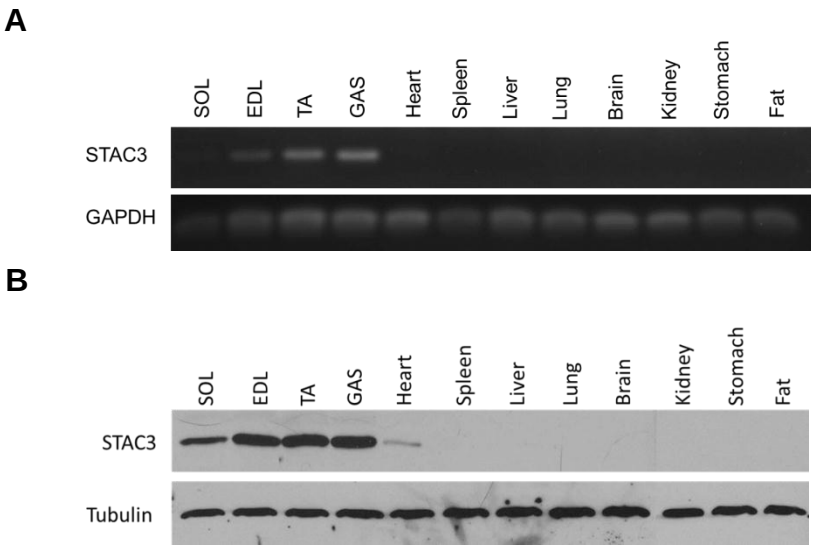


Fig. 4.1. STAC3 expression profile in mouse tissues. (A) STAC3 mRNA levels in different mouse tissues were determined by semiquantitative RT-PCR using GAPDH as internal control. (B) STAC3 protein levels in different mouse tissues were determined by western blot analysis. Tubulin expression was used as internal control.

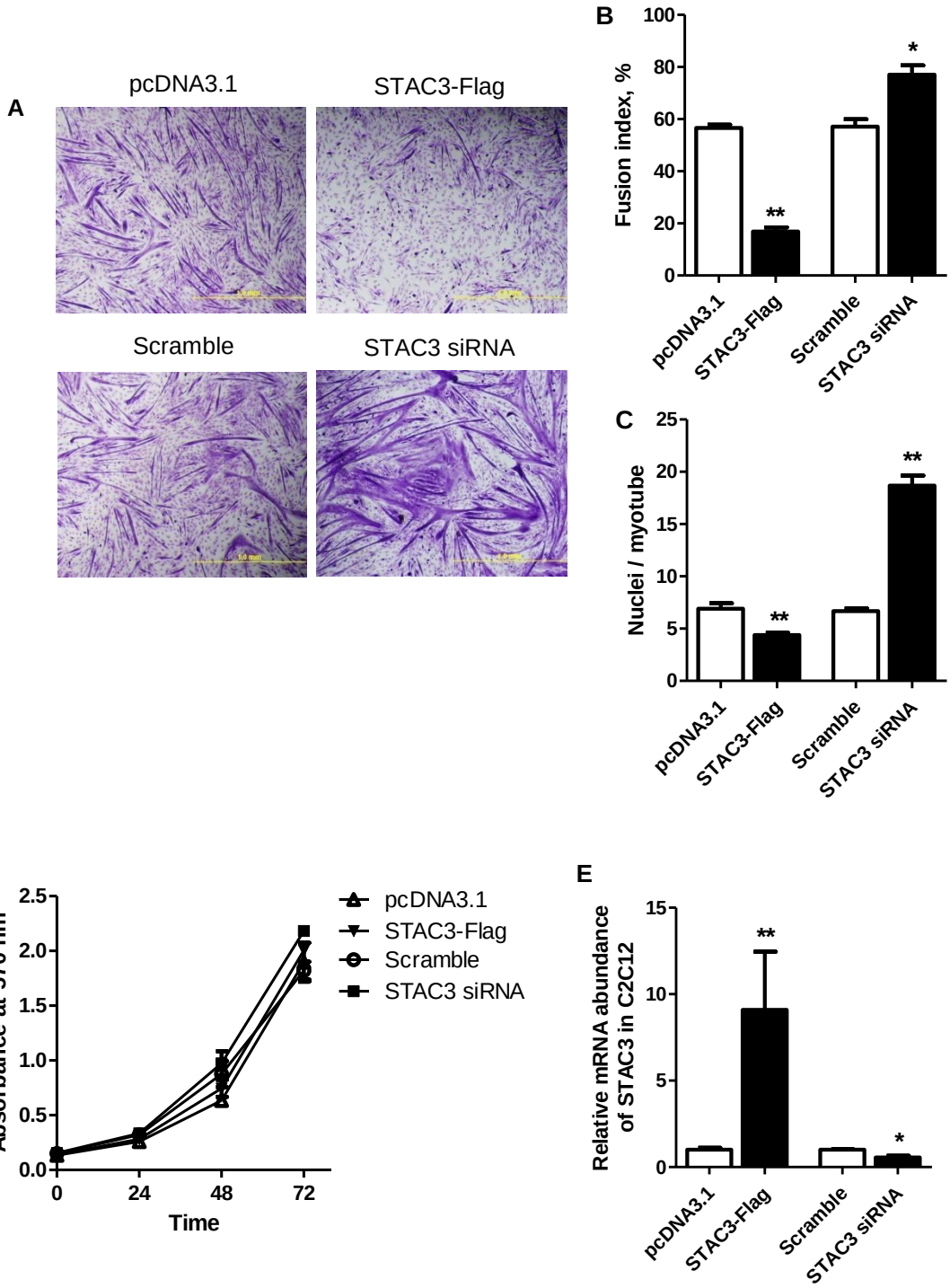

A
pcDNA3.1
STAC3-Flag
Scramble
STAC3 siRNA
B
Fusion index, %
100
80
60
40
20
0
*
**
pcDNA3.1
STAC3-Flag
Scramble
STAC3 siRNA
C
Nuclei / myotube
25
20
15
10
5
0
**
**
pcDNA3.1
STAC3-Flag
Scramble
STAC3 siRNA
D
Absorbance at 570 nm
2.5
2.0
1.5
1.0
0.5
0.0
0
24
48
72
Time
pcDNA3.1
STAC3-Flag
Scramble
STAC3 siRNA
E
Relative mRNA abundance of STAC3 in C2C12
15
10
5
0
**
*
pcDNA3.1
STAC3-Flag
Scramble
STAC3 siRNA

Fig. 4.2. Transient overexpression or knockdown of STAC3 affected fusion but not proliferation of C2C12 cells. (A) Morphology of C2C12 transiently transfected with pcDNA3.1-hygro (+), STAC3-Flag/pcDNA3.1-hygro (+), Scramble and STAC3 siRNA, and differentiated for 72 h before Giemsa staining. Pictures were taken under 20×magnification. (B, C) Transfected C2C12 cells were induced to differentiate for 72 h. After staining of nuclei, the fusion index and the nuclear number per myotube were calculated. Histograms are means ± SEM. * represents $P < 0.05$ and ** represents $P < 0.01$ comparing to corresponding control, and n = 4. (D) Equal numbers of transfected C2C12 cells were cultured in growth medium for 24, 48, and 72 h before the numbers of viable cells were determined. The absorbance at 570 nm on the y-axis represents the number of viable cells (n = 4). (E) The STAC3 mRNA levels in transfected cells were assessed by real-time PCR. Histograms are means ± SEM. * represents $P < 0.05$ and ** represents $P < 0.01$ comparing to corresponding control, and n = 4.

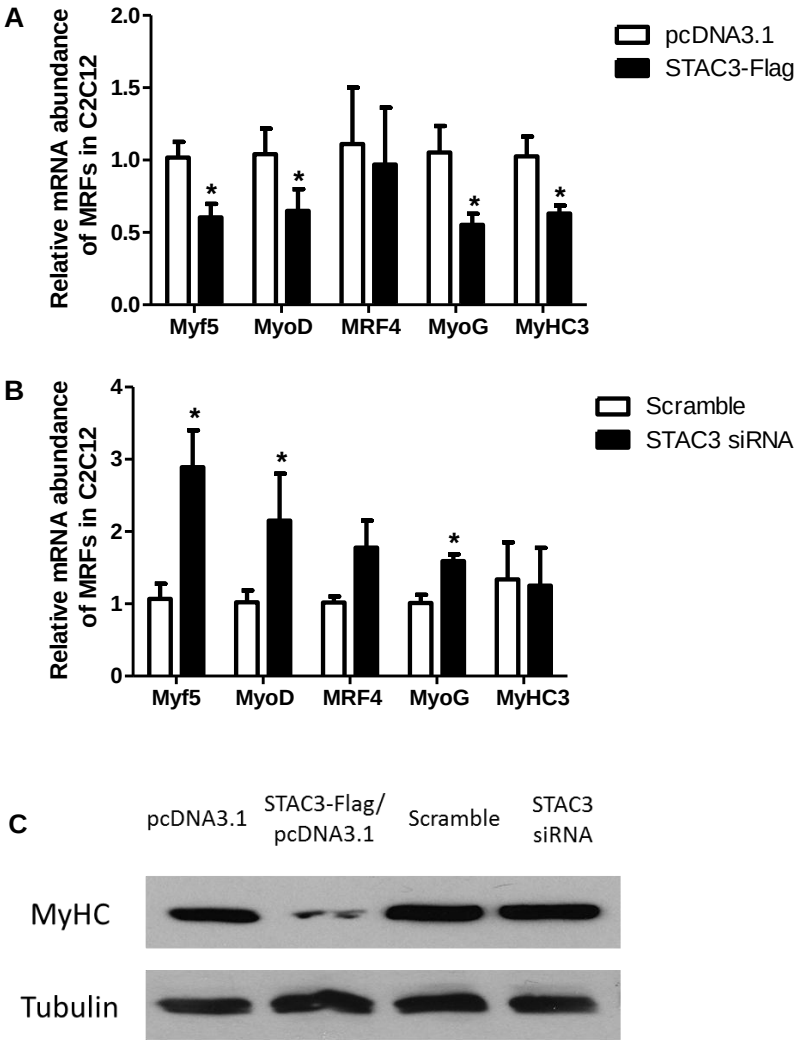

A
Relative mRNA abundance of MRFs in C2C12
2.0
1.5
1.0
0.5
0.0
Myf5
MyoD
MRF4
MyoG
MyHC3
pcDNA3.1
STAC3-Flag
B
Relative mRNA abundance of MRFs in C2C12
4
3
2
1
0
Myf5
MyoD
MRF4
MyoG
MyHC3
Scramble
STAC3 siRNA
C
pcDNA3.1
STAC3-Flag/ pcDNA3.1
Scramble
STAC3 siRNA
MyHC
Tubulin

Fig. 4.3. Transient overexpression or knockdown of STAC3 affected C2C12 differentiation. (A, B) mRNA levels of MRFs and MyHC3 in C2C12 transfected with STAC3, control plasmid, STAC siRNA or scramble. Total RNA was extracted from C2C12 cells in differentiation medium for 48 h, and subjected to real-time PCR using 18s as internal control. Histograms are means ± SEM. * represents $P < 0.05$ within genes, n = 4. (C) Representative picture of total MyHC western blot. Total protein was isolated from C2C12 cells transfected and differentiated for 48 h. Tubulin was the control.

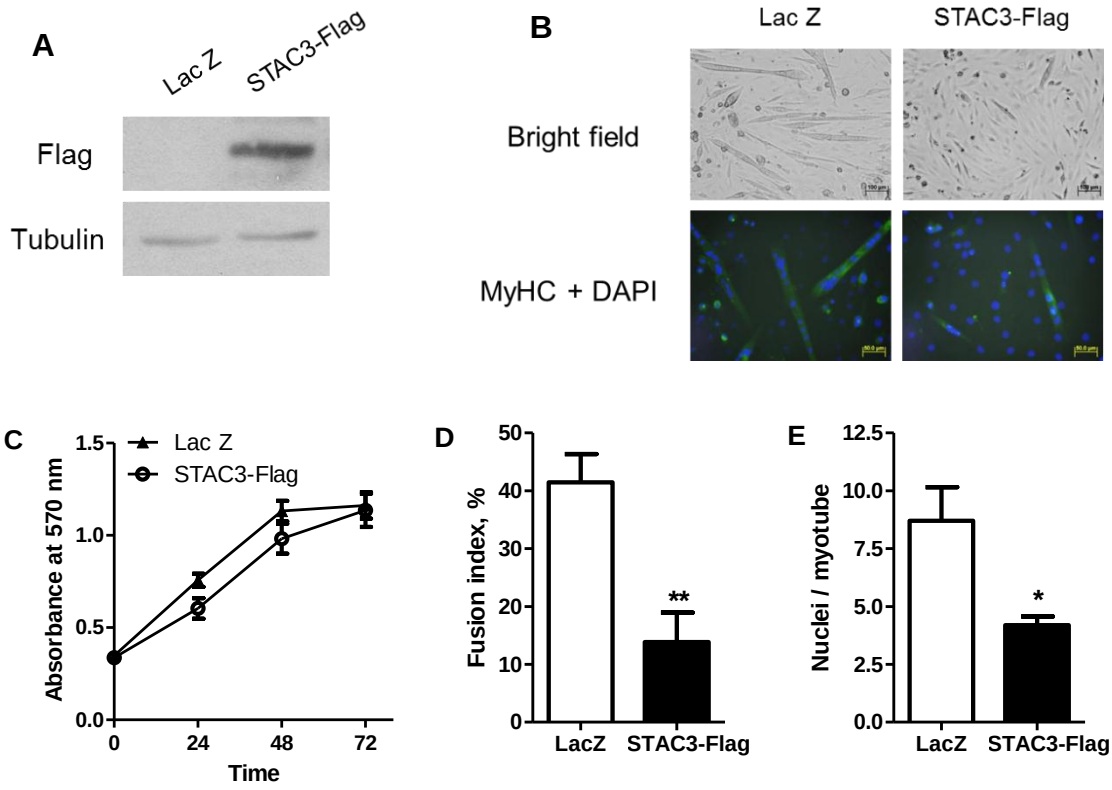
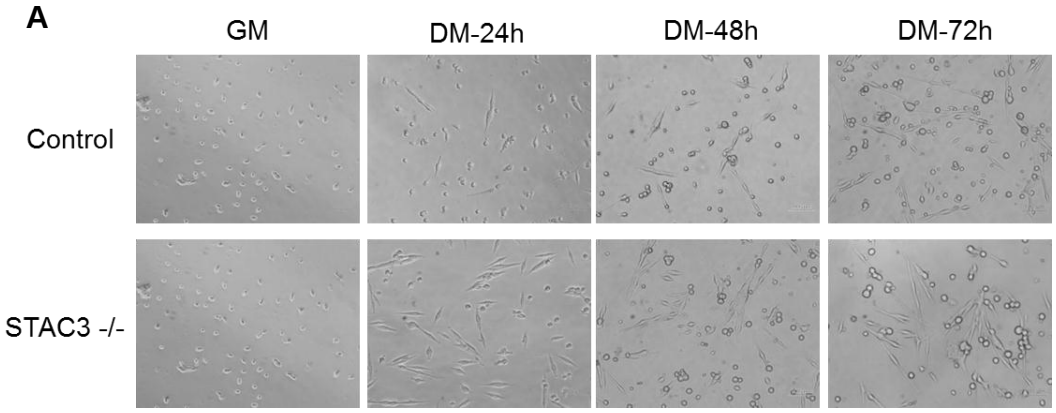
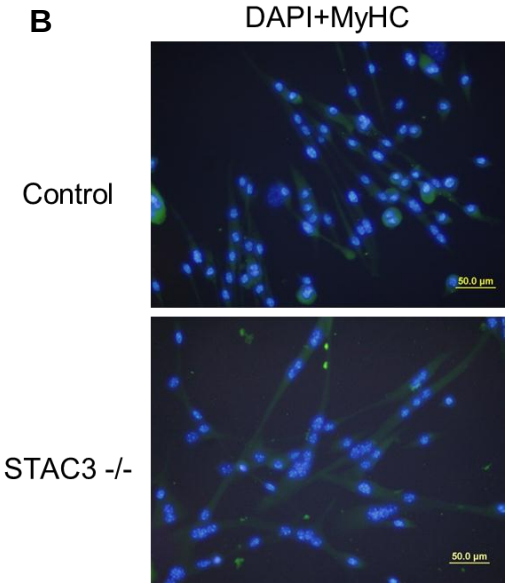
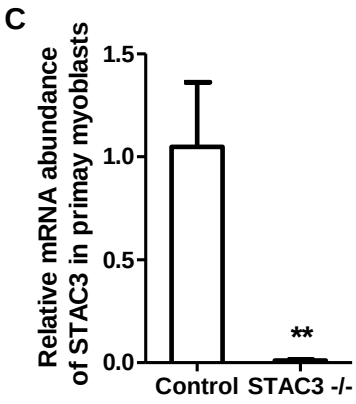
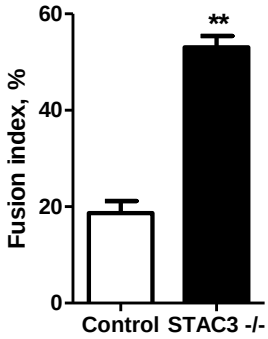

A
Lac Z
STAC3-Flag
Flag
Tubulin
B
Lac Z
STAC3-Flag
Bright field
MyHC + DAPI
C
Lac Z
STAC3-Flag
Absorbance at 570 nm
1.5
1.0
0.5
0.0
0
24
48
72
Time
D
Fusion index, %
50
40
30
20
10
0
**
LacZ
STAC3-Flag
E
Nuclei / myotube
12.5
10.0
7.5
5.0
2.5
0.0
*
LacZ
STAC3-Flag

Fig. 4.4. Stable STAC3 overexpression inhibited differentiation and fusion without affecting proliferation of C2C12 cells. (A) Western blot analysis of expression level of Flag-tagged STAC3 in C2C12 cells over-expressing Lac Z or STAC3 with tubulin as control. (B) Morphology and immunocytochemistry of established cell lines. Representative images of C2C12 cells over-expressing Lac Z or STAC3. Myotubes derived from Lac Z cells or STAC3 cells were stained with anti-MyHC antibody and DAPI. Pictures were taken under a phase contrast microscope, or a fluorescence microscope (20×magnification). (C) Equal numbers of STAC3 and LacZ overexpressing C2C12 cells were cultured in growth medium for 24, 48, and 72 h before the numbers of viable cells were determined. The absorbance at 570 nm on the y-axis represents the number of viable cells. There is no difference between STAC3 and Lac Z cell lines in proliferation (n = 4). (D, E) STAC3 and LacZ overexpressing C2C12 cells were induced to differentiate for 72 h. After staining of nuclei, the fusion index and the nuclear number were calculated. Histograms are means ± SEM of four clones from either STAC3 or LacZ stable cell line. * represents $P < 0.05$, ** represents $P < 0.01$, and n = 4.


A
GM
DM-24h
DM-48h
DM-72h
Control
STAC3 -/-


B
DAPI+MyHC
Control
STAC3 -/-
50.0 µm
50.0 µm


C
Relative mRNA abundance of STAC3 in primay myoblasts
1.5
1.0
0.5
0.0
**
Control
STAC3 -/-


Fusion index, %
60
40
20
0
**
Control
STAC3 -/-

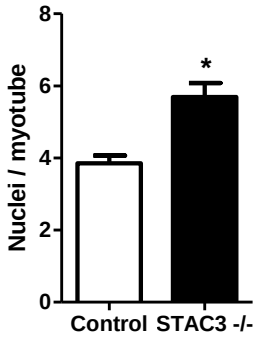

Nuclei / myotube
8
6
4
2
0
*
Control
STAC3 -/-

Fig. 4.5. Effects of STAC3 deletion on differentiation of mouse embryonic myoblasts. (A) Morphology of STAC3 deficient myoblasts and control myoblasts. Pictures were taken before differentiation and differentiated for 24 h, 48 h and 72 h. (B) Immunocytochemistry of STAC3 deficient myoblasts and control myoblasts. Myotubes were stained with anti-MyHC antibody and DAPI. Pictures were taken under a fluorescence microscope (20×magnification). (C) The STAC3 mRNA levels in myoblasts were assessed by real-time PCR using 18s as internal control. Histograms are means ± SEM. ** represents $P < 0.01$, and n = 3. (D, E) STAC3 deficient myoblasts and control myoblasts were induced to differentiate for 72 h. Nuclei were stained, and the fusion index and the nuclear number per myotube were calculated. Histograms are means ± SEM. * represents $P < 0.05$, ** represents $P < 0.01$, and n = 3.

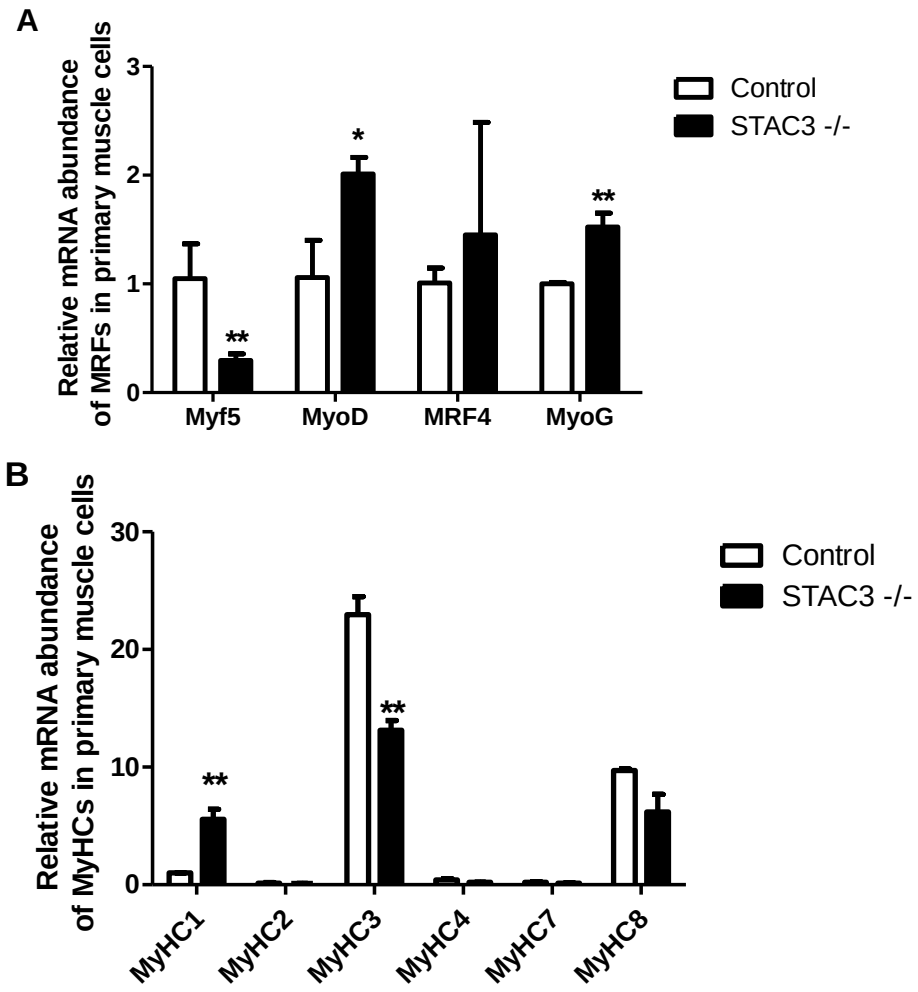

A
Relative mRNA abundance of MRFs in primary muscle cells
3
2
1
0
**
*
**
Myf5
MyoD
MRF4
MyoG
Control
STAC3 -/-
B
Relative mRNA abundance of MyHCs in primary muscle cells
30
20
10
0
**
**
MyHC1
MyHC2
MyHC3
MyHC4
MyHC7
MyHC8
Control
STAC3 -/-

Fig. 4.6. Effects of STAC3 deletion on expression of MRFs and MyHC mRNAs. (A) mRNA levels of MRFs in STAC3 -/- myoblasts and control myoblasts. Total RNA was extracted from myoblasts in differentiation medium for 48 h, and subjected to real-time PCR using 18s as internal control. Histograms are means ± SEM. * represents $P < 0.05$ and ** represents $P < 0.01$ within genes, and n = 3. (B) mRNA levels of six isoforms of MyHC in STAC3 -/- and control myoblasts. Total RNA was extracted from myoblasts in differentiation medium for 48 h, and subjected to real-time PCR using 18s as internal control. Histograms are means ± SEM. * represents $P < 0.05$ and ** represents $P < 0.01$ within genes, and n = 3.

Chapter V

Conclusions and Future Directions

Conclusions

Skeletal muscle mass is regulated by the number and the size of muscle fibers. The number of muscle fiber is determined during skeletal muscle development at prenatal stage, whereas the size of myofiber is due to changes at postnatal stage in response to environmental and physiological demands. Mature skeletal muscle, however, has the ability to incorporate new DNA at postnatal stage. The size of muscle fiber is regulated dynamically by the balance between anabolic and catabolic metabolism in the muscle. These processes contributing to skeletal muscle mass are controlled by a network of factors including transcription factors, hormones, and many signaling molecules. In this dissertation project, I conducted three independent studies to understand the roles of two hormone GH and IGF-I, and a potentially novel signaling protein STAC3 in skeletal muscle growth and development.

In the first study (Chapter II), the cellular effects of GH and IGF-I on skeletal muscle growth were compared using bovine satellite cells. The purpose of this study was to determine if GH stimulates skeletal muscle growth through IGF-I-independent direct mechanisms. I discovered that overall GH has different effects from IGF-I in skeletal muscle. Whereas GH had only effect on protein synthesis in bovine myotubes, IGF-I not only stimulated protein synthesis but also inhibited protein degradation in myotubes as well as stimulated myoblast proliferation. I also found that the anabolic effect of GH was unlikely mediated by local IGF-I as GH did not alter IGF-I mRNA expression in those cells.

In the second study (Chapter III), I investigated the signaling pathways responsible for various effects of IGF-I that lead to skeletal muscle growth. I again used the bovine satellite cells as the muscle cells model in this study. I discovered that IGF-I stimulated proliferation of bovine myoblasts and protein synthesis in bovine myotubes through both the PI3K/AKT and the MAPK signaling pathways, and that IGF-I inhibited protein degradation in bovine myotubes through the PI3K/AKT pathway only. This study also suggested that signaling from the PI3K/AKT or the MAPK signaling pathway leads to increased expression of CCND2, thereby mediating IGF-I stimulation of proliferation of bovine myoblasts.

In the third study (Chapter IV), I determined the potential role of STAC3 in the different steps of myogenesis, including myoblast proliferation, differentiation, and fusion, using the mouse myoblast cell line C2C12 cells as well as myoblasts directly derived from mouse embryos. Through gene overexpression, knockdown, and knockout, I discovered that STAC3 is a negative regulator of myoblast differentiation and fusion while it has no effect on proliferation of myoblasts.

Future Directions

Roles of GH and IGF-I in skeletal muscle growth

The following studies can be conducted to further understand the mechanisms by which GH and IGF-I stimulate skeletal muscle growth.

1) The signaling pathways by which GH stimulates protein accumulation in bovine myotubes. In my dissertation research, I showed that GH increased protein mass in myotubes without increasing IGF-I mRNA level. The signaling pathways by which GH stimulates protein synthesis need to be further investigated. One possibility is that GH signaling directly stimulates mTOR activity in bovine myotubes. Another possibility is that factors instead of IGF-I mediate the growth promoting effect of GH in bovine myotubes. One of such factors could be is NFATc2 as there was one study finding that GH increased myoblast fusion through NFATc2 but not IGF-I in mice.

2) The role of CCND2 in proliferation of bovine myoblasts. In my dissertation research, I noticed that IGF-I-stimulated proliferation of bovine myoblasts was associated with increased expression of CCND2 mRNA. This raises the possibility that CCND2 mediates IGF-I stimulation of myoblast proliferation. This possibility can be tested by CCND2 overexpression or knockdown in myoblasts.

Role of STAC3 in skeletal myogenesis

My studies have shown that STAC3 plays an important role in myogenic differentiation and fusion. Many studies could be conducted to further understand this role.

1) Expression level of STAC3 during muscle development in embryos. Skeletal muscle development includes a series of well-defined events. In mice, skeletal muscle is established between day 8.5/9 (E8.5/9) to day 18.5 (E18.5) at embryonic stage, and it further matures during the first 2~3 weeks of postnatal life. The primary fibers form before E 14.5, and, the secondary fibers form between E 14.5 and E18.5. The STAC3 deficient mouse embryos are lethal and likely because of skeletal muscle dysfunction. The dynamic change of expression level will give us some indication about at what stage STAC3 is possibly involved in muscle development.

2) Subcellular localization of STAC3 in myoblasts and myofibers. In order to perform their expected function, proteins need be localized at their proper subcellular compartment. Cell signaling proteins tend to stay in cytoplasm, while transcription factors can translocate from cytoplasm into nucleus. STAC3 appears to be a cytoplasmic signaling protein based on its molecular structure. It contains the SH3 domain and phorbol esters/diacylglycerol binding C1 domain.

3) Interacting proteins of STAC3. To fully understand the mechanism of STAC3 in myogenesis, it is important to identify the potential protein-protein interactions. Protein-protein interaction mediates most physiological process, such as subcellular structure assembly, regulation of gene expression and cell signal transduction. The identification of STAC3 interacting proteins will suggest its biological functions. Thus, identification and characterization of physical protein-protein interactions are essential for understanding the biological functions and underlying mechanisms of STAC3.