Roles of Oxytocin, Arachidonic Acid, and Proteasome in Bovine Myoblast Proliferation and Differentiation

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ABSTRACT

The overall objective of this dissertation project was to identify factors and mechanisms that control bovine myoblast proliferation, differentiation, and fusion. Three studies were conducted during this project. The objective of the first study was to determine the effect of oxytocin (OXT) on myoblast proliferation, differentiation and fusion. Treating primary bovine myoblasts in culture with 10 nM and 100 nM OXT for 24 h increased their proliferation rate by 7% ($P < 0.05$) and 10% ($P < 0.05$), respectively. Treating bovine myoblasts with either concentration of OXT for 48 h had no effect on their differentiation and fusion, as indicated by no changes in mRNA expression of selected myoblast differentiation markers and fusion index. The objective of the second study was to determine the effects of arachidonic acid (AA) and its major metabolites prostaglandin E_2 (PGE_2), PGF_{2a}, and PGI_2 on myoblast proliferation, differentiation and fusion. Treating myoblasts with 10 μM AA, 1 μM PGE_2, 1 μM $PGF_{2\alpha}$, and 1 μM PGI_2 for 24 h each increased the number of proliferating cells by 13%, 24%, 16%, and 16%, respectively, compared to the control ($P < 0.05$). At the same concentrations, AA, PGE_2, and PGF_{2a} stimulated myoblast differentiation and PGE_2 improved myoblast fusion ($P < 0.05$). Treating myoblasts with AA and the cyclooxygenase (COX)-1 and COX-2 inhibitor indomethacin or the COX-2-specific inhibitor NS-398 reversed the stimulatory effect of AA on myoblast proliferation ($P < 0.05$). The objective of the third study was to determine the role of the proteasome in bovine myoblast differentiation and fusion. It was found that the proteasome activity increased ($P < 0.05$) during myoblast differentiation and fusion. Adding 5 μM lactacystin, a specific inhibitor of the proteasome, to the differentiation medium nearly completely blocked myoblast differentiation and fusion. Inhibitor of DNA-binding 1 (ID1) is known to inhibit myoblast differentiation and to be degraded by the proteasome in some cells. Both ID1 protein and mRNA expression were found to decrease during myoblast differentiation

and fusion, and the decrease in ID1 protein but not ID1 mRNA was reversed ($P < 0.05$) by treating the cells with lactacystin. In summary, this project reveals that OXT and AA are stimulators of bovine myoblast proliferation and that AA is a stimulator of bovine myoblast differentiation. This project also indicates that the proteasome plays a positive role in bovine myoblast differentiation and fusion, and that it does so perhaps by reducing the accumulation of the ID1 protein.

GENERAL AUDIENCE ABSTRACT

Myoblast proliferation, differentiation, and fusion are key steps in skeletal muscle formation and growth. Three studies were conducted to identify factors and mechanisms that control these steps in cattle, agriculturally important animals. The objective of the first study was to determine the effect of oxytocin on proliferation, differentiation and fusion of myoblasts isolated from adult cattle. Treating bovine myoblasts in culture with oxytocin increased their proliferation rate but had no effect on their differentiation and fusion. The objective of the second study was to determine the effects of arachidonic acid and its major metabolites prostaglandin E2 (PGE_2), PGF_{2a}, and PGI_2 on proliferation, differentiation and fusion of bovine myoblasts. All of these chemicals were found to stimulate myoblast proliferation; AA, PGE2, and PGF2a to stimulate myoblast differentiation; and PGE_2 to improve myoblast fusion. The objective of the third study was to determine the role of the proteasome in bovine myoblast differentiation and fusion. The proteasome activity was found to increase during myoblast differentiation and fusion. Furthermore, the proteasome activity was found to promote myoblast differentiation and fusion. These results imply that skeletal muscle development and growth in cattle could be potentially improved by administrating cattle with oxytocin or prostaglandin or by altering the activity of the proteasome in cattle skeletal muscle.

DEDICATION

This work is dedicated to Wanru Wang (汪琬如), my grandmother who raised me and has been a strong female figure in my life.

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Chapter 1 Literature Review

1.1 Introduction

Skeletal muscle, a type of striated muscle, contracts under the control of the somatic nervous system, also known as voluntary control. Nearly 40% of the total body weight and 50-75% of all body protein in mammals is skeletal muscle. Skeletal muscle has four major properties: excitability, contractility, extensibility, and elasticity. These features ensure that skeletal muscle functions during movement, posture, joints stabilization, and heat generation. The mass of skeletal muscle is determined mainly by protein synthesis and protein degradation, which are affected by many factors including nutrient, hormone, exercise, injury, and disease.

The process of skeletal muscle formation, or myogenesis, can be generally divided into two phases: embryonic myogenesis and postnatal muscle development and growth. Both stages of muscle development and growth are tightly controlled. For years, postnatal muscle growth and muscle regeneration are studied using muscle cell lines and primary muscle cells. Satellite cells, the main type of muscle progenitor cells in adulthood, provide an invaluable model for recapitulation of embryonic and postnatal muscle development and muscle regeneration.

In this review, I will summarize and discuss current understanding of the key steps in skeletal muscle development, growth, and contraction, and the key factors and mechanisms that control these steps.

1.2 Skeletal muscle organization and physiology

1.2.1 Skeletal muscle structure

Skeletal muscles are separated from surrounding tissues by a dense irregular layer of connective tissue, epimysium. A thicker connective tissue layer, perimysium, holds groups of

muscle fibers forming fascicles. Within each fascicle are individual muscle fibers, known as myofibers. Each muscle fiber is surrounded by an even thicker connective tissue layer, endomysium. Epimysium, perimysium, and endomysium merge at the ends of muscles to form either tendons or aponeuroses that attach to bones (Akers and Denbow, 2013; Hall, 2015).

Muscle fibers are large, multinucleated cells. One muscle fiber can spread across an entire skeletal muscle and contains hundreds of nuclei. The nuclei are located closely to the basal side of the sarcolemma, the plasma membrane of muscle fibers. Each muscle fiber is made of hundreds or thousands of myofibrils. A myofibril is mainly made of thick and thin filaments formed by contractile proteins, myosin and actin (**Figure 1.1**). A myofibril is a tandem of sarcomere, the basic contractile unit of skeletal muscle. Under the microscope, sections of skeletal muscle show alternating bands (A band, I band) and distinct lines (M line, Z line). A sarcomere is the muscle tissue between two neighboring Z lines. An A-band contains entire thick filaments and parts of thin filaments overlapping with thick filaments. A light region that can be seen in the center of an A band in resting muscle is called an H zone, and contains only thick filaments. A M line is a line

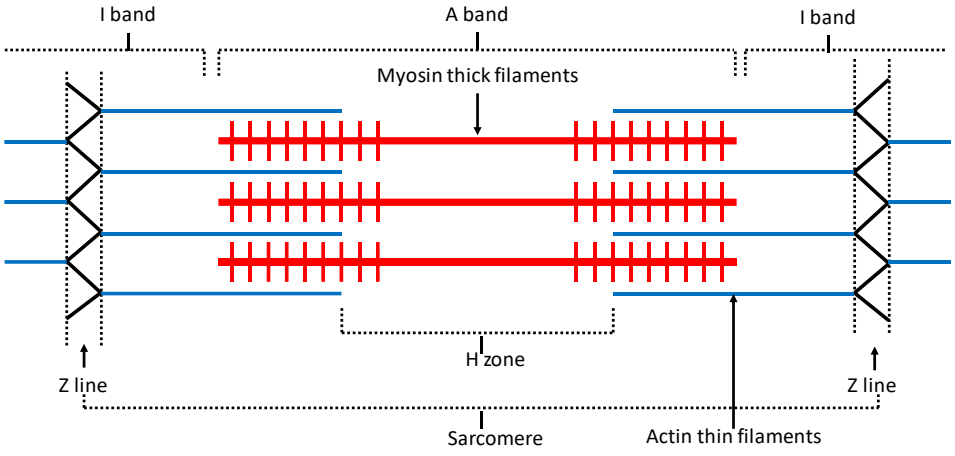

Figure 1.1 Basic myofibril and sarcomere structure. Myofibril consists of two types of myofilaments, myosin thick filaments and actin thin filaments. A sarcomere, the functional unit of skeletal muscle, runs from one Z line to next Z line. The various segments of the myofibril and sarcomere are identified in the graph (Adapted from Akers, R. M., and D. M. Denbow. 2013. Anatomy and Physiology of Domestic Animals. Wiley)

in the middle of an H zone and is formed by proteins that crosslink thick filaments. An I band is the sarcomere region between two A bands and contains only thin filaments. A Z line is made of discs bisecting I bands and serves as anchoring points for thin filaments. Each sarcomere is composed of a half of the first I band, an A band, and a half of the second I band (Hopkins, 2006; Akers and Denbow, 2013; Hall, 2015).

1.2.2 Skeletal muscle excitation and contraction

As mentioned at the beginning of this review, skeletal muscle contracts in response to signals from the nervous system. The α motor neurons project axons from the central nervous system (CNS) to innervate skeletal muscle at neuromuscular junctions. Each myofiber is innervated by a neuron, while a single neuron may innervate multiple myofibers though branching at perimysium. Each neuron branch end is a synaptic terminal that forms a synapse with its target myofiber. When a motor neuron fires, it generates action potentials, and the action potentials travel along the axon of the motor neuron causing the release of the neurotransmitter acetylcholine (ACh) from the axon terminals into the neuromuscular junctions. Diffusing across the synaptic cleft, ACh binds to the nicotinic receptor on the sarcolemma, which leads to the opening of ligand-gated ion channels. Abundant Na^+ ions entering the myofiber through these channels cause the generation of action potentials on the sarcolemma. Propagating through activated voltage gated Na^+ channels, action potentials spread through the sarcolemma and reach the invaginations in the sarcolemma called transverse-tubules (T-tubules). T-tubules run perpendicular to the sarcolemma and deep into the sarcoplasm. Sarcoplasmic reticulum (SR), the muscle-specific endoplasmic reticulum, stores intercellular Ca^{2+}. At the A-I band junction, the t-tubule is associated with a terminal cisterna of SR on each side, forming a three-component structure called triad. The triads play a critical role

in excitation-contraction coupling. At the triads, the dihydropyridine receptors (DHPR), a voltage sensor and an L-type calcium channel located on the T-tubules, physically interacts with type 1 ryanodine receptors (RyR1), also a type of Ca^{2+} channel located on the terminal cisternae. When action potentials transmit from the sarcolemma to the T-tubules, they cause conformational changes in the DHPR. These changes lead to the opening of the RyR1s and the efflux of Ca^{2+} from the SR (Hernández-Ochoa et al., 2016; Dulhunty et al., 2017).

The major proteins that are responsible for muscle contraction are contractile proteins actin and myosin, regulatory proteins tropomyosin and troponin, and other structural proteins (e.g., titin, myomesin, and dystrophin). In a thick filament, myosin molecules, which have long "tails" and globular structured "heads," are arranged to project outward the thick filament and toward the neighboring thin filament. The globular protein on the myosin head has ATPase activity and can bind to the actin filament. A thin filament is mainly a double helix of filamentous actin (F actin) that is a chain of globular actin (G actin) molecules. The thin filament also contains tropomyosin, a protein that runs along the double helix of F actin and that covers seven myosin-binding sites on F actin. A third protein of the thin filament is the globular protein troponin, which is typically located near the myosin binding sites on F actin. Troponin is a complex of three subunits: troponin T (TnT), which binds to tropomyosin; troponin I (TnI), which binds to G actin; and troponin C (TnC), which binds to Ca^{2+} (Hopkins, 2006; Akers and Denbow, 2013; Hall, 2015; Polster et al., 2015).

When the muscle is not contracting, the myosin-actin binding is blocked by TnI and tropomyosin. Upon the release of Ca^{2+} from the SR into the sarcoplasm, Ca^{2+} binds to TnC, causing an allosteric change in TnI. The conformational change in TnI leaves the space for tropomyosin to slide into the groove created by F actin strands, exposing the myosin binding sites. Based on the

"sliding filament theory" (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954), heads of myosin filaments bind to the myosin-binding sites on G actin, liberating ADP and inorganic phosphate (Pi). The power stroke of myosin pulling actin filaments towards the M-line initiates contraction. Myosin heads remain attached to F actin until ATP attaches to the myosin head and breaks the myosin-actin binding. Subsequently, the myosin head returns to its original position, hydrolyzing ATP to ADP and Pi. This cycle of sliding filaments repeats itself as long as the cytosolic Ca^{2+} concentration is abundant, myosin-actin binding sites are exposed, and sufficient ATP is present. Eventually, with the help of ATP, Ca^{2+} is removed from the sarcoplasm and transported back to the SR through the calcium active transport pump, also known as sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, located on the SR. Once the cytosolic Ca^{2+} level drops, Ca^{2+} dissociates from TnC, troponin returns to its resting configuration, and tropomyosin and TnI again cover the myosin-actin binding sites (Hopkins, 2006; Akers and Denbow, 2013; Hall, 2015; Polster et al., 2015).

1.2.3 Stac3 in skeletal muscle EC coupling

The Src homology three (SH3) domain and cysteine rich (C1) domain 3 (Stac3) gene has recently been found to play an essential role in skeletal muscle contraction. Stac3 belongs to the Src homology three (SH3) domain and cysteine rich (C1) (STAC) gene family that also includes Stac1 and Stac2 (Iftinca and Altier, 2017). Stac1 and Stac2 have been found to be specifically expressed in neurons (Suzuki et al., 1996; Kawai et al., 1998) and work as markers for different subsets of dorsal root ganglia (Legha et al., 2010). Stac3 is highly expressed in skeletal muscle (Nelson et al., 2013; Reinholt et al., 2013) and is localized to the triad (Nelson et al., 2013; Campiglio and Flucher, 2017). Stac3 knockout (KO) mice showed 100% fatality after birth with

the inability of respiration and pathological morphology, suggesting defects in muscle development, neuromuscular junction function, or muscle contraction (Nelson et al., 2013; Reinholt et al., 2013). Further studies revealed that Stac3 KO muscle lost its contractile ability under electrostimulation, yet that it contracted in response to the RyR agonist 4-CMC or caffeine. (Horstick et al., 2013; Nelson et al., 2013; Cong et al., 2016b). However, with the treatment of RyR agonists, only 60% of the contractile ability of Stac3 KO hindlimb muscle was resumed, suggesting that post EC coupling defects play a role in reduced contractility (Cong et al., 2016a). A more recent study reveals that $Ca_v1.1$, the alpha 1A subunit of DHPR, is the major interaction partner of Stac3 in skeletal muscle EC coupling (Campiglio and Flucher, 2017).

1.2.4 Skeletal muscle fiber type classification

Skeletal muscle fibers are structurally and functionally heterogeneous. Muscle fibers differ in diameter (ranging from 10 to 100 μM), in color (varying from red to pink to white), and in the amounts of myoglobin, capillaries, and mitochondria (Schiaffino and Reggiani, 2011; Akers and Denbow, 2013). Muscle fibers can also differ in contractile ability (fast or slow) and metabolic mechanism (aerobic/oxidative and anaerobic/glycolytic) (Blaauw et al., 2013). Fast-twitch fibers usually fatigue quickly with high maximum shortening velocity, use predominantly glycolytic metabolism, have less mitochondria and myoglobin content, and are relatively pale colored. Slow-twitch fibers are more fatigue-resistant with low mechanical power output, use aerobic metabolism, have more mitochondria and myoglobin content, and are red. The third fiber type is the intermediate fast fiber that features a blend of fast and slow twitch fibers characteristics

(Blaauw et al., 2013). However, the categorization of this muscle fiber type is somewhat ambiguous due to the continuum nature of skeletal muscle fiber arrangement.

The most widely used classification of fiber type at the molecular level is based on the expression level of myosin heavy chain (MyHC) protein isoforms. There are four major adult skeletal muscle MyHC isoforms: type I, type IIA, IIX (or IID), and IIB. The slow-twitch fiber has predominantly type I fiber (encoded by myosin heavy chain 7, MYH7, gene) while the fast-twitch fiber type IIB fiber (encoded by MYH4). The IIA (encoded by MYH2) and IIX (or IID) (encoded by MYH1) fibers are intermediate between type I and type IIB (Pette and Staron, 2001). Additionally, other MyHC isoforms are identified in developing muscle (MyHC-emb and MyHC-neo, encoded by MYH3 and MYH8, respectively), cardiac muscle (MyHC-α and MyHC-β, encoded by MYH6 and MYH7, respectively), and extraocular or jaw muscles (MyHC-EO and MyHC-M, encoded by MYH13 and MYH16, respectively) (Schiaffino and Reggiani, 2011). Although most fibers express only one MyHC isoform, hybrid fibers contain two or more MyHC isoforms (Weiss and Leinwand, 1996). The pure and hybrid fibers in muscle can switch or transit among close isoforms in the following order: I ↔ IIA ↔ IIX (or IID) ↔ IIB. Although two neighboring fiber types can be co-existing in the same muscle, their "adaptive range" is limited. Slow fibers typically transit in the range of I ↔ IIA ↔ IIX (or IID) while fast fibers in the range of IIA ↔ IIX (or IID) ↔ IIB (Pette and Staron, 2001; Schiaffino and Reggiani, 2011; Blaauw et al., 2013).

Another common way of fiber classification is based on the ATP generation paths and the abundance of various enzymes for ATP generation. The fastest ATP supply is from direct phosphorylation of creatine phosphate (CP) and ADP by creatine kinase. This process is more active in fast than in slow fibers. The glycolytic pathway utilizes glucose-6-P derived from

sarcoplasm and blood flow with the need of pyruvate-to-lactate conversion under lactate dehydrogenase (LDH). This pathway is twice as active in fast fibers as in slow fibers, providing fast but not as long time of ATP supply. The oxidative pathway with ATP regeneration through mitochondria is more effective in slow fibers than in fast fiber. Slow fibers show a significantly higher amount of mitochondria along with more enzymes participating in the TCA cycle, such as succinate dehydrogenase (SDH) and nicotinamide adenine dinucleotide (NADH). In the oxidative pathway, ATP supply is slow but long lasting (Gerrard and Grant, 2003; Blaauw et al., 2013). Based on metabolic enzymes and activities, fibers are categorized as slow type I oxidative fibers, fast type IIA oxidative fibers, fast IIX oxido-glycolytic fibers, and fast IIB glycolytic fibers.

Fibers can be further specified according to regulatory and structural proteins such as myosin light chains (MYL), troponins, and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), which have various isoforms (Schiaffino and Reggiani, 2011; Blaauw et al., 2013). The combinations of various myosin proteins and non-myosin proteins form diverse muscle fibers that meet distinct functional demands of different muscles.

Cattle, as large ruminant animals, have different physiology from non-ruminant mammals. Thus, the MyHC isoform composition in bovine skeletal muscle differs from that of many other mammals. Understanding the fiber isoform composition in meat animals like cattle helps control meat quality (Xiong, 1994). A fragment of *bovine* fetal MYH gene isoform was first sequenced in 1994 (Young et al., 1994). Subsequent sequencing led to the identification of more bovine MYH genes, including those encoding the MyHC-IIA, -IIX, and - I fibers. Significant difference in MYH isoform composition is found between in the same named *bovine* and *porcine* muscles; for example, MYH4, is not found in *bovine* muscles (Tanabe et al., 1998; Chikuni et al., 2004). Several studies demonstrated that the bovine MYH4 is expressed in the extraocular muscles but not in the

limb or trunk muscles (Maccatrozzo et al., 2004; Toniolo et al., 2005). Picard et al. detected mRNA expression of MYH4 only in five of the twenty two *Blonde d'Aquitaine* bulls used in their study indicating complication in MYH4 expression regulation in cattle (Picard and Cassar-Malek, 2009). All three fast MyHC isoforms, MyHC-IIA, -IIX, and -IIB, were detected from bovine muscle on SDS-PAGE with a special running buffer (Picard et al., 2002; Picard et al., 2011). Clearly, the continuum of muscle fiber composition and transition makes the myosin heavy chain isoform identification more complicated. Only the combination of traditional histochemical or immunohistochemical techniques and molecular biology techniques (i.e., qPCR, western blotting) ensures the clarification of fiber type identification.

1.3 Skeletal muscle myogenesis

Skeletal muscle development and growth in animals can be divided into two stages: stage one, the growing of muscle fiber number, known as hyperplasia; and stage two, the growing of muscle fiber size, also known as hypertrophy (Gerrard and Grant, 2003; Akers and Denbow, 2013). In most mammals, the total number of muscle fibers is fixed before birth, which means postnatal muscle growth is mainly the result of hypertrophy (Picard et al., 2002). Both hyperplasia and hypertrophy in skeletal muscle growth and development are aided by muscle progenitor cells providing nuclei and genetic material. Satellite cells are the most widely studied muscle progenitor cells.

1.3.1 Embryonic myogenesis

In the early stage of embryogenesis, an embryo will develop into three layers: endoderm, mesoderm, and ectoderm. The mesoderm further divides into paraxial, intermediate, and lateral

mesoderm. Paraxial mesoderm is closely aligned on both sides of the axial organs, the neural tube and notochord. The somite is subsequently derived from the paraxial mesoderm along the anterior-posterior axis (Bentzinger et al., 2012). The cyclic genes involved in somitogenesis are under the control of Notch signaling, which is further under the control of Wnt and fibroblast growth factors (FGFs) (Hofmann et al., 2004). A part of somite dissociates ventrally and develops into mesenchymal sclerotome, while the remaining somite dorsal epithelial roof becomes the dermomyotome. Dermomyotome, containing multipotent progenitor cells, is the origin of trunk and limb muscles, dermis, endothelial cells, brown fat tissue, vascular smooth muscle, and cartilage of the scapula blade (Buckingham, 2006).

Myogenic precursor cells (MPCs) undergo an epithelial-mesenchymal transition (EMT) in response to environmental cues. The MPCs from dorsomedial dermomyotomal border translocate to the ventral position under dermomyotome and form primary myotome consisting of primitive muscles. This primary myotome serves as scaffold for a second wave of myotome growth by adding MPCs from the cranial and caudal dermomyotomal borders (Gros et al., 2004; Pu et al., 2013). Sonic hedgehog (SHH), secreted from the floor plate and notochord, facilitates the development of epaxial (dorsal) dermomyotome into epaxial myotome where back muscles originate (Borycki et al., 1999). Hypaxial (ventral) dermomyotome produces hypaxial myotome under the regulation of the Wnt pathway and bone morphogenetic protein (BMP) 4 from the ectoderm and lateral plate mesoderm separately. Ventral myotome produces limbs, diaphragm, and body wall muscles (Cheng et al., 2004).

All MPCs in the dermomyotome express paired box gene transcription factors Pax3. Once entering the myotome, MPCs quickly undergo myogenic determination and become myoblasts. Pax3 expression level gradually decreases with the increased expression of myogenic regulatory

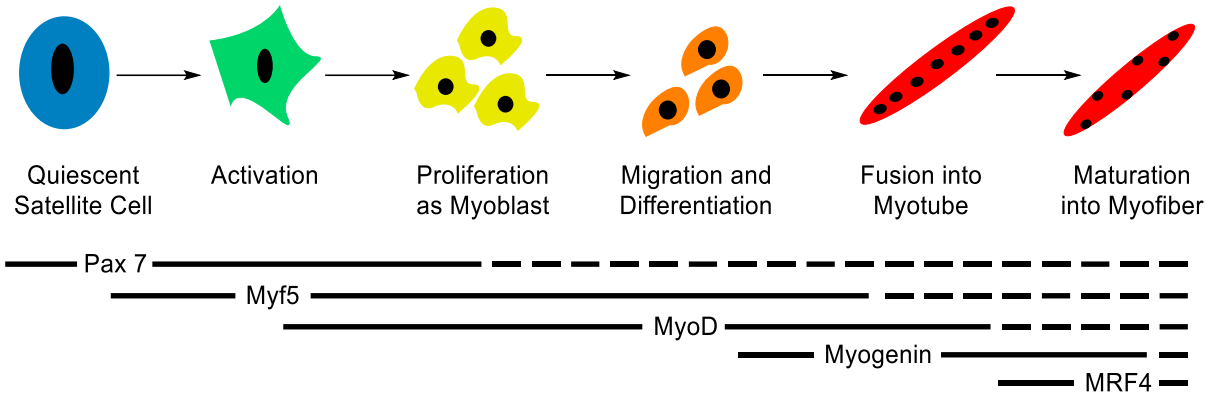

Figure 1.2 The expression pattern of transcription factors during myogenesis. The overall myogenic differentiation pathway includes the activation of quiescent satellite cells, proliferation as myoblasts, commitment to differentiation, fusion to form myotubes, and ultimately maturation into myofibers. The various transcription factors expressed at different stages of myogenesis are shown in the graph. A solid line indicates the expression of a transcription factor; a dotted line indicates the diminished expression of a transcription factor. Pax7, paired box 7; Myf5, myogenic factor 5; MyoD, myogenic determination 1; MRF4, myogenic regulatory factor 4. (Adapted from Buckingham, M. 1994. Muscle Differentiation: Which myogenic factors make muscle? Curr. Biol. 4:61–63. doi:10.1016/S0960-9822(00)00014-2.)

factors (MRF) myogenic factor 5 (Myf5) (Kiefer and Hauschka, 2001) and myoblast determination protein (MyoD) (Yusuf and Brand-Saberi, 2006). MyoD and Myf5 are markers for committed muscle lineage cells (Pownall et al., 2002); and MyoD expression occurs sequentially after Pax3 and Pax7 (Rudnicki et al., 1993; Maroto et al., 1997). $MyoD^{+}/Myf5^{+}$ cells will withdraw from cell cycle and form embryonic muscle compartments. Eventually, those myoblasts will elongate and start to fuse and form myotubes, expressing myogenin (MyoG) (Hasty et al., 1993) and myogenic regulatory factor 4 (MRF4; also known as Myf6) (Zhang et al., 1995; Rawls et al., 1998). Some of the Pax3 and Pax7 positive cells added to myotome during the second wave of development keep $Pax3^{+}/Pax7^{+}$ signal and continuously proliferate and replenish the progenitor cell pool (Gros et al., 2005) (**Figure 1.2**).

Another group of hypaxial dermomyotome MPCs in the adjacent limb regions leave dermomyotomal epithelial structure after EMT and migrate towards limb buds. These MPCs only express Pax3 with negative MRFs expression to retain their proliferation state during the

migration. Once reaching the muscle forming area in limb buds, these MPCs start to express MRFs and differentiate into myofibers with downregulation of Pax3 expression. Interestingly, the expression of hepatocyte growth factor (HGF) and its receptor c-met is also associated with MPCs migration. HGF mRNA is found predominantly at adjacent limb mesenchyme while c-met is co-expressed with Pax3 in migrating MPCs (Dietrich et al., 1999). Mice with no HGF/c-met expression show muscle-free limbs due to ceased MPCs migration from dermomyotome to limb buds during embryonic development (Bladt et al., 1995; Dietrich et al., 1999).

The formation of multinucleated myotubes is followed by the hypertrophy of the musculature system to form functional myofibers during fetal development. In cattle, myotube formation during muscle development has three distinct phases (Picard et al., 2002). The first generation of myotubes is based on embryonic myoblasts, giving rise to primary muscle fibers. Primary myotubes have limited contribution to mature muscle fibers, but they serve as the scaffold for the formation of secondary muscle fibers from fatal myoblasts. The third generation fibers are small in diameter, express developmental MYH, and are closely associated with secondary fibers. The third generation of fibers are also considered a partial reason of large muscle fiber formation. The third wave of fiber generation exists not only in cattle but also in other large animals such as sheep, pigs, and humans. It is believed that the number of primary myofibers is under the genetic control in cattle while the number of secondary myofibers is more influenced by nutrition and growth factors (Picard et al., 2002). Primary and secondary fibers both first express MyHC-slow, -fast, -emb, -neo, and -α. The MyHC expression pattern in primary fibers is gradually changing toward only MyHC-slow in type I muscle fiber, but MyHC-fast can be predominantly expressed in fast fiber-only muscles through cattle maturation. Most of the secondary fibers develop into type II fibers with a small amount of type I. By the end of gestation, all developmental MyHC isoforms

(MyHC-emb and -neo) are replaced with adult isoforms (MyHC- I, IIA, IIX). (Picard et al., 1994; Gagniere et al., 1999).

Another population of myoblasts does not participate in the prenatal myofiber formation but stays in close proximity to formed muscle fibers. Those cells that keep the whole set of genetic information and that are able to serve as the postnatal nuclei source for muscle growth are hypothesized to be adult satellite cells. Satellite cells are believed to be originated from a subpopulation of $Pax3^{+}/Pax7^{+}$ cells from the central dermomyotome between the primaxial and abaxial regions (Gros et al., 2005; Schienda et al., 2006; Messina and Cossu, 2009). By down-regulating MyoD expression and inhibiting MyoG induction, Pax7 manages to prevent satellite cells from undergoing myogenesis (Olguin et al., 2007). Adult satellite cells serve as the nuclear resource of myofibers under muscle "wear and tear" by adding nuclei into existing myofibers or fusing into new myofibers to replace the damaged myofibers (Yablonka-Reuveni, 2011). A subpopulation of adult satellite cells also undergo self-renewal to replenish the satellite reservoir (Chakkalakal et al., 2014). Thus, satellite cells are not only important for postnatal muscle growth but also for muscle repair and regeneration in adulthood.

1.3.2 Postnatal skeletal muscle growth and development

1.3.2.1 The roles of satellite cells in skeletal muscle postnatal growth

Even though DNA dividing and replicating ability is lost in myofibers after birth in most mammals (Staun, 1963; Rowe and Goldspink, 1969) including cattle (Picard et al., 1994; Gagniere et al., 1999), the DNA content in healthy skeletal muscle increases substantially during postnatal development (Moss, 1968). Satellite cells contribute up to 30% of the total nuclei in skeletal muscle after birth, although this percentage decreases to 2-7% with aging (Yin et al., 2013). They

are believed to be responsible for the addition of nuclei to existing muscle fibers (Moss and Leblond, 1971). The mononucleated myoblasts developed from satellite cells are capable of DNA renewal and fusion into myotubes in vitro (Stockdale and Holtzer, 1961).

A. Satellite cell as muscle progenitor cells and muscle stem cells

Adult vertebrate skeletal muscle shows tremendous ability of to regenerate after injury or atrophy. First observed in 1961, satellite cells were found lying in basal lamina, the niche between the sarcolemma and the extracellular matrix (ECM). Mauro proposed that satellite cells were dormant embryonic myoblasts that could rapidly become activated under muscle injury and function similarly as the myoblasts during embryonic myogenesis (Mauro, 1961). The engraftment of single myofiber and its satellite cells into radiation-ablated muscle successfully restored the regeneration ability of the muscles with no indigenous satellite cell (Collins et al., 2005; Rocheteau et al., 2012). The ablation of Pax7 (a satellite cell marker) positive cell pool in adult skeletal muscle fully abolished the regeneration ability of adult muscles (Lepper et al., 2011; Sambasivan et al., 2011). These findings suggest that satellite cells are indispensable myogenic progenitor cells for muscle regeneration.

A group of satellite cells has the ability to expand the satellite cell population for muscle regeneration through self-renewal. Indeed, a series of studies have proven that the Pax7 positive satellite cells can undergo asymmetric cell division (Kassar-duchossoy et al., 2005; Cinnamon et al., 2006; Shinin et al., 2006). In their quiescent state, a subpopulation of satellite cells with high levels of Pax7 and lower metabolic rate are less primed to myogenic commitment under activation signal with longer response time to enter cell cycle. Even though the subsequent cell cycle elapse is the same in Pax7-high cells as the remaining satellite cells, the Pax7-high population is only

replenished by Pax7-high decedents (Rocheteau et al., 2012). Satellite cells that commit to proliferation and/or differentiation as myoblasts express Myf5 with a portion of $Pax7^{+}$ cells withdrawing from cell cycle and maintaining the full set of genetic information of the animal (Rocheteau et al., 2012). Approximately 10% of Pax7+ satellite cells never express Myf5. Those Pax7+/Myf5- satellite cells can asymmetrically give rise to Pax7+/Myf5+ cells that undergo differentiation and Pax7+/Myf5- cells that contribute to maintaining satellite cell population (Kuang et al., 2007). Thus, satellite cells are considered heterogeneous myogenic progenitor cells with a subpopulation of committed stem cell fate and a subpopulation of committed myogenic terminal differentiation fate.

The distinction of definitions between muscle progenitors and muscle stem cells have gradually become somewhat vague in postnatal animals. Muscle progenitor cells are thought to be leftover muscle lineage committed cells during embryonic development and exhibit the ability to divide for limited generations and differentiate into muscle fibers. Muscle stem cells, on the other hand, should have infinite self-renewal as well as muscle or other types of tissue regeneration ability (Robey, 2000). Upon severe muscle injury under either physiological or pathological conditions, muscle regeneration relies on satellite cell function and goes through a process involving myofiber necrosis, inflammatory response, activation of satellite cells, proliferation and differentiation of satellite cell-derived myoblasts (Kardon et al., 2002). Therefore, skeletal muscle satellite cells can be considered muscle progenitor and muscle stem cells because of their dormant nature under normal condition, ability to activate upon stimulation, and ability to replenish the satellite cell pool through self-renewal.

B. Satellite cell quiescence

In healthy resting muscle, satellite cells usually remain in a dormant state known as quiescence, referred to as G_0 in the cell cycle (Cheung and Rando, 2013). Cell quiescence is a reversible process. Quiescent cells can be identified by low content of RNA, lack of cell proliferation markers, and a low turnover rate indicated by retention of labels like 5'-bromo-2'-deoxyuridine (BrdU) or H2B-GFP/YFP (Tumbar et al., 2004; Shinin et al., 2006; Fukada et al., 2007; Buczacki et al., 2013). Rapidly dividing cells lose the labeling signal much faster than quiescent cells, which have a relatively slow metabolic rate. Quiescent satellite cells can be also identified by lineage tracing (Buczacki et al., 2013). Lineage tracing can help distinguish low-turnover stem cells like fibrogenic and adipogenic progenitor cells from functionally different muscle stem cells (Joe et al., 2010; Uezumi et al., 2010). Quiescent muscle stem cells were purified by fluorescence-activated cell sorting (FACS) based on the SM/C-2.6+ cell surface marker in murine (Fukada et al., 2007).

Microarray analyses of more than 500 genes in quiescent muscle stem cells showed low-level expression of cyclins, cyclin-dependent kinases (CDKs), and checkpoint kinases (CHKs), yet high-level expression of negative cell cycle regulators CDK inhibitors 1B (CDKN1B; also known as p27 or $p27^{Kip1}$) and 1C (CDKN1C; also known as p57 or $p57^{Kip2}$), retinoblastoma tumor suppressor protein (Rb; also known as Rb1), regulator of G-protein signaling 2 and 5 (Rgs2 and Rgs5), peripheral myelin protein 22 (Pmp22), and the negative regulator of FGF signaling sprouty 1 (Spry 1) (Fukada et al., 2007; Shea et al., 2010; Liu et al., 2013). In quiescent satellite cells, upregulated Notch signaling activates the Notch target genes that repress the expression of MyoD, one of the satellite cell myogenic fate determinants, but promote the Pax7 expression for preserving muscle lineage and rapid reentry into cell cycle (Bjornson et al., 2012; Wen et al.,

2012). Deletion of the transcription factor Forkhead box protein O3 (FOXO3) in quiescent satellite cells led to decreased expression of Notch 1 and 3 receptors and reduced satellite cell number but increased differentiation of committed muscle progenitor cells. Thus, FOXO3 regulates satellite cell quiescence through Notch signaling (Gopinath et al., 2014). microRNAs (miRNAs) are also involved in maintaining stem cell quiescence. For instance, miR-489, which is highly expressed in quiescent satellite cells, post-transcriptionally represses the oncogene *Dek* and thereby prevents satellite cells from entering cell cycle (Cheung et al., 2012).

C. Satellite cell activation

Satellite cells are activated and proliferate rapidly during neonatal growth to support the gaining of muscle mass (Schultz, 1996; Mesires and Doumit, 2002). Satellite cells become mitotically quiescent in adult skeletal muscle, maintaining their G_0 state. During muscle damage, exercise, or disease, satellite cells are activated to proliferate as myoblasts. Following muscle injury, satellite cells enter a G_{Alert} state with larger shape, higher metabolic activity, and increased cell cycle gene expression level, but do not proliferate. Satellite cells in intermediate G_{Alert} state have a greater potential for muscle regeneration due to their faster cell division speed compared to cells in G_0 state (Rodgers et al., 2014). Upon activation, satellite cells express the myogenic progenitor markers, MyoD, desmin, and MyoG (Smith et al., 1994).

Damage of muscle fibers destructs the niches for satellite cells and promotes the mobility and activation of satellite cells. At the same time, local damage to sarcolemma and basal lamina, combined with cation channel couplings, allow the increase of intracellular calcium. Calcium binds to calmodulin and activates nitric oxide synthase (NOS). Elevating NOS leads to increased nitric oxide (NO) production, which in turn triggers extracellular matrix (ECM) to release

hepatocyte growth factor (HGF) (Tatsumi et al., 2006; Tatsumi et al., 2009; M. Hara et al., 2012). It is believed that HGF binding to its receptor c-Met initiates the satellite cell activation signal. HGF from local injury areas activates mTORC1 in distant quiescent satellite cells and promotes their G_0 to G_{Alert} transition and the re-entry of cell cycle (Allen et al., 1995; Tatsumi et al., 1998; Rodgers et al., 2014). Satellite cells also produce HGF; thus, HGF could work in either endocrine or autocrine manner (Sheehan et al., 2000). Though the molecular mechanism of HGF activation of satellite cells is not yet clear, HGF may work through its downstream tyrosine kinase pathway to change the expression of cell cycle related genes.

Besides HGF, other growth factors released from damaged muscles can also activate signaling pathways related to cell cycle reentry. For example, insulin-like growth factor-1 (IGF-1) inactivates Forkhead box protein O1 (FOXO1), leading to down regulation of cell cycle inhibitor CDKN1B/$p27^{Kip1}$ and eventually cell cycle reentry. IGF-1 also activates Myf5 expression through the calcium-calmodulin pathway, the phosphatidylinositol-4,5-bisphosphate 3-kinase/ protein kinase B (PI3K/Akt) pathway, or the extracellular signal–regulated kinases (ERK) pathway. HGF, IGF, and FGF family growth factors activate the P38α/β MAPK pathway, which has proven to play a role in satellite cells activation. Satellite cells with inhibited P38α/β MAPK show elevated cell cycle exit and tendency to remain quiescent (Jones et al., 2005). FGF2 on the other hand is able to push the cell cycle phase Gap1 (G_1) to phase Synthesis (S) transition through the ERK1/2 pathway, thereby reinforcing satellite cell activation and myogenic decision (Yablonka-Reuveni et al., 1999; Jones et al., 2001). A more recent study demonstrated that FGF2 increases intracellular calcium level, thus enhancing NFATc2 and NFATc3 translocation into nucleus and MyoD expression (Liu and Schneider, 2014).

D. Satellite cell self-renewal

Activated satellite cells can undergo both asymmetric (in $Pax7^+/Myf5^-$ satellite cells only) and symmetric (in both $Pax7^+/Myf5^-$ and $Pax7^+/Myf5^+$ satellite cells) division. During asymmetric division, the $Pax7^+/Myf5^-$ satellite cell can give rise to a daughter $Pax7^+/Myf5^-$ satellite stem cell and a daughter $Pax7^+/ Myf5^+$ satellite myogenic cell. $Pax7^+/ Myf5^-$ satellite stem cells serve as the foundation of satellite cell self-renewal and reconstitute the satellite cell compartment (Kuang et al., 2007).

Recent *in vitro* studies showed that activated satellite cells can return to quiescence. The majority of proliferating myoblasts in cell culture are MyoD+ after 24 h, and most of the MyoD+ cells will transit from $Pax7^+/MyoD^+$ to $Pax7^-/MyoD^-/MyoG^+$ upon differentiation signal. A small group of myoblasts with down-regulated MyoD but up-regulated Pax7 withdraw from cell cycle and become reserved cells (Zammit et al., 2004). The down-regulation of MyoD expression is promoted by Notch signaling through Notch3 (Kuang et al., 2007). As a receptor tyrosine kinase inhibitor, Spry1 expression in myogenic progenitors elevates after cell cycle withdrawal, and this elevation in turn reduces the ERK signaling and enhances cell cycle withdrawal (Shea et al., 2010). The transcription factor CCAAT/enhancer binding protein beta (C/EBPβ) is expressed in $Pax7^+$ satellite cells and down-regulated in differentiating satellite cells. C/EBPβ up-regulates Notch2 to repress satellite cell differentiation and restore satellite cell self-renewal (Lala-Tabbert et al., 2016). Spry1 and C/EBPβ are essential for satellite cell self-renewal and satellite stem cell population maintenance. Myostatin, also known as growth and differentiation factor (GDF-8), a member of the TGFβ superfamily, negatively regulates G_1 to S phase progression in satellite cells, thus maintaining satellite cells in quiescence. Myostatin-deficient muscles show increased number

of actively proliferating satellite cells, which indicates the negative regulator role of myostatin in satellite cell renewal (McCroskery et al., 2003).

1.3.2.2 Other myogenic precursor cells in skeletal muscle

Several studies have demonstrated the existence of nonsatellite cells that serve as myogenic progenitors in myogenesis. Muscle-derived stem cells (MDSCs) are slow adhesion cells with self-renewing capability. MDSCs have multipotency in regeneration of muscle, bone, and blood vessels (Lee et al., 2000; Qu-Petersen et al., 2002). Interestingly, myogenic oriented MDSCs are CD45 negative and this subpopulation of MDSCs can only gain myogenic capability under forced expression of Pax7 or influence of satellite cell niche (Peng and Huard, 2004).

Muscle side population (SP) cells are a group of heterogenic $Pax7^{-}/Myf5^{-}$ cells located at skeletal muscle interstitium neighboring blood vessels (Asakura et al., 2002). When transplanted intravenously into dystrophin mutated *mdx* mice, muscle SP cells are able to migrate through blood stream and fulfill the role of dystrophin regeneration (Gussoni et al., 1999). A small subpopulation of $CD45^{+}$ muscle SP cells can be induced into myogenic cells through co-culture with primary myoblasts or Pax7/MyoD transfection (Asakura et al., 2002; Seale et al., 2004). However, somatic hypaxial $Pax3^{+}$ cells-originated $CD45^{-}$ SP cells are believed to have more myogenic potential compared to muscle-derived $CD45^{+}$ SP cells (Schienda et al., 2006).

Interstitial cells with PW1 expression show myogenic potential *in vitro,* but the absence of Pax7 compromises the myogenic potential of PW1+ interstitial cells (Mitchell et al., 2010). Originated from embryonic dorsal aorta, mesoangioblasts are able to contribute progenitor cells to mesoderm-originated tissues such as bone, cartilage, and all types of muscles (Minasi et al., 2002).

Mesoangioblasts used to be named vessel-associated mesodermal progenitor cells. Though their role in healthy muscle development is controversial, mesoangioblasts facilitate muscle regeneration under pathological conditions such as dystrophic muscle, perhaps by traveling through bloodstream (Sampaolesi, 2003). MyoD is involved in mesoangioblasts-based myogenic regeneration (Morosetti et al., 2006). Interestingly, mesoangioblasts also express PW1, which is important for maintaining stemness (Bonfanti et al., 2015).

Pericytes are a type of contractile cells encircling the endothelial cells that form the inner lining of blood vessels. Originated from sclerotome, pericytes are pluripotent stem cells that can differentiate into myofibers, adipocytes, chondrocytes, and osteoblasts. $Pax7^-/MyoD^-/Myf5^-$ pericytes can be induced into $Pax7^+$ satellite cells and generate functional myofibers (Crisan et al., 2008; Birbrair et al., 2015). More recent research indicated the essential role of pericytes in skeletal muscle formation and satellite cell niche maintenance (Kostallari et al., 2015).

CD133 is an expression marker for a population of heterogeneous blood and muscle-derived myogenic cells. $CD133^+/Pax7^+$ cells can be found in muscle interstitium and basal lamina of myofibers (Bauer et al., 2008). Due to their good regeneration capability in intramuscular transplantation therapy, $CD133^+/Pax7^+$ cells are considered promising non-gene therapy treating Dunchenne muscular dystrophy (Meng et al., 2014). Identified by expression of PDGFR-α, CD34, and stem cell antigen-1 (Sca1), the skeletal muscle non-myogenic population consists of fibro-adipogenic/mesenchymal progenitors (FAPs/MPs). It is believed that through myogenic regeneration, these cells are the source of pro-differentiation signals for myoblasts and can differentiate into new myofibroblasts, adipocytes, osteocytes, and chondrocytes (Tedesco et al., 2017).

1.3.2.3 Myoblast proliferation

A. Cell cycle

Upon activation, satellite cells will re-enter cell cycle and start to proliferate as myogenic progenitor cells myoblasts. Cell cycle consists of four different sequential phases: Gap 1 (G_1), Synthesis (S), Gap 2 (G_2), and Mitosis (M) (Alberts et al., 2014). After leaving resting G_0 state, satellite cells enter G_1 phase to resume cell cycle. Cells at G_1 phase show increased size, metabolism rate, organelles number and activities, and protein synthesis. G_1 phase is separated into early and late G_1 phases by the "restriction point" or "G_1/S checkpoint". As cells enter G_1 phase, depending on internal and external growth factors, cell procession can either be delayed at G_1 checkpoint and return to G_0 state or pass the checkpoint and commit to fulfill the entire cell cycle. From now on, no growth factor stimuli are required to complete cell division.

DNA replication occurs during S phase, accompanied by low RNA transcription and protein synthesis (with the exception of histone production). After DNA replication, G2 phase is a cell growth period for mitotic protein production. During G2, the DNA damage checkpoint ensures cell cycle arrest when DNA damage presents until the damage is fixed. The highly regulated M phase is about nuclear division and a checkpoint in the middle makes sure the cell is ready for complete division. During cytokinesis afterwards, a mother cell divides nucleus, cytoplasm, organelles and cell membrane into about equally two parts, and eventually separates the cell into two daughter cells. Phases G1, S, and G2 are also termed interphase while M phase is considered cell division phase (Crosby, 2007; Alberts et al., 2014).

B. Cell cycle regulation

Multiple checkpoints in cell cycle ensure that division only occurs under sufficient cell growth, faithful DNA replication, and favorable condition. Numerous numbers of proteins are involved in this process of regulation and coordination, among which cyclins and cyclin dependent kinases (CDKs) play key roles. Each cyclin can form a cyclin-CDK complex with its specific CDKs while each cyclin-CDK complex can modify a specific group of proteins. Cyclins A, B, D, and E and their corresponding CDKs (mainly 1, 2, 4, and 6; CDK1 is also known as cell cycle controller 2, CDC2) are involved directly in cell cycle regulation (Crosby, 2007).

During the early G1 phase, with stimulation from growth factors, cyclin D binds to CDK4 and CDK6. These complexes phosphorylate the target genes, initiate the S phase progression, and trigger the cyclin E-CDK2 complex formation. Cyclin E-CDK2 complex regulates the G_1 phase transition into S phase by activating more S phase genes (Bertoli et al., 2013). Then Cyclin A in association with CDK2 are essential for the progression through S phase. Ultimately, cyclins A and B teaming up with CDK1 are necessary for the G_2 phase through mitosis (Morgan, 1995).

In the meanwhile, inhibitors of cyclin-CDK complexes (CDKIs) are functioning as negative regulators of cell cycle progression and positive regulators of cell arrest. CDKIs can be categorized into two families: INK4 family ($p16^{INK4a}$, also known as p16 or CDKN2A; $p15^{INK4b}$, also known as p15 or CDKN2B; $p18^{INK4c}$, also known as p18 or CDKN2C; and $p19^{INK4d/ARF}$, also known as p19 or CDKN2D) and Cip/Kip family ($p21^{Cip1/Waf1/Sdi1}$, also known as p21 or CDKN1A; $p27^{Kip1}$, also known as p27 or CDKN1B; and $p57^{Kip2}$, also known as p57 or CDKN1C). INK4 inhibitors bind to CDK4 and CDK6 and block their interaction with cyclin D while Cip/Kip family targets all cyclin/CDK complexes (Besson et al., 2008).

Ubiquitin-proteasome dependent protein degradation also controls the progression of cell cycle through cyclin-CDKs or CDKIs. E3 ubiquitin ligase Anaphase-promoting complex/cyclosome (APC/C) combining with coactivator fizzy-related 1(FZR1) can associate with Skp1, Cullin, and F-box containing complex (SCF). SCF can recruit either recognition factor F-box/WD repeat-containing protein 7(Fbw7) or S-phase kinase-associated protein 2 (Skp2). When APC/C-FZR1 in association with SCF-Fbw7, cyclin E is targeted for degradation, cell cycle progression is inhibited. In contrast, APC/C-FZR1 working with SCF-Skp2 leads to degradation of p21 and p27 and the entry of cell cycle (Bassermann et al., 2014). The pocket protein family member retinoblastoma protein (Rb) can prevent the G_1 to S phase transition by binding and inhibiting E2 promoter-binding-protein-dimerization partner (E2F-DP) (Goodrich et al., 1991). Upon growth factor stimulation, Cyclin D-CDK4/6 phosphorylates and activates Rb first followed by cyclinE-CDK2 (Goodrich et al., 1991). Phosphorylated Rb (pRb) releases E2F which in turn promotes cell cycle transiting into S phase and activates more cyclins for mitosis (Duronio et al., 1995).

C. Regulation of muscle specific genes in cell cycle

During proliferation, myoblasts show distinct expression profiles of MyoD and Myf5 at different phases of the cell cycle. MyoD expression is absent at G_0, up-regulated by mid-G_1, decreased by G_1-S phase, and increased again from the S to M phase. In contrast, Myf5 expression is high at G_0, low at the beginning of G_1 but up-regulated by the end of G_1, and remains high until the completion of mitosis (Kitzmann et al., 1998). The distinguishing expression profiles of Myf5 and MyoD indicate their different roles in myogenesis. Indeed, studies support that Myf5

predominance leads to enhanced proliferation and delayed differentiation while predominant MyoD expression results in early differentiation (Montarras et al., 2000; Rudnicki et al., 2008).

Expression of a number of cell cycle regulator genes, including cyclins A, B, D, E, F, and G, is augmented in activated myoblasts. MyoD activity through G_1 phase is known to be suppressed by cyclin D1-CDK4 complex (Skapek et al., 1995; Zhang et al., 1999). Expression level of cyclin D1 increases under the regulation of transcription factor c-Jun, which in turn is phosphorylated by c-Jun N-terminal kinase (JNK), under growth factor stimulation (Schreiber et al., 1999; Wisdom et al., 1999). In contrast, while stimulating proliferation, cyclin D3-CDK4/6 complex also participates in cell cycle arrest, transits rapidly through G_1 phase, and enhances cell cycle exit (De Luca et al., 2013). Cyclin D family proteins repress the creatine kinase, muscle (CKM) activity through pocket protein/E2F- dependent pathway while cyclins A and E only inhibit CKM expression when they are co-expressed in the form of cyclin A/E-CDK2 complexes (Guo and Walsh, 1997). Once cyclin D1 degradation begins, MyoD is freed from regulation by cyclin - CDK complexes and able to bind to E protein and initiate myogenic gene transcription and differentiation (Weintraub, 1993).

1.3.2.4 Myoblast differentiation and fusion

During embryonic myogenesis, undifferentiated $Pax3^+/Pax7^+$ myogenic precursor cells migrate from somite to primary myotome or limb bud mesenchyme and subsequently differentiate and fuse to form myotubes, which further develop into individual muscles in limb and trunk (Yin et al., 2013). During the migration, proliferating myogenic cells start to exit cell cycle, synthesize myofibrillar proteins, and prepare for myoblast fusion. In myogenic cell lines or primary satellite cells, the process of myoblast differentiation can be recapitulated when culture medium is switched

from high serum (mitogen rich) to low serum (mitogen low) (Cheng et al., 2014). Extrinsic factors like cytokines, mitogens, growth factors, Notch, Wnt/Wg, Hedgehog, and TGFβ-BMP ligands together with intrinsic cell signals such as transcription factors and cell cycle regulators lead to proliferation to differentiation transition.

A. Cell cycle withdrawal

During proliferation, myoblasts lack CDKIs or high levels of CDKs and their cyclins, and undergo a relative short G_1 phase. Once under induction of differentiation or developmental transition, the CDK-cyclin associated kinase activities decrease while CDKIs expression increases (Ciemerych and Sicinski, 2005). Consequently, upon induction of differentiation, myoblasts proceed through a longer cell cycle with an extended G_1 phase, which allows more time to respond to external signals and to accumulate differentiation-related transcription factors (Lange and Calegari, 2010). In the meanwhile, transcription factors that regulate the expression of muscle-specific genes also control the expression of cell cycle regulators. MyoD, the master regulator of myogenesis, up-regulates the expression of cell cycle inhibitors p21 and p57 by associating with their promoter sequences and activating their transcription during myoblast differentiation (Ruijtenberg and van den Heuvel, 2016). p21 and p57 working together induce cell cycle arrest and trigger myogenic terminal differentiation (Zhang et al., 1998).

B. Myogenic terminal differentiation

As a highly ordered temporal event, myogenic terminal differentiation is promoted by MyoD and other bHLH transcription factors like MRF4 and MyoG. Inhibitor of DNA binding (Id) proteins negatively regulate differentiation by inhibiting their binding of bHLH transcription

factors to regulatory elements on muscle specific genes. Expression levels of Id proteins decline in cells following high serum withdrawal, which promotes myogenic bHLH transcription factors binding to E proteins and interaction with the muscle specific genes (Benezra et al., 1990). Upon differentiation induction, p38 MAPK phosphorylates BAF60C, MEK2, and scaffolding protein KAP. Each of these proteins can form functional protein complexes that activate MyoD and/or myocyte enhancer factor 2 (MEF2) bound promoters, recruiting co-activators while releasing existing co-repressor (Rampalli et al., 2007; Puri and Mercola, 2012; Ruijtenberg and Van Den Heuvel, 2015; Singh et al., 2015).

Synthesis of MyoG, a marker of myoblast commitment to differentiation, is activated and up-regulated by MyoD (Blais et al., 2005). During differentiation, a significant group of $MyoG^+$ mononucleated myogenic cells still undergo DNA synthesis, indicating MyoG expression is not sufficient for cell cycle arrest/withdrawal. In the meanwhile, MyoD transactivates p21 in a p53-independent manner and promotes the upregulation of p21, which in turn downregulates CDK2/4/6 activities (Guo et al., 1995). $MyoG^+$ and $p21^+$ mononucleated cells lose their ability to incorporate BrdU even under high-serum re-stimulation, demonstrating that p21 is the marker for myogenic cell post-mitotic state and that $MyoG^+$ and $p21^+$ myogenic cells permanently withdrew from cell cycle (Andres and Walsh, 1996). Upon differentiation, mRNA level of pRb increases and the active form of pRb is required to maintain the post-mitotic state and promote the expression of MyHC (Novitch et al., 1996). The expression levels of myosin heavy and light chains are both elevated under the regulation of MyoD and MyoG, and so are other muscle specific contractile proteins such as actin, troponin, etc. (Olson, 1992).

C. Fusion

Skeletal muscle is composed of bundles of multinucleated myofibers that are each fused from hundreds or thousands of myoblasts. The first type of myoblast fusion during myogenesis is myoblast-to-myoblast fusion to form nascent myotubes. This is followed by myoblasts fusing into existing myotubes to form larger myotubes. Fusion begins with cell-to-cell adhesion that aligns the membranes of two cells in close proximity. This is followed by pore formation in membranes and membrane vesiculation, which works to recycle membranes and expand pores. Cytoplasmic continuity between the myoblasts is thus achieved. This process is accompanied by the actin-cytoskeletal remodeling that helps merge the two myoblasts. Cell membrane adhesion proteins β1-integrin, VLA-4, VCAM, and caveolin-3, extracellular MMPs, intracellular calcium and calcium binding proteins as well as lipids and phospholipase are believed to play critical roles in myoblast to myoblast fusion (Horsley and Pavlath, 2004).

In vitro studies revealed that nuclear factor of activated T cells C2 (NFATC2) is critical for myoblast to nascent myotube fusion (Horsley et al., 2001). The activity of NFATC2 is controlled by calcium and calmodulin, which is activated by $PGF_{2\alpha}$ (Horsley and Pavlath, 2003). When activated, NFATC2 regulates the secretion of cytokine interleukin-4 (IL-4), and IL-4 works through its receptor IL4R in an autocrine manner to increase fusion of myoblast into myotubes (Horsley et al., 2003). Mammalian target of rapamycin (mTOR) regulates both myoblast-myoblast fusion and later-stage myotube maturation independent of NFATC2. Upon differentiation initiation, mTOR upregulates the transcription of insulin-like growth factor 2 (IGF2) independent of its kinase activity, and IGF2 in turn controls myoblast differentiation through the PI3K/Akt pathway. In later stage fusion, mTOR inhibits the activity of histone deacetylase (HDAC) through

MyoD stabilization and ultimately elevates the expression of follistatin, which can induce myogenesis (Hay and Sonenberg, 2004; Park and Chen, 2005; Ge and Chen, 2012).

Membrane protein myoferlin from the ferlin family is highly expressed in immature myoblasts. Expression of dysferlin from the same ferlin family is upregulated during myoblast differentiation with high level at T-tubule, a structure that is absent in myoblasts. Myoferlin- or dysferlin-null myoblasts have fusion defects and reduced myofiber size while myoferlin/dysferlin double deletion leads to enhanced muscle dystrophy, T-tubule defects, and lipid extrusion from the sarcolemma (Posey et al., 2011; Demonbreun et al., 2015). A transmembrane protein named myomaker is more recently identified as a highly conservative muscle-specific protein that is required for myoblast fusion (Millay et al., 2013). Myomaker expression is regulated by MyoG in embryonic developing muscle, injured muscle, or myotubes formed in vitro from activated satellite cells, but it is undetectable in mature muscle. Although the capability of proliferation and differentiation is not affected, fusion is completely blocked in myomaker-null satellite cells (Millay et al., 2013; Millay et al., 2014; Demonbreun et al., 2015).

1.3.2.5 Protein balance in skeletal muscle

Skeletal muscle provides the largest protein reservoir in vertebrates, and protein balance is important for the maintenance of metabolic homeostasis. The net protein balance is defined as the difference between skeletal muscle protein synthesis (MPS) and breakdown (MPB). The increase in MPS (anabolism) and/or decrease in MPB (catabolism) leads to positive net protein balance and thus accumulation of skeletal muscle protein (Breen and Phillips, 2011). In skeletal muscle, muscle hypertrophy involves the increase of myofiber size that consists of both accretions of myofibrillar

protein content and sarcoplasmic volume. Muscle atrophy, on the opposite, is considered an excessive pathological decrease in muscle mass (Ransone, 1996).

A. Factors involved in skeletal muscle protein balance

Muscle protein synthesis rate is controlled primarily at the initiation step of mRNA translation, partially through binding of initiator methionyl-tRNA (met-tRNA) and binding of messenger RNA (mRNA) to 40S ribosomal subunit mediated by eukaryotic initiation factor 4 (elF4) and activation of ribosomal protein S6 kinase beta1 (S6K1) (Jefferson and Kimball, 2001). Though excessive proteolysis is harmful to muscle function, protein degradation under physiological conditions benefits the maintenance of cellular function and muscle health. Proteolysis in skeletal muscle is mainly mediated by the ubiquitin proteasome system (UPS), autophagy lysosome pathway, calcium-dependent calpain system, and caspases (Pasiakos and Carbone, 2014).

A number of extracellular factors affect muscle protein balance, including nutrition, hormones, energy states, and cytokines. The PI3K/Akt pathway is the key intracellular signaling pathway that coordinates the effects of these extracellular factors on muscle protein balance. During anabolism, insulin/IGF1 activates PI3K/Akt signaling, which in turn inhibits glycogen synthase 3β (GSK3β), triggers mTOR complex 1 (mTORC1) activation, and decreases the activities of FOXO family transcription factors. GSK3β is a negative regulator of protein translation initiator eIF2B, transcription factor NFAT, and actin polymerization. Inhibition of GSK3β activity through Akt-mediated phosphorylation improves myoblast differentiation and myotube formation. (Glass, 2010; Schiaffino and Mammucari, 2011). There are two mTOR complexes. mTORC1 contains Raptor and is sensitive to rapamycin; mTORC2 contains Rictor

and is insensitive to rapamycin (Zoncu et al., 2011). Akt activates mTORC1 by phosphorylating tuberous sclerosis complex (TSC) 2 (Huang and Manning, 2009), and activated mTORC1 in turn activates S6K and inhibits eIF4E-binding proteins (4EBPs) through phosphorylation, leading to translation initiation and protein synthesis (Zoncu et al., 2011). Differently, mTORC2 is found to enhance Akt signaling and phosphorylation of PKCα, facilitating Akt activation of transcription factors from the FOXO family (Guertin et al., 2006).

FOXO proteins are essential for transcription of muscle-specific ubiquitin ligases, atrogin1 (also called muscle atrophy F-box, MAFbx) and muscle ring finger protein1 (MuRF1), which are responsible for the degradation of myosin heavy chain and other thick filament proteins (Koyama et al., 2008; Foletta et al., 2011). FOXO3 is also required for the upregulation of microtubule-associated protein 1A/1B-light chain3 (LC3) and BCL2-interacting protein 3 (Bnip3), marker proteins in the autophagy-lysosome pathway (Mammucari et al., 2007). Thus, the PI3K/Akt pathway promotes positive protein balance through increase of MPS and decrease of MPB at the same time. The PI3K/Akt/mTOR pathway mediates the anabolic effect of growth hormones, amino acids, and β-agonists. The PI3k/Akt/mTOR/FOXO pathway mediates the catabolic effects of fasting, low protein diet, glucocorticoids, inflammatory cytokines, and myostatin (Anthony, 2016). When myostatin, a negative regulator of myogenesis, is overexpressed, PI3K/Akt/mTOR signaling is down-regulated as well as IGF1 mediated muscle hypertrophy. In the contrary, the inhibition of myostatin promotes Akt-dependent hypertrophy (Rodriguez et al., 2014).

B. Satellite cells in muscle hypertrophy

The increase of total mRNA and protein synthesis in skeletal muscle can be achieved either by the incretion of mRNA and protein synthesis in existing nuclei or by the addition of new nuclei

from satellite cells to myofibers with stable mRNA and protein levels in existing nuclei. It has long been believed that in adult skeletal muscle, the size of myonuclear domain (i.e., the amount of cytoplasm surrounding each nucleus) is constant; thus, the increase of myofiber size requires extra nuclei in order to keep the myonuclei domain constant (Pavlath et al., 1989; Allen et al., 1999). The experiments tracing satellite cells incorporation into hypertrophic myofibers demonstrated the contribution of satellite cells in developing muscles (Schiaffino et al., 1976; Bruusgaard et al., 2010).

However, results from some studies questioned the necessity of satellite cells to keeping the homeostasis in myonuclear domain and muscle hypertrophy. In transgenic mouse models, rapid skeletal muscle hypertrophy could be induced without incretion of BrdU+ nuclei (Blaauw et al., 2009; McCarthy et al., 2011). It is possible that stem cells other than satellite cells take over the satellite cells' role in hypertrophy during satellite cell ablation (Losick et al., 2011). A more recent study suggests that using muscle mass as criteria for measuring muscle hypertrophy is not sufficient, and that it is difficult to prove that the definite absence of satellite cells leads to functional, sustainable hypertrophy in non-regrowth models (Egner et al., 2016). In summary, there is no satisfactory evidence that can clarify the exact roles or mechanisms of muscle stem cells in functional muscle hypertrophy in adult animals.

1.4 Hormonal regulation of skeletal muscle development and growth

1.4.1 Growth Hormone

Growth hormone (GH), also known as somatotropin (ST), is a peptide hormone synthesized and secreted by somatotropic cells in the anterior pituitary. The secretion of GH is stimulated by growth hormone releasing hormone (GHRH) but inhibited by somatostatin from the

hypothalamus (Gardner and Shoback, 2007). Growth hormone plays a key role in regulating postnatal growth in animals. Mice with GH deficiency or growth hormone receptor (GHR) mutation show retarded growth and dwarfism (Beamer and Eicher, 1976; Zhou et al., 1997). Recombinant GH treatment in GH-deficient patients significantly increases their skeletal muscle mass and strength (Sartorio and Narici, 1994). Administration of GH to pigs and cattle increased body weight, average daily gain (ADG), and protein content but decreased fat composition (Etherton, 2001; Schlegel et al., 2006).

The GHR is a type of cytokine receptors consisting of a single transmembrane glycoprotein with a hydrophobic transmembrane domain (Gardner and Shoback, 2007). In cattle, GHR mRNA is expressed in many organs and tissues such as liver, skeletal muscle, fat, and mammary gland, among which liver has the highest GHR mRNA abundance (Lucy et al., 1998). GHR1A mRNA is the liver-specific GHR mRNA variant with GHR1B and 1C mRNAs being available in most other tissues (Jiang and Lucy, 2001). Humans and rodents have GH binding proteins (GHBP), which lack a membrane anchoring domain compared to GHR. In humans GHBP is derived from proteolytic cleavage of the GHR protein, but in rodents GHBP is generated from alternative splicing during GHR gene expression (Baumann, 2002). In domestic animal like cattle, GHBP-like proteins have been proposed to be present in plasma and milk (Davis et al., 1992; Devolder et al., 1993), though further detailed analysis of such proteins is absent.

Upon GH binding, two GHR molecules form a homodimer that activates the receptor-associated cytoplasmic tyrosine kinase, Janus Kinase (JAK) 2 (He et al., 2003). Activated JAK2 phosphorylates itself and GHR, which in turn triggers phosphorylation of several proteins (**Figure 1.3**). These proteins include signal transducers and activators of transcription (STAT) family transcription factor 1, 3, and 5; Shc adapter proteins that connect to the Ras/Raf/MEK/ERK

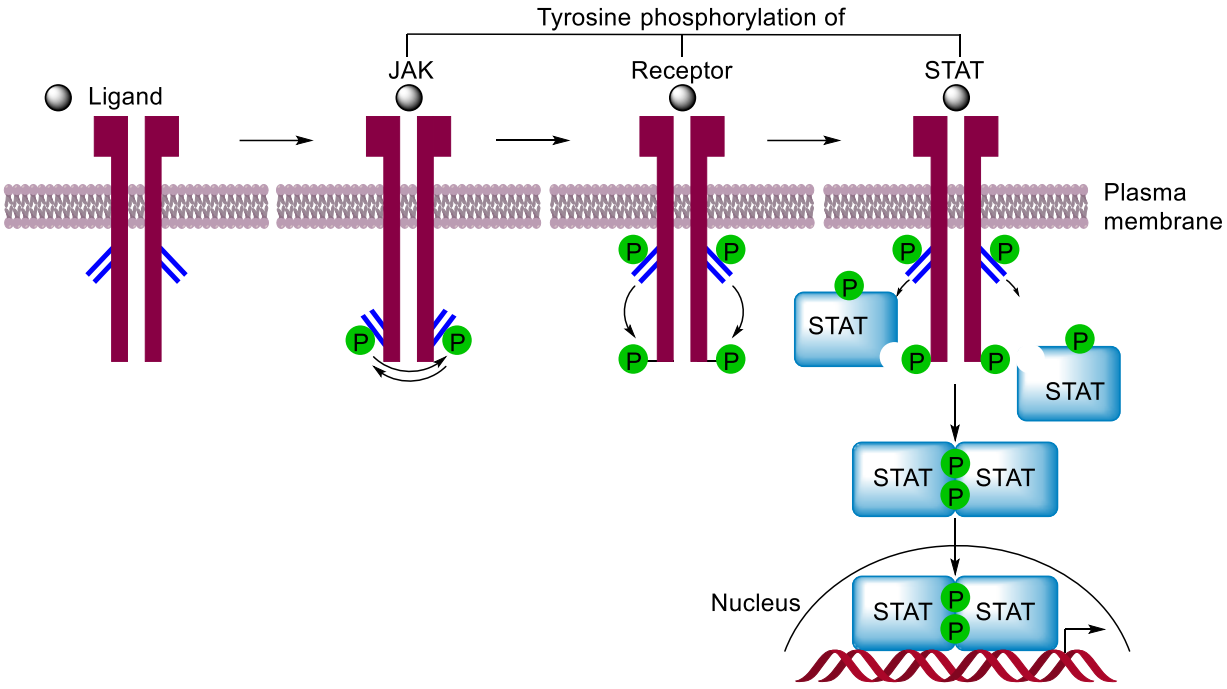

Figure 1.3 Simplified schematics for cytokine receptor signaling through the JAK family tyrosine kinase. Cytokines such as growth hormone induce dimerization of their tyrosine kinase associated receptors. JAKs cross-phosphorylate each other on tyrosine residues. Activated JAKs phosphorylate both cytokine receptor subunits on tyrosine residues, which then act as docking sites for STAT proteins. STATs become phosphorylated by JAKs, which then allows the formation of STAT dimers. STAT dimers translocate to the nucleus and initiate transcription of target genes. P, phosphate; JAK, Janus kinase; STAT, Signal Transducer and Activator of Transcription. (Adapted from Buckingham, M. 1994. Muscle Differentiation: Which myogenic factors make muscle? Curr. Biol. 4:61–63. doi:10.1016/S0960-9822(00)00014-2.)

pathway; and insulin receptor substrate proteins that initiate phosphatidylinositol-3'-kinase (PI3K)/Akt pathway (Sotiropoulos et al., 2006). Among the STAT family transcription factors, STAT5 controls the transcription of genes like insulin-like growth factor (IGF) 1 and its binding protein component acid labile subunit (ALS) in liver (Herrington et al., 2000).

Growth hormone exerts its stimulatory effects on growth mainly through IGF1. Evidence from gene knockout mouse models indicates that GH increases the production of liver-derived IGF1 (endocrine IGF1), which partially mediates GH stimulation on muscle hypertrophy (Yakar et al., 2002; Stratikopoulos et al., 2008; Wu et al., 2009). IGF1 produced in non-hepatic tissues (autocrine or paracrine IGF1) is also thought to facilitate GH stimulation of tissue growth (D'Ercole et al., 1980; Isaksson et al., 1987; Roberts Charles T. et al., 1987). However, little

evidence exists that supports GH stimulating IGF1 production in muscle during muscle growth in cattle, sheep, pigs, and humans (Grant et al., 1991; Pell et al., 1993; Dunaiski et al., 1999; Jørgensen et al., 2006; Ge et al., 2012; Ge et al., 2013). Growth hormone also has IGF1-independent effects on growth (Waters et al., 1999). GH facilitates the fusion of myoblasts into nascent myotubes independent of IGF1 in mice but has no effect on myoblast size, myoblast proliferation, or myoblast-to-myoblast fusion (Sotiropoulos et al., 2006). Growth hormone increases protein synthesis in cultured bovine myoblasts or myotubes without upregulating IGF1 gene transcription (Ge et al., 2012; Ge et al., 2013), but GH itself has no effect on bovine myoblast proliferation or differentiation (Ge et al., 2012).

1.4.2 Growth factors

Growth factors (GFs) are polypeptides that stimulate cell proliferation and/ or differentiation by binding to specific high-affinity cell membrane receptors. Unlike classical hormones that are produced by specific endocrine organs, GFs are usually produced in a number of tissues. GFs secreted by certain cells influence the same cells or neighboring cells. Some GFs enter the circulation system in the form of GF/GF binding protein complex and impact cells/tissues in an endocrine manner (Goustin et al., 1986; Dodson et al., 1996).

Typical GF receptors, such as the insulin receptor, IGF1 receptor, and hepatocyte growth factor (HGF) receptor, also known as receptor tyrosine kinases (RTKs), are single transmembrane polypeptides that consist of an extracellular ligand-binding domain, a hydrophobic membrane-spinning intracellular domain, and a cytoplasmic domain with tyrosine kinase activity (Allen et al., 1995) (**Figure 1.4**). Ligand binding to extracellular domain of the receptor triggers the dimerization of receptors. This leads to the autophosphorylation of specific tyrosine residues on

the receptor, which creates binding sites for Src homology (SH) 2 domain and sometimes SH3 domain. The phosphorylated sites on the receptors recruit and phosphorylate intracellular substrates including growth factor receptor-bound protein (GRB)2, PI3K, phospholipase Cγ (PLCγ), GTPase-activating protein (GAP), and the Src family non-receptor tyrosine kinases

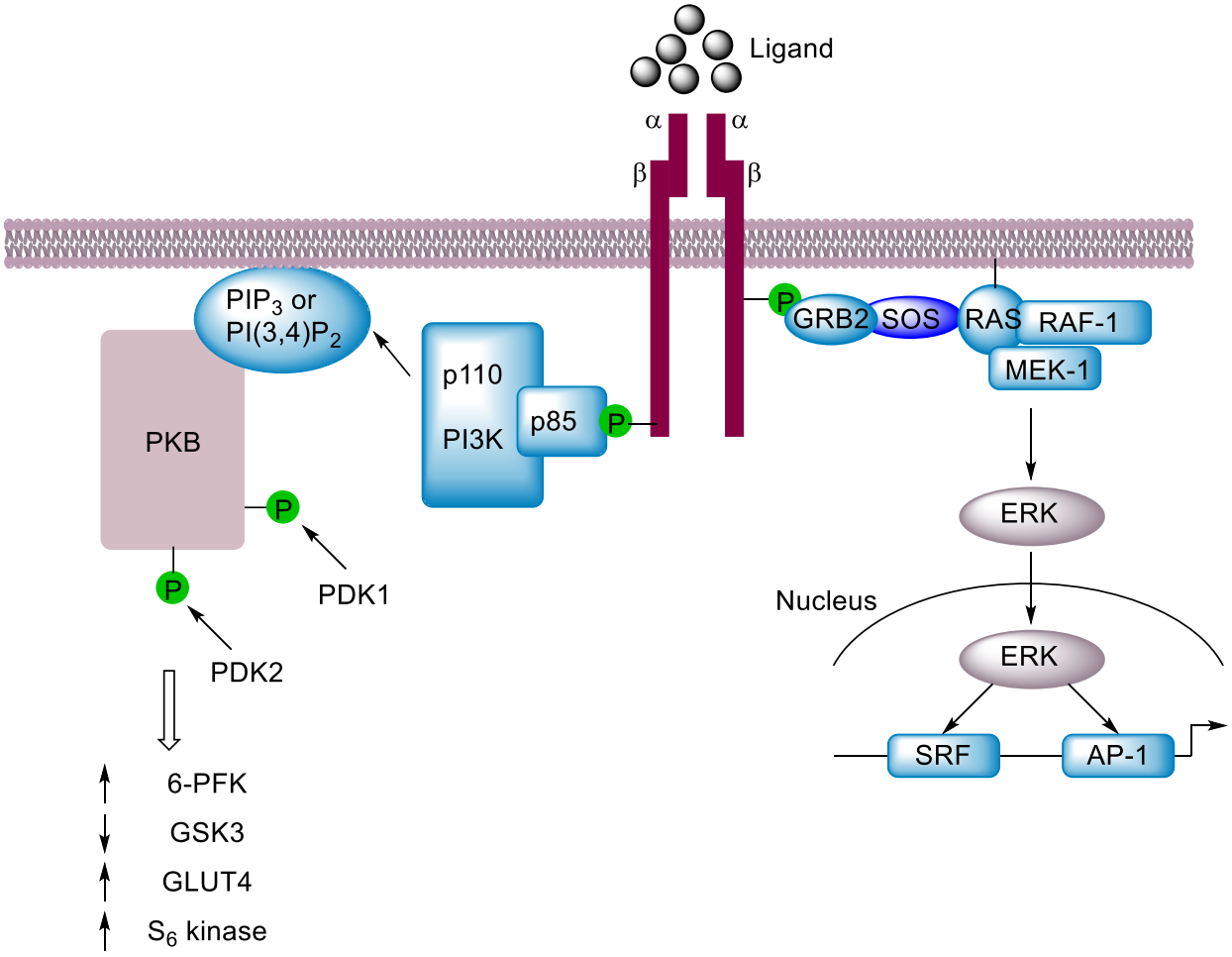

Figure 1.4 Simplified schematics for signaling from receptor tyrosine kinases Binding of ligands to receptor tyrosine kinases activates two major pathways: the RAS/RAF/MEK/ERK pathway and the PI3K/Akt pathway, which mediate a number of cellular responses that are related to cell survival, proliferation, and protein synthesis. GRB-2, growth factor receptor-bound protein 2; SOS, son of sevenless; RAS, a small GTPase; RAF-1, mitogen-activated protein kinase kinase kinase (MAP3K); MEK-1, mitogen-activated protein kinase kinase (MAP2K); ERK, extracellular signal-regulated kinase; SRF, serum response factor; AP-1, activator protein 1; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; $PI(3,4)P_2$, phosphatidylinositol (3,4)-bisphosphate; PKB, protein kinase B; PDK1 or 2, phosphoinositide-dependent protein kinase 1 or 2; 6-PFK, 6-phosphofructokinase; GSK3, glycogen synthase kinase 3; GLUT4, glucose transporter type 4. (Adapted from Gardner, D., and D. Shoback. 2007. Greenspan's Basic ; Clinical Endocrinology: Eighth Edition. McGraw-Hill Companies,Incorporated.)

(nRTKs) (Gardner and Shoback, 2007). GRB2 and PI3K are examples of cytoplasmic substrates serving as scaffolds for building cytoplasmic signaling cascades. Upon growth factor binding to its receptor, the receptor-GRB2 complex will be associated with protein son-of-sevenless (SOS) through the SH3 domain. SOS in turn signals to protein Ras and then Raf kinase, which phosphorylates downstream targets like mitogen-activated protein kinase (MAPK) kinases (MEKs). MEKs phosphorylate and activate p42 and p44 of MAPKs (also called extracellular signal-regulated kinases; ERKs). ERKs can activate a variety of downstream substrates including ribosomal protein S6 and some transcription factors (Guan, 1994; Avruch et al., 2001). Growth factor binding to its receptor also leads to the phosphorylation of the catalytic subunit p110 of PI3K. Activated P13K stimulates the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) and phosphatidylinositol-3,4-bisphosphate (PI[3,4]P2), which in turn sequester protein kinase B (Akt). Subsequently, phosphorylation by PIP3-dependent kinase (PDK1 and PDK2) occurs at two different sites on Akt. Phosphorylated Akt stimulates various downstream targets including 6-phophofructo-2-kinase, GLUT4, p70S6 kinase, and glycogen synthase kinase (GSK) 3.

Members of the transforming growth factor beta (TGFβ) super family bind to receptors with transmembrane serine/threonine kinase activity, termed serine/threonine kinase receptors. Like tyrosine kinase receptors, serine/threonine kinase receptors are single transmembrane polypeptides with extracellular, transmembrane, and intracellular domains (Dijke et al., 1994). TGFβ super family receptors transduce signal from the ligands through heteromeric complexes comprising of type I, type II, and/or type III serine/threonine kinase receptors. In vertebrates, seven type I, five type II, and two type III receptors have been discovered. The typical TGFβ signaling in skeletal muscle initiates with the binding of dimeric ligands to the extracellular domain of

serine/threonine kinase type II receptors (Miyazono et al., 1994). This induces the formation of multimeric receptors composed of two type I and two type II receptors. Type II receptors phosphorylate type I receptors in the juxtamembrane region and activate type I receptors. Activated type I receptors can specifically phosphorylate the Receptor-regulated Smad (R-Smad; Smad1, 2, 3, 5, and 8/9), which in turn form a heteromeric complex with the Common-mediator Smad (Co-Smad) Smad 4. The Smad complexes then shuttle into nuclei by nuclear transport proteins and target specific promoters to activate transcription (Weiss and Attisano, 2013). The third group of Smads, inhibitor Smads (I-Smad; Smad 6 and Smad 7), repress TGF-β signaling through an auto-inhibitory feedback loop (Jayaraman and Massagué, 2000) (**Figure 1.5**).

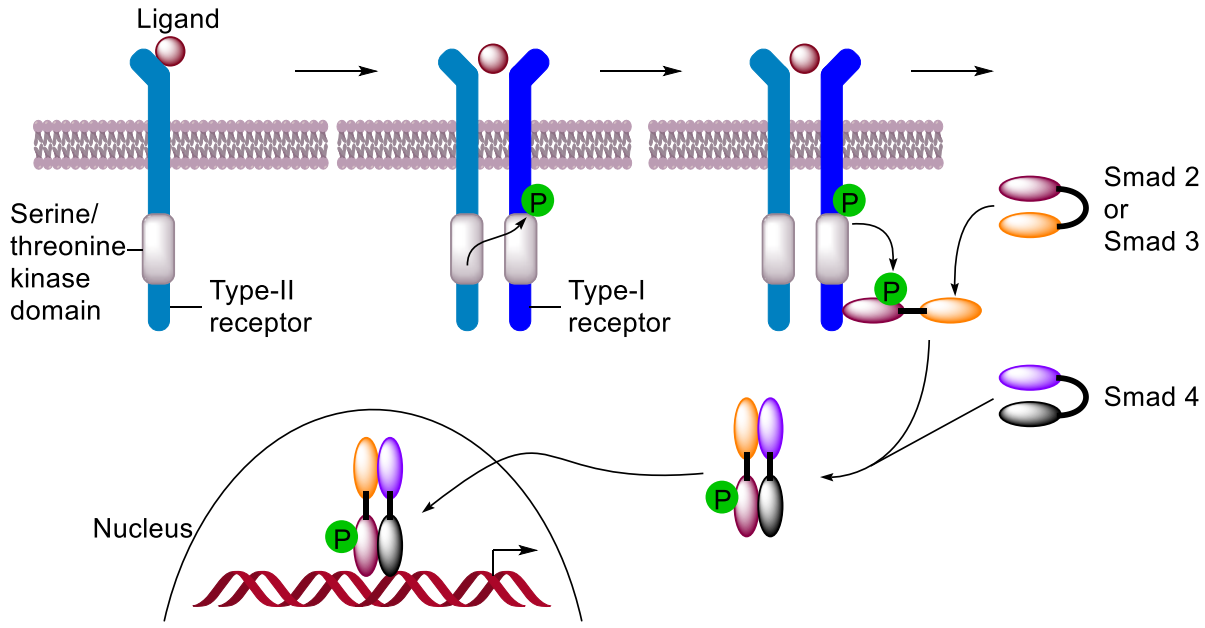

Figure 1.5 Simplified schematics for signaling from the serine/threonine kinase receptors Ligand binding to a type II receptor causes the receptor to recruit and phosphorylate a type I receptor. Phosphorylated type I receptor recruits and phosphorylates Smad2 or Smad3. Phosphorylated Smad2 or Smad3 dissociates from the receptor and forms a complex with Smad4. The complex migrates into nucleus, recruiting other gene regulatory proteins and activating transcription of specific gene targets. (Adapted from Natale, D. A., C. N. Arighi, W. C. Barker, J. Blake, T.-C. Chang, Z. Hu, H. Liu, B. Smith, and C. H. Wu. 2007. Framework for a Protein Ontology. BMC Bioinformatics. 8:S1–S1. doi:10.1186/1471-2105-8-S9-S1.)

1.4.2.1 Insulin-like growth factors (IGFs)

Insulin-like growth factors are polypeptides having highly similar sequences to insulin (Werner et al., 2008). Insulin-like growth factor-1 (IGF1) is important for postnatal growth and development while IGF2 plays a more significant role in fetal development (Hossner et al., 1997). Serum IGF1 is mainly produced by the liver under the stimulation of GH. Insulin-like growth factor-1 gene transcription in the liver is stimulated by GH through the JAK2-STAT5 pathway (Wang and Jiang, 2005; Rotwein, 2012). In blood circulation, the majority of IGFs form ternary complexes with IGF binding protein (IGFBP) 3 and acid labile subunit (ALS) or binary complexes with one of the IGFBPs (IGFBP1-6) to prolong their half-lives in blood (Hossner et al., 1997; Twigg and Baxter, 1998; Boisclair et al., 2001; Buckway et al., 2001). Depending on the cell type and physiological situation, IGFBPs can also modulate the binding affinity of IGFs to their receptors, thereby stimulating or attenuating IGF effects on cells (Hossner et al., 1997).

Insulin-like growth factors can bind to three different receptors, type 1 IGF receptor (IGF1R), type 2 IGF receptor (IGF2R), and insulin receptor with different binding affinities. Insulin-like growth factor-1 binds to IGF1R more efficiently than to IGF2R or insulin receptor while IGF2 binds to IGF2R more efficiently than to IGF1R or insulin receptor. IGF1R is a type of receptor tyrosine kinase with 2 alpha subunits containing IGF binding sites and 2 beta subunits with intrinsic tyrosine kinase activity. IGF binding to IGF1R activates a number of signaling pathways, among which the SOS/Ras/Raf/MEK/ERK pathway and the PI3K/Akt/mTOR/p70S6 pathway mediate most of the action of IGFs on growth and metabolism (Coolican et al., 1997; Menetrey et al., 2000; Fernández et al., 2002). Different from IGF1R, IGF2R is a single chain transmembrane glycoprotein that is also known as mannose-6- phosphate receptor (Kornfeld,

1992). IGF2R facilitates lysosome enzyme transportation and internalization, M-6-P protein regulation, and IGF2 degradation (Kornfeld, 1992; O'Dell and Day, 1998).

When IGFs were first discovered, it was believed that IGFs only play an endocrine role in mediating GH actions. Subsequent studies have demonstrated the effects of IGFs as autocrine and paracrine hormones (See 1.4.1). During skeletal muscle growth, IGF1 stimulates both myoblast proliferation and differentiation while IGF2 is only involved in myoblast differentiation (Pesall et al., 2001). It is believed that the stimulatory effect of IGF1 on myoblast proliferation is carried out through the MEK/ERK pathway (Coolican et al., 1997; Clemmons, 2009). Through this pathway, IGF1 binding to its receptor leads to the phosphorylation of ERK1/2; ERK1/2 controls the increase of cyclin D expression and the formation of cyclin D-CDK4 complexes, accelerating cell proliferation (Jones et al., 2001; Meloche and Pouysségur, 2007; Clemmons, 2009).

By binding to IGF1R, both IGF1 and IGF2 are able to promote myoblast differentiation. Insulin-like growth factor-2 signaling through IGF1R activates PI3K/Akt pathway promotes MyoD signaling for early differentiation initiation. MyoD in turn further induces the production of IGF2 locally during the late stage of differentiation and keeps the IGF-2/IGF1R/PI3K/Akt pathway active (Wilson et al., 2003; Wilson and Rotwein, 2006). Type 1 IGF receptor activated PI3K stimulates the Akt phosphorylation, which in turn increases the mTOR/p70S6 kinase activity, triggering myoblast differentiation (Park et al., 2005). The IGF-controlled PI3K/Akt pathway modulates myoblast differentiation both upstream and downstream of MyoG (Tureckova et al., 2001). Akt also mediates IGF effects on increased protein synthesis in muscle fibers through the Akt/mTOR/p70S6 and Akt/mTOR/GSK3 pathways (Rommel et al., 2001) and on decreased protein degradation through FOXO1 and FOXO3A phosphorylation (Coolican et al., 1997; Hribal et al., 2003). When the mTOR inhibitor rapamycin is administered during skeletal muscle

development, both myoblast differentiation and myofiber hypertrophy are ceased (Willett et al., 2009). In genetically engineered mouse models, Akt increases muscle mass and force without increasing satellite cell activation (Blaauw et al., 2009). FOXO1 and FOXO3A are transcription factors of the ubiquitin ligases MurF1 and Atrogin1 (Sandri, 2008); FOXO3A also acts as a transcription factor for the autophagy related genes like BCL2/adenovirus E1B 19k Da interacting protein (BNIP)3 (Mammucari et al., 2007). Phosphorylation of FOXO1 and FOXO3A by Akt leads to inhibited nucleus translocation of these transcription activators (Milan et al., 2015).

1.4.2.2 Fibroblast growth factors (FGFs)

The fibroblast growth factor (FGF) family consists of 22 known members classified into 7 subfamilies sharing conserved sequences. The FGFs are essential for embryonic development, metabolism, body homeostasis, angiogenesis, and wound healing. Among the 22 FGFs, 18 are considered canonical FGFs with the ability to bind and activate one of the four FGF receptor (FGFR1-4) tyrosine kinases. Fourteen of the canonical FGFs are autocrine or paracrine factors that function with co-factor heparin/heparan sulfate. The other 4 canonical FGFs are endocrine FGFs working with co-factor Klotho, a transmembrane protein. The rest of the 4 non-canonical FGFs are intracellular non-signaling proteins that do not work through FGFRs but serve as co-factors for voltage gated sodium channels or other molecules (Ornitz and Itoh, 2015).

Fibroblast growth factor receptors are proteins with three immunoglobulin-like extracellular domains, a single transmembrane domain, and an intracellular split tyrosine kinase domain. Fibroblast growth factor receptor-1 and -4 are highly expressed in satellite cells while FGFR-2 and -3 expression levels in satellite cells are low (Yablonka-Reuveni et al., 1999). Fibroblast growth factor receptor-1 is thought to prevent terminal differentiation while fibroblast

growth factor receptor-4 seems to participate in cell fate determination during embryonic development (Kästner et al., 2000). Compared to FGFR1, FGFR4 mediates weak to no phosphorylation of phospholipase Cγ, SHC adaptor protein, Ras GTPase-activating protein, Raf, or MAPK. The association of GRB2 adaptor protein SH2 domain with FGFR4 is also undetectable (Vainikka et al., 1994). Inhibition of FGFR1 activity leads to ceased progenitor cell migration, loss of myoblasts, reduced skeletal muscle mass and myofiber density in chickens (Flanagan-Steet et al., 2000).

Fibroblast growth factors have stimulatory effects on myoblast proliferation, migration, and self-renewal. In the mouse skeletal muscle cell line MM14, proliferating cells express FGF-1, -2, -6, and -7 mRNAs while differentiated myotubes express FGF-5 and -7, but a reduced level of FGF-6 mRNA (Hannon et al., 1996). FGF-1, -2, -4, -6, and -9 stimulate the proliferation of cultured rat satellite cells (Sheehan and Allen, 1999). Fibroblast growth factor-1 and -4 are detectable in developing muscle and isolated individual myofibers, and FGF-1 is also found in injured adult skeletal muscle. But neither FGF-1 nor -4 is expressed in uninjured adult muscle tissue or during muscle regeneration (Niswander and Martin, 1992; Conte et al., 2009).

Fibroblast growth factor-2 and -6 are located within extracellular matrix and basal lamina of intact skeletal muscle and are ready to participate in muscle regeneration (Dimario et al., 1989). Produced by myofibers, satellite cells, and fibroblasts (Hannon et al., 1996; Chakkalakal et al., 2012; Rao et al., 2013), FGF-2 accelerates satellite cell proliferation with the presence of serum but further myogenesis is not affected under the aborted FGF2 function (Kudla et al., 1998; Yablonka-Reuveni et al., 2015). Fibroblast growth factor-2 expression level is elevated during muscle regeneration while addition of exogenous FGF-2 enhances muscle regeneration in muscular dystrophic mice (Pawlikowski et al., 2017). Observed in embryonic skeletal muscles and

isolated myofibers, FGF-6 is also produced by fast twitch fibers in adult skeletal muscle (Han and Martin, 1993; Ollendorff et al., 1993; Kästner et al., 2000). Though the expression level of FGF6 increases after injury (Floss et al., 1997), the role of FGF6 in muscle regeneration needs more clarification because existing data is contradictory (Floss et al., 1997; Fiore et al., 2000; Armand et al., 2005). However, it is thought that the transient upregulation of FGF6 following muscle injury helps with the proliferation and migration of myogenic stem cells as well as the muscle progenitor cell pool maintenance (Armand et al., 2006).

1.4.2.3 Hepatocyte growth factor (HGF)

Hepatocyte growth factor, also called scatter factor (SF), is originally found in sera of partially hepatectomized rats and considered to be a mitogen for cultured human hepatocytes (Nakamura et al., 1986; Nakamura et al., 1989). Secreted HGF is in its inactive form as a single peptide known as pro-HGF. After proteolytic cleavage, active HGF is generated, which is a heterodimer composed of an alpha chain and a beta chain linked by a disulfide bond (Michalopoulos and Zarnegar, 1992). Due to its mitogenic and motogenic abilities, HGF is widely linked to growth and regeneration of various tissues and cells including skeletal muscle and its progenitor cells, satellite cells (Zarnegar and Michalopoulos, 1995). HGF mRNA and protein expression levels are increasing in injured skeletal muscle during the early stage of muscle regeneration (Jennische et al., 1993). HGF expression levels in spleen, kidney, and serum elevate within one hour of muscle injury (Suzuki et al., 2002). The presence of HGF has also been detected in intact skeletal muscle, where it facilitates satellite cell activation from quiescence.

HGF is the only known growth factor that can activate satellite cells from quiescence to enter cell cycle *in vitro* and *in vivo* (Allen et al., 1995; Tatsumi et al., 1998). Besides its effect on

satellite cell activation, HGF promotes myoblasts proliferation but inhibits myoblasts differentiation. The expression of HGF and its receptor, c-met, are detected in myofibers and satellite cells (Anastasi et al., 1997; Cornelison and Wold, 1997). Upon muscle stretch or injury, HGF is released from extracellular matrix through the regulation of a cascade of calcium-dependent signals. Specifically, influx of extracellular calcium binding to calmodulin activates nitric oxide synthase (NOS). NOS released from basal lamina synthesizes a burst of nitric oxide (NO), which is diffused into extracellular environment. NO then activates matrix metalloproteinase-2 (MMP2) and releases HGF from its binding partner heparan sulfate proteoglycans (HSPGs) in the extracellular matrix, leading to activation of HGF signaling (Tatsumi et al., 2002; Tatsumi et al., 2009; Minako Hara et al., 2012).

C-met, as the only known receptor for HGF, is a type of tyrosine kinase. The dual effects of HGF on promoting satellite proliferation but inhibiting differentiation are mediated by c-met phosphorylation on different tyrosine residues. Upon HGF binding, specific tyrosine kinase phosphorylation recruits GRB2 and activates its downstream signaling cascade Ras/Raf/MEK/ERK which facilitates cell proliferation. When coupling with the p85 subunit from PI3K, c-met can activate the PI3K/Akt pathway and stimulate cell cycle withdrawal and cell differentiation. The comprehensive dual effects of HGF can only be carried out when c-met/GRB2 functions predominantly while c-met/PI3K coupling is low (Leshem et al., 2000). Moreover, c-met also mediates the Ras and calcium activated Ral pathway as well as Wiskott-Aldrich syndrome protein (WASP) N-WASP and WAVE2 downstream of PI3K. These signals are involved in HGF-induced cell lamellipodial formation and chemotactic migration (Suzuki et al., 2000; Kawamura et al., 2004).

HGF stimulates the proliferation of chicken and turkey satellite cells in vitro by enhancing the DNA synthesis in satellite cells (Gal-Levi et al., 1998; Zeng et al., 2002). HGF suppresses MyoD and MyoG gene expression and thereby disrupts the MyoD/E protein or MyoG/E protein heterodimer. This leads to the impaired expression of muscle specific genes like MYH or CKM and inhibition of muscle differentiation (Gal-Levi et al., 1998; Miller et al., 2000). The repression of HGF on MRFs also involves the increased expression of bHLH inhibitor Twist. The reduced cyclin-dependent kinase inhibitor p27 level under HGF regulation leads to accumulated hyper-phosphorylated retinoblastoma protein (pRb), which pushes G_1 phase progression and differentiation inhibition (Leshem et al., 2000; Leshem and Halevy, 2002).

1.4.2.4 Transforming growth factor beta-1 (TGFβ-1)

Transforming growth factor beta-1 (TGFβ-1) is a member of TGFβ superfamily consisting of over 50 structurally related peptides that regulate cell proliferation, differentiation, adhesion, migration, and apoptosis (Massagué, 2000). TGFβ-1 has cell type- and species- dependent effects that profoundly affect development and mass in skeletal muscle (Kollias and Mcdermott, 2008).

TGFβ1 signals through the TGFβ superfamily receptor serine/threonine kinase. Both TGFβ1 and its receptor are widely expressed in different types of cells and tissues including skeletal muscle and myogenic precursor cells (Kingsley, 1994; Alexandra C McPherron et al., 1997). TGFβ1 binds to TGFβ1 receptor II (TGFBR2), which activates type I receptor activin A receptor type II like 1 (ACVRL1) or TGFβ receptor I (TGFB1) (Ten Dijke et al., 1994). Activated TGFβ1 receptors phosphorylate Smad2 and Smad3, which then form complexes with Smad4. These Smad complexes will translocate into the nucleus and inhibit the transcriptional activities

of MRFs by binding to the E box motif of MRF target genes, without affecting the DNA binding ability of the bHLH MRF/E protein heterodimers (Brennan et al., 1991; Martin et al., 1992).

Different from the stimulatory effects on fibroblasts and bone precursor cells, TGFβ1 inhibits the proliferation of turkey satellite cells, cattle satellite cells, pig embryonic myoblasts, and pig satellite cells. Moreover, TGFβ1 decreases the differentiation abilities of turkey, chicken, sheep, and cattle satellite cells (Dodson et al., 1996). The transcription of MyoD gene is down-regulated by TGFβ signaling (Brennan et al., 1991). Smad3 alone inhibits MyoD functions by interacting with the bHLH domain of MyoD, interfering with the ability of MyoD to form heterodimers with E protein and to bind E box motif of its target DNA sequences (Liu et al., 2001). Furthermore, Smad3 *in vitro* also shows its inhibitory effects on myocyte-specific enhancer factor (MEF) 2C transcriptional activities, which are important in late stage muscle differentiation (Liu et al., 2004).

1.4.2.5 Myostatin (MSTN)

Myostatin (MSTN), also known as growth differentiation factor (GDF) 8, is a protein of the TGFβ superfamily that is specifically expressed in developing and adult muscle (A C McPherron et al., 1997). As a myokine, MSTN is produced and secreted by muscle cells and acts on muscle cells in autocrine, paracrine, and endocrine manners (Gao et al., 2013; Lee et al., 2016). The absence of functional MSTN in MSTN-null mice or Belgian blue and Piedmontese cattle leads to a distinguishable muscular hypertrophic phenotype with increased muscle mass called “double muscling” (Grobet et al., 1997; Kambadur et al., 1997; A C McPherron et al., 1997).

In skeletal muscle cells, MSTN inhibits cell proliferation, differentiation, and protein synthesis through different mechanisms. The actions of MSTN are mainly dependent on the MSTN

receptor serine/threonine kinase. Myostatin preferentially binds to type II serine/threonine kinase receptor activin A receptor, type IIB (ACVR2B), recruiting and phosphorylating type I receptor activin A receptor, type IB (ACV21B) or TGFβ receptor I (TGFB1) (Lee and McPherron, 2001).

Myostatin negatively regulates satellite cell activation and self-renewal by hindering the G_1 to S phase progression and down-regulating Pax7. Larger numbers of satellite cells are detected in MSTN deficient mice, accompanied with higher proliferation rate and differentiation ability (McCroskery et al., 2003; Magee et al., 2006). However, some studies have reported that the number and proliferation rate of satellite cells in MSTN deficient mice are not changed compared to wild-type littermates. The post-developmental muscle hypertrophy in MSTN-null animals without functioning satellite cells suggests that larger myonuclear domain rather than increased myoblasts fusion into existing myofibers contributes to muscle hypertrophy in this model (Rodriguez et al., 2011; Lee et al., 2012). When MSTN expression is inhibited, the activation of a small amount of satellite cells is detected after but not before the onset of muscle hypertrophy in adult mice (Wang and McPherron, 2012).

Myostatin inhibits DNA synthesis and cell proliferation in C2C12 cells through accumulation of hypo-phosphorylated Rb resulting from up-regulation of cyclin-dependent kinase inhibitor p21 and down-regulation of CDK2 and CDK4 (Thomas et al., 2000; Taylor et al., 2001; Joulia et al., 2003). The decreased CDK4 level is linked to increased degradation of cyclin D1 through GSK3β phosphorylation. This process involves the serine/threonine kinase receptor ACVR2B and PI3K/Akt pathway activation but not Smad3 (Yang et al., 2007). In human myoblasts, MSTN inhibits cell proliferation through up-regulation of p21 and cell differentiation through MyoD inhibition. MSTN is also found to promote the Notch signaling pathway and

Notch1-intracellular domain association with Smad3, which in turn inhibits the myogenic activity of MyoD (McFarlane et al., 2011).

During C2C12 myoblast differentiation, MyoD, Myf5, and MyoG expression and activities are negatively influenced by MSTN (Joulia et al., 2003). Upon MSTN receptor activation, increased phosphorylated Smad3 associates with MyoD, thereby interfering with its expression level and activity (Langley et al., 2002). Upregulation of the Ras/Raf/MEK/ERK signaling pathway is detected under MSTN treatment through ACVR2B activation in differentiating C2C12 cells, indicating that MSTN-stimulated activation of ERK negatively regulates myogenic differentiation (Yang et al., 2006). Furthermore, MSTN decreases fusion index, myotube diameter, and creatine kinase activity in human myoblasts by inhibiting the Akt/mTOR pathway. Inhibition of regulatory-associated protein of mTOR (RAPTOR) and mammalian target of rapamycin complex (mTORC)-1 reinforces the MSTN receptor activated Samd2 phosphorylation (Trendelenburg et al., 2009).

1.4.3 Steroid hormones

Derived from metabolites of cholesterol, steroid hormones can be categorized into two classes: corticosteroids, typically produced in the adrenal gland, and sex steroids, typically made in the gonads or placenta. Depending on the type of receptors they bind, steroid hormones can also be classified into five types: glucocorticoids (corticosteroids; binding to glucocorticoid receptor, GR), mineralocorticoids (corticosteroids; binding to mineralocorticoid receptor, MR), androgens (sex steroids; binding to androgen receptor, AR), estrogens (sex steroids; binding to estrogen receptor, ER), and progestogens (sex steroids; binding to progesterone receptor, PR) (Nussey and Whitehead, 2013). Steroid hormones play important roles in metabolism, immune response,

osmoregulation, and sexual development (Funder et al., 1997; Gupta and Lalchhandama, 2002; Frye, 2009). Among steroid hormones, androgen, estrogen, and glucocorticoids are also known for their critical regulatory roles in the healthy growth and development of skeletal muscle (Schakman et al., 2013; Sato and Iemitsu, 2015).

Hydrophobic in nature, steroid hormones are transported in the form of steroid hormone/carrier protein complex in circulation for higher solubility. Sex hormone-binding globulin (SHBG) is a glycoprotein binding to androgen and estrogen while corticosteroid-binding globulin (CBG) is an alpha-globulin binding to glucocorticoids (Avvakumov et al., 2010; Hammond, 2016). Based on the free hormone hypothesis, only free steroids that are not bound by serum carrier proteins can passively diffuse through the cell membrane (Mendel, 1989; Hammond, 2016).

Different from growth factors that act through cell membrane receptors, steroid hormones exert their effects through intracellular/nuclear receptors that are located in the cytoplasm or nucleus associated with heat shock proteins (HSP) (Kumar and Thompson, 1999). Nuclear receptors (NRs) are a class of proteins that share great similarity in structure and sequence. NRs can directly bind to DNA and function as transcription factors. Each NR consists of a ligand binding domain (LBD), a hinge region, a DNA binding domain (DBD), and a variable region. Ligand binding to NR results in conformational changes that can accommodate binding of co-regulatory proteins to ligand-dependent activation function (AF)-2 and small molecule modulators to binding function (BF)-3. Furthermore, the surfaces of LBD are also involved in dimerization of receptor monomers (Buzón et al., 2012). Connecting LBD to DBD is the hinge region where the nuclear translocation sequence is located. The hinge region is essential for the translocation of ligand bound receptors from cytoplasm to nucleus. DBD is highly conserved with two zinc fingers

mediating homo- and hetero-dimerization and binding of NR-ligand to DNA. NRs typically bind to specific DNA regions called hormone response elements (HRE). HRE sequences with inverted repeats of half-site sequences AGAACA or AGGTCA are the targets of AR and GR or ER, respectively (Malkoski and Dorin, 1999; Gruber et al., 2004; De Bruyn et al., 2011). The N-terminal variable region is the largest but least conserved domain for steroid hormones. This regulatory domain contains the ligand-independent activation function (AF)-1 that can carry out weak transcription activity without ligand activation. When collaborating with AF-2 and LBD, AF-1 can ensure the robust upregulation of target gene expression (Wärnmark et al., 2003).

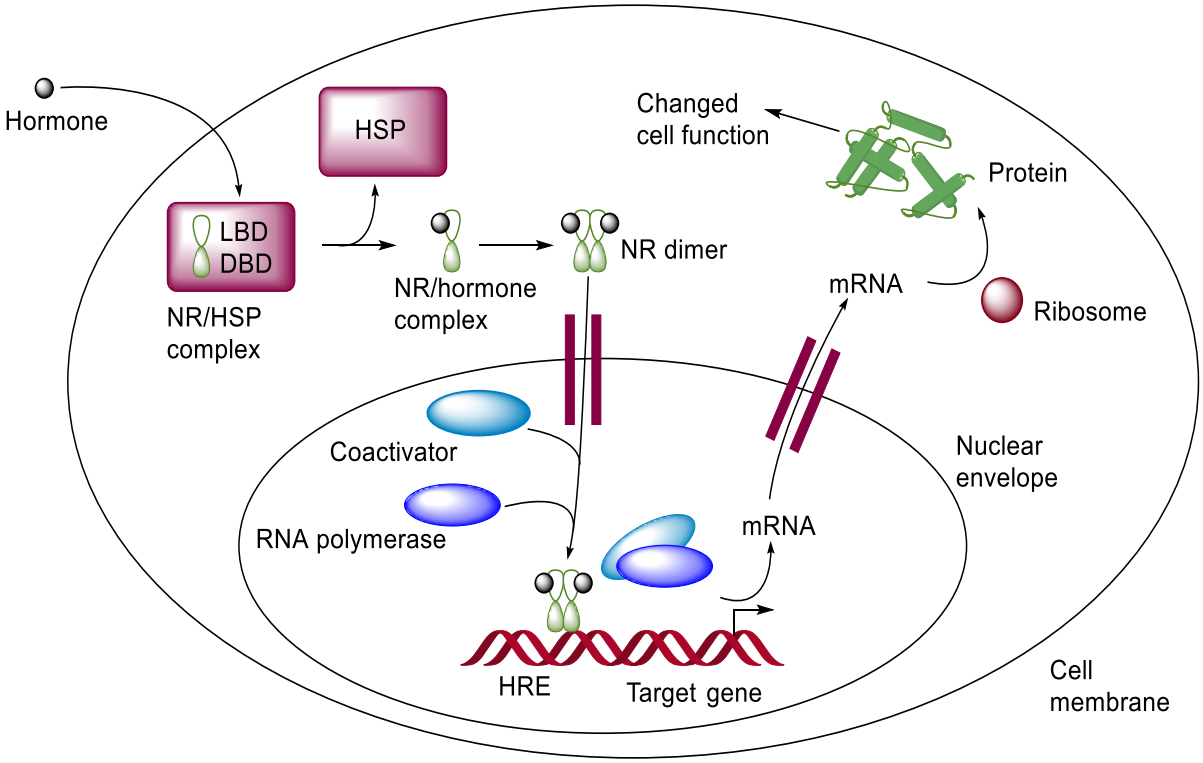

Figure 1.6 Simplified schematics for signaling pathways from steroid hormone receptors Steroid hormone diffuses through plasma membrane into cytoplasm. . Hormone binds with nuclear receptor in cytoplasm, replacing HSP and forming a nuclear receptor/hormone complex dimer. The complex dimer enters the nucleus, recruiting coactivator and RNA polymerase and initiating gene transcription. Transcribed mRNA is translated into proteins that change cell functions. LBD, ligand binding domain; DBD, DNA binding domain; NR, nuclear receptor; HSP, heat shock protein; HRE, hormone response element. (Adapted from Natale, D. A., C. N. Arighi, W. C. Barker, J. Blake, T.-C. Chang, Z. Hu, H. Liu, B. Smith, and C. H. Wu. 2007. Framework for a Protein Ontology. BMC Bioinformatics. 8:S1–S1. doi:10.1186/1471-2105-8-S9-S1.)

Steroid hormone receptors are type I nuclear receptors binding with HSP in the cytosol. Upon hormone competitively binding NR in the cytosol, the hormone/NR complex releases HSP and forms a homodimer with another identical hormone/NR complex. Under the facilitation of nuclear translocation signal, the hormone/NR homodimer translocates into nucleus through nuclear pore. In the nucleus, the homodimer binds to the hormone-specific HREs on target DNA and recruits coactivators and other proteins for activation of transcription (Mangelsdorf et al., 1995; Nussey and Whitehead, 2013) (**Figure 1.6**).

1.4.3.1 Androgen and Estrogen

An androgen is a steroid hormone that regulates the development and maintenance of male characteristics in vertebrates. Testosterone (T) is the major androgen along with two other important members dihydrotestosterone (DHT) and androstenedione (Mooradian et al., 1987; Rommerts, 1998). Testosterone has been closely studied and linked to skeletal muscle development for years (Florini, 1987). Testosterone is able to reverse the reduced percentage of proliferating satellite cells in castrated pigs (Mulvaney et al., 1988). In the early study model of levator ani muscle, T increases satellite cell proliferation, number of myonuclei, and hypertrophy during early development (Joubert and Tobin, 1989; Joubert et al., 1994). Perinatally, T is also responsible for the higher myofiber number and greater cross-section area in male than female (Joubert et al., 1994). Postnatally, T increases the levator ani muscle hypertrophy and satellite cell proliferation during days 2 and 3 of administration but has no effect on muscle fiber number (Joubert and Tobin, 1989). However, only 30% of the satellite cell population is affected by T treatment (Joubert and Tobin, 1995). Satellite cell activation under denervation is impaired in

levator ani muscle in castrated males, suggesting T involvement in satellite cell activation (Nnodim, 2001).

Estrogen is the primary female sex hormone that is responsible for the development and regulation of female reproductive system and secondary sex characteristics (Nilsson et al., 2001). In non-pregnant females, estrone (E1), estradiol (E2), and estriol (E3) are the three major forms of estrogen among which E2 has the strongest estrogenic effects and is the most studied (Shimizu, 2005). E2 administration prevents exercise-induced muscle damage in ovariectomized female and male rats with the elevation of activation and numbers of proliferating satellite cells in soleus and white vastus muscles (Bär et al., 1988; Tiidus et al., 2005; Enns and Tiidus, 2008). Additionally, E2 administration counteracts muscle atrophy caused by muscle immobilization (Sugiura et al., 2006).

For nearly 60 years, both androgen and estrogen have been widely applied in beef cattle production to promote feed efficiency, rate of gain, and muscle growth (Hancock et al., 1991). Trenbolone acetate (TBA), a synthetic testosterone analog that is not subject to aromatization, has long been used as a growth promoter in cattle (Galbraith and Watson, 1978). It was demonstrated later that the combination of TBA with E2 implant can further improve protein composition, feedlot performance, and carcass protein accretion in cattle (Hayden et al., 1992; Johnson et al., 1996). Steers implanted with TBA+E2 combination not only show increased ADG and feed efficiency but also exhibit increased circulating IGF-1 concentrations. Bovine satellite cells (BSC) isolated from steers implanted with TBA and E2 have higher proliferation rate and greater fusion index compared to controls (Johnson et al., 1998). More detailed studies on yearling steers indicated that IGF1 mRNA level increased by the 7th day after TBA+E2 implantation, and that IGF1 mRNA level continued to increase and reached 3 times higher than pre-implant 28 d after

implantation. mRNA levels of myostatin, IGFBP3, and HGF were not affected by implantation (Pampusch et al., 2003). *In vitro,* either TBA or E2 treatment also significantly increased IGF1 mRNA expression in either myoblasts or myotubes accompanied by increased expression of estrogen receptor α (ESR1) or AR (Kamanga-Sollo et al., 2004).

Estrogen receptor α (ESR1) and estrogen receptor β (ESR2) are the main players in E2 transcriptional activation of IGF1 mRNA in non-muscle tissues. However, the effect of E2 on promoting IGF1 mRNA level in longissimus muscle in steers or BSC is believed to be carried out through an unconventional E2 signaling pathway (Dayton et al., 2013). G protein-coupled receptor (GPR) 30, also known as G protein-coupled estrogen receptor (GPER1), is identified in skeletal muscle as the potential mediator of E2 action on IGF1 expression (Prossnitz et al., 2008). GPER1 agonist, G1, was able to elevate the IGF1 mRNA level in BSC culture but not to increase BSC proliferation rate, and E2-stimulated BSC proliferation was reversed with the presence of E2 antagonists (Kamanga-Sollo et al., 2008a). Even in BSC showing no sign of increased IGFs and IGFR1, E2 treatment still stimulated BSC proliferation (Kamanga-Sollo et al., 2008b). These findings suggest that E2 stimulates IGF1 mRNA expression via GPER1, which is a different mechanism from how E2 stimulates BSC proliferation, which may involve ESR. Application of ESR1- or IGFR1-specific siRNAs completely abolished E2-mediated BSC proliferation (Kamanga-Sollo et al., 2013). These results indicate that ESR1, not ESR2, is responsible for E2 stimulated BSC proliferation.

IGF1R is another essential component for carrying out the stimulatory effect of E2 on BSC proliferation. A more recent study has shown an essential role of epidermal growth factor receptor (EGFR) in E2-stimulated BSC proliferation because inhibiting or silencing EGFR led to suppression of E2 stimulation on BSC proliferation. EGFR is known to regulate IGF1R

expression; EGFR silencing might lead to post-translational modification that decreases IGF-1Rβ protein level and indirectly influences the E2-stimulated BSC proliferation (Reiter et al., 2014).

TBA increases IGF1 mRNA expression in BSC through AR (Kamanga-Sollo et al., 2008b). The TBA-increased BSC proliferation was negatively impacted by AR inhibitors (Kamanga-Sollo et al., 2004). An androgen response element (ARE) is later identified on IGF1 DNA upstream promoter region, indicating that androgen stimulates IGF1 mRNA expression likely through androgen/AR complex interaction with this ARE (Wu et al., 2007). The addition of MEK1 inhibitor, PD98059 or PI3K inhibitor, wortmannin to E2- or TBA-treated BSC culture suppressed the stimulatory effects of E2 and TBA on cell proliferation. These data suggest that both the MAPK pathway and the PI3K pathway are necessary for E2/TBA-mediated BSC proliferation and that IGF1 is at least partially responsible for the stimulatory effects of E2/TBA on BSC proliferation.

Additionally, E2 or TBA treatment increased protein synthesis rate in fused BSC in a dose-dependent manner (Kamanga-Sollo et al., 2011). The ESR antagonist or AR antagonist reversed the E2 or TBA stimulated protein synthesis and decreased protein degradation in fused BSC, indicating ESR1 and 2, and AR are responsible for E2 mediated protein turnover rate in fused BSC (Kamanga-Sollo et al., 2011). Increased phospho-Akt protein level in E2- or TBA-treated fused BSC implies that PI3K/Akt pathway is involved in E2- or TBA-altered protein turnover rates (Thornton et al., 2016; Kamanga-Sollo et al., 2017).

In E2-treated proliferating BSC or fused BSC, inhibition of GPER1, matrix metalloproteinases 2 and 9 (MMP2/9), and heparin binding epidermal growth factor-like growth factor (hbEGF) by their specific inhibitors diminished the stimulatory effects of E2 on BSC proliferation and suppressed E2-stimulated protein synthesis without affecting decreased protein

degradation rate (Kamanga-Sollo et al., 2014; Kamanga-Sollo et al., 2017). These results suggest a non-canonical pathway of E2 stimulates BSC proliferation by activating GPER1 and EGFR.

1.4.4 Thyroid hormone (TH)

Thyroid hormones (TH), thyroxine/ tetraiodothyronine (T4), and triiodothyronine (T3), are hydrophobic hormones made in the thyroid gland. Thyroid hormone production is controlled by thyroid-stimulating hormone (TSH) that is produced and secreted by the anterior pituitary gland. The production of TSH is under the regulation of thyrotropin-releasing hormone (TRH) made and secreted by the hypothalamus. In the thyroid gland, T4 is produced as a prohormone with four iodine atoms attached to its tyrosine residues while a small amount of T3 is also made directly in the thyroid gland with three iodine atoms in its structure. The majority of active free T3 is converted from T4 by type 2 iodothyronine deiodinase (DIO2) (Köhrle, 2000). T3 is the more active thyroid hormone. Inactivation of thyroid hormones is carried out by type 3 iodothyronine deiodinase (DIO3), which removes an iodine atom on the inner ring and coverts T4 to reverse T3 (rT3) and T3 to diiodothyronine (T2) (Bianco and Kim, 2006; Nussey and Whitehead, 2013).

Thyroid hormones essentially affect every cell in the body. Thyroid hormones control metabolism, homeostasis, and growth and development in skeletal muscle and other organs throughout the body. Specifically, T3 exerts its activity in skeletal muscle through TH nuclear receptors α and β (THRA and THRB) (Brent, 2012). Thyroid hormone receptors are considered type II nuclear receptors sharing high structural similarity with steroid hormone receptors. The major difference between them is that the variable region of the thyroid hormone receptor is smaller in size compared to the steroid hormone receptor (Amoutzias et al., 2007). In skeletal muscle, it is THRA1 that mainly mediates the T3-dependent activation or repression of gene

transcription (Milanesi et al., 2016). The thyroid hormone receptor is retained in the nucleus regardless of its ligand binding status. When a ligand is not available, THR binds to thyroid hormone responsive elements on target DNA, forming a heterodimer with retinoic acid receptor

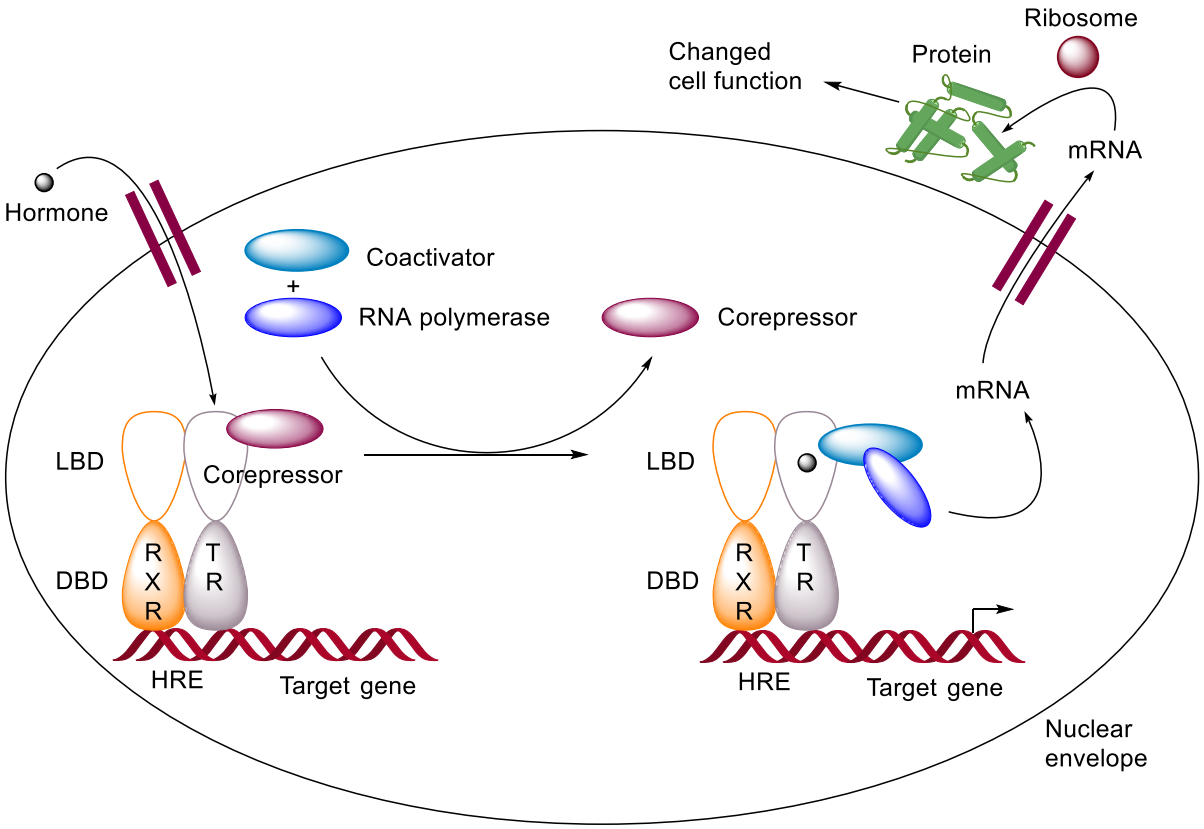

Figure 1.7 Simplified schematics for signaling pathways from the thyroid hormone receptor Thyroid hormone enters the cytosol and then the nucleus. In the nucleus, thyroid hormone binds to thyroid hormone receptor, releasing the corepressor on the receptor complex and recruiting coactivator and RNA polymerase. Activated receptor-hormone complex alters target gene mRNA transcription, which leads to the altered specific protein synthesis through ribosome. LBD, ligand binding domain; DBD, DNA binding domain; RXR, retinoid X receptor; TR, thyroid hormone receptor; HRE, hormone response element. (Adapted from Brent, G. A. 2012. Mechanisms of thyroid hormone action. J. Clin. Invest. 122:3035–3043. doi:10.1172/JCI60047.)

RXR. Corepressor NCoR2 and histone-modifying enzymes are among the components complexed with the THR/RXR heterodimer. This THR protein complex actively represses target gene transcription. Upon T3 binding to THR, corepressors are replaced by coactivators so that RNA polymerase is recruited to stimulate gene transcription (Yen, 2001; Brent, 2012) (**Figure 1.7**).

In skeletal muscle, the expression of a number of proteins is transcriptionally regulated by T3-mediated signaling. T3 up-regulates the expression of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) 1a and 2a (Hartong et al., 1994; Simonides et al., 1996), uncoupling protein 3 (UCP3) (Solanes et al., 2005), GLUT4 (main glucose transporter in muscle) (Zorzano et al., 2005), NADP-dependent malic enzyme (ME1; catalyze the NADPH-dependent decarboxylation of lactate) (Desvergne et al., 1991), and muscle glycerol-3-phosphate dehydrogenase (mGPDH; dominantly expressed in mitochondria of fast muscle fibers) (DÜMMLER et al., 1996), but down-regulates the expression of MyHC slow protein (encoded by MYH7 gene) (Morkin, 2000). These expression changes in structural and metabolic proteins show the slow to fast fiber shifting as well as increased glycolytic and oxidative metabolism capacity in skeletal muscle (Salvatore et al., 2014). T3 up-regulates the expression of MyoD (Muscat et al., 1994) and MyoG (Downes et al., 1993). Working as a downstream effector of T3, MyoD stimulates the expression of fast fiber proteins MyHC IIA (encoded by MYH2), MyHC IIX (encoded by MYH1), and MyHC IIB (encoded by MYH4) (Muller et al., 1996; Kraus and Pette, 1997; Wheeler et al., 1999; Allen et al., 2001; Aguiar et al., 2013). T3 is also able to induce the slow-to-fast fiber transition in the pattern of MYH7 → MYH2, MYH2→MYH1, and MYH1→MYH4 (Simonides and van Hardeveld, 2008). Furthermore, calcineurin as an important signaling mediator for maintenance of slow type fibers in skeletal muscle is down-regulated by T3 (Simonides and van Hardeveld, 2008). Overall, T3 modulates the skeletal muscle plasticity through transcriptional activation or repression of various genes.

During myogenesis, the intracellular level of T3 is critical to cell cycle progression in myogenic precursor cells like satellite cells. DIO3 is highly expressed in activated proliferating satellite cells to keep TH at a relatively low level. Attenuated TH signal ensures the amplification of satellite cells without withdrawing from cell cycle or tuning to terminal differentiation (Dentice

et al., 2014). Once differentiation progress initiates, DIO3 level decreases while DIO2 expression increases (Dentice et al., 2010). The changes of these DIO enzyme levels alter the TH from inactive T4 to active T3 and increase in the cell T3 level. Active T3 is able to stimulate the transcription of MRFs like MyoD and its downstream myogenic factors through THRA1 not THRB. Activated THRA1 is able to up-regulate the Wnt/β-catenin signaling through transcriptional activation and influence satellite cell proliferation and differentiation through this pathway (Milanesi et al., 2016). High levels of DIO2 and T3 in neonates are crucial for muscle fiber type shifting from embryonic myosin heavy chain isoforms to adult myosin heavy chain isoforms (Simonides and van Hardeveld, 2008). In bovine fetuses, rT3 and T4 blood levels gradually increase from development day 110 to day 210 and development day 110 to day 260, respectively. However, the blood T3 level increase is not observed until development day 180 and onwards. On development day 260, high blood levels of rT3 and T4 are detected in contrast to low T3 blood level (Cassar-Malek et al., 2007). However, postnatal cattle show decreased T4 level as well as increased T3 despite a surge of T4 level observed right after birth (Hernandez et al., 1972). Overall, the low level of T3 in early stage of prenatal development seems to be essential for precursor cells proliferation and survival; postnatal increase of T3 is critical for the full development of body and muscle fiber type phenotypes.

1.5 Conclusion

Skeletal muscle is a dynamic tissue with great hypertrophic ability during postnatal life and remarkable regeneration capacity in adulthood. Though the number of myofiber is largely determined prenatally, the postnatal addition of myonuclei and increase of individual myofiber size are critical for the continuous building of skeletal muscle size and strength.

Satellite cells are the main source of postnatal myogenic precursor cells, and their activation, proliferation, and differentiation are regulated primarily by transcription factors, including Pax7, Myf5, MyoD, MyoG and MRF4, and hormones such as insulin-like growth factors, hepatocyte growth factor, and fibroblast growth factors. Clearly, a better understanding of the roles of transcription factors and hormones in myogenesis can help design strategies for enhancing muscle growth and regeneration and preventing muscle atrophy in humans and for improving meat production efficiency and quality in agricultural animals.

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Chapter 2 Effect of oxytocin on bovine myoblasts proliferation, differentiation, and fusion

Abstract

Oxytocin (OXT) is a hormone that is best known for its roles in lactation and parturition. Recent studies have shown that OXT stimulates the proliferation of murine satellite cells and differentiation of human satellite cells. The objective of this study was to determine if OXT has similar effects on bovine satellite cells. Using RT-PCR, we showed that OXT receptor (OXTR) mRNA was expressed in bovine skeletal muscle, myoblasts, and myotubes derived from bovine satellite cells. Using Western blotting, we detected OXTR protein in both bovine myoblasts and myotubes. Using EdU labeling, we found that treating bovine myoblasts with 10 and 100 nM OXT for 24 h increased their proliferation rate by 7 and 10%, respectively ($P < 0.05$). Myogenin (MYOG), creatine kinase (CKM), myosin heavy chain 3 (MYH3), and myoglobin (MB) are markers of differentiated myoblasts and fused myotubes. Treating bovine myoblasts during induced differentiation with 10 nM and 100 nM oxytocin for 48 h had no effect on the expression of these four genes except MB. Staining the myoblasts at the end of 48 h differentiation with Giemsa and counting the nuclei located in myotubes showed that the fusion index was not different between OXT-treated and control myoblasts ($P > 0.05$). These results indicate that OXT has no effect on the differentiation and fusion of bovine myoblasts. We lastly determined if OXT stimulates bovine myoblast proliferation through ERK1/2. Treating bovine myoblasts with the ERK1/2 inhibitor U0126 completely reversed the stimulatory effect of OXT on myoblast proliferation ($P < 0.05$). However, based on Western blotting, OXT did not change the phosphorylation level of ERK1/2 in bovine myoblasts ($P > 0.05$). These results support the

possible participation of ERK1/2 in OXT-stimulated proliferation in bovine myoblasts but this participation does not involve ERK1/2 phosphorylation

2.1 Introduction

Oxytocin (OXT) is known as a nonapeptide synthesized by neurons located in the supraoptic and paraventricular nuclei within the hypothalamus and released by the posterior pituitary (Standring, 2015). Oxytocin has a wide range of central and peripheral effects among which the best known are stimulation of uterine contractions during parturition and milk release during lactation (Hauth et al., 1986). Oxytocin also regulates various social behaviors as well as cardiovascular functions (Petersson, 2002; Bartz et al., 2011).

The actions of oxytocin are mediated by the oxytocin receptor (OXTR), a transmembrane receptor belonging to the G protein-coupled receptor superfamily (Gimpl and Fahrenholz, 2010). This receptor is functionally coupled to $G\alpha_{q/11}$ class GTP-binding proteins, which activates phospholipase C (PLC) isoforms upon binding of OXT to OXTR (Devost et al., 2008). Activated PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the secondary messengers inositol trisphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 triggers Ca^{2+} release from endoplasmic reticulum (ER) to the cytosol. Both DAG and intracellular Ca^{2+} activate protein kinase C (PKC). Activated PKC and increased intracellular Ca^{2+} stimulate a series of cellular events including the formation of Ca^{2+}-calmodulin complex that initiate smooth muscle contraction in myometrium or mammary gland (Masso-Welch et al., 1998; Sanborn et al., 1998). Binding of OXT to OXTR also activates the extracellular signal-regulated kinases 1/2 (ERK1/2) in myometrial cells (Ohmichi et al., 1995).

Recent studies have shown additional functions of OXT in stimulating osteogenesis and cardio-myogenesis but inhibiting adipogenesis (Tamma et al., 2009; Noiseux et al., 2012; Beranger et al., 2014).Oxytocin has been also linked to skeletal muscle. Oxytocin increases the proliferation of muscle stem cells in mice and enhances the fusion of human myoblasts in vitro (Breton et al.,

2002; Elabd et al., 2014). These newly discovered functions indicate the possible roles of OXT as a general growth and differentiation factor.

A role of oxytocin in skeletal muscle growth and development in cattle is suggested by the finding that anabolic steroids-increased muscle growth in cattle was associated with increased plasma concentration of oxytocin and skeletal muscle expression of oxytocin mRNA (De Jager et al., 2011). Therefore, the objective of this study was to determine the effects of oxytocin on the proliferation, differentiation, and fusion of bovine primary myoblasts in vitro.

2.2 Material and methods

2.2.1 Isolation of bovine satellite cells

Satellite cells were isolated from extensor carpi radius muscle from Angus or Angus crossbred steers using a previously published protocol (Ge et al., 2012). Briefly, muscle was dissected to remove connective tissues and intramuscular fat. Muscle was then cut into small pieces and ground through a meat grinder. Ground muscle was digested in 1 mg/mL PRONASE protease, *Streptomyces griseus* (Calbiochem, San Diego, CA) in PBS for 1 h at 37℃ in a shaker incubator. The digested muscle was firstly centrifuged at 1,500 g for 10 min at room temperature. The pellet was resuspended in PBS and centrifuged at 400 g at room temperature for 5 min. From this centrifugation, the supernatant was collected and the pellet was discarded. This two-speed (first 1,500 g and then 400 g) centrifugation cycle was repeated 3 more times to enrich satellite cells. A final 1,500 g centrifugation was performed to collect the purified satellite cells. The isolated satellite cells were cultured in growth medium containing Dulbecco's Modified Eagle Medium (**DMEM**; CORNING, Corning, NY), 10% fetal bovine serum (**FBS**; Atlanta Biologicals, Lawrenceville, GA), 2% L-glutamine (CORNING), and 1% ABAM for up to 10 days before being

used for the following proliferation and differentiation experiments. At least 90% of satellite cells isolated this way were Pax7+ cells determined by an immunocytochemistry method.

2.2.2 Proliferation experiments

Bovine myoblasts were seeded in growth medium at a density of 2×10^4 cells/well in 24-well plates. Approximately 4 hours after seeding, when most myoblasts would have attached to the plates, growth medium was removed. Cells were washed twice with warm (37℃) PBS, and then cultured in proliferation medium containing 2.5% FBS, 2% L-glutamine, and 1% ABAM overnight.

To determine the effects of oxytocin, myoblasts were cultured with10 nM and 100 nM oxytocin (OXT) (Bachem, Bubendorf, Switzerland), and 200 ng/mL insulin-like growth factor I (Research and Diagnostic Systems, Inc, Minneapolis, MN) as a positive control for 24 h. To determine the signaling mechanism that mediated the effect of oxytocin on myoblast proliferation, myoblasts were treated with 10 nM OXT and 10 μM U0126 (Cell Signaling Technology, Danvers, MA) or 1 μM BIM-1 (Biovision, Milpitas, CA) for 48 h. U0126 is an inhibitor for both MEK1 and MEK2 (Favata et al., 1998). BIM-1 is a bisindolylmaleimide-based protein kinase C (PKC) inhibitor (Toullec et al., 1991). Medium and treatment were refreshed every 24 h.

2.2.3 EdU Proliferation Assay

Myoblast proliferation rate was determined through EdU assay (Salic and Mitchison, 2008). Following an initial overnight culture in proliferation medium, half of the culture medium was replaced with freshly made proliferation medium containing treatment and 20 μM EdU (ThermoFisher Scientific, Waltham, MA), and myoblasts were further cultured for 24 h. Myoblasts

were fixed in 4% paraformaldehyde (PFA) (ThermoFisher Scientific) for 15 min and then permeabilized in 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for 20 min. Myoblasts were then incubated in Click-iT reaction cocktail with fluorescence dye Alexa flour 594 (ThermoFisher Scientific) for 30 min at room temperature. Finally, myoblasts were incubated with 5 μg/ml Hoechst 33342 (FisherThermo Scientific) in PBS for 30 min at room temperature to stain DNA.

Stained cells were photographed with a fluorescence microscope (Nikon, Tokyo, Japan). Ten randomly selected images were taken from each well at 10× magnification. Numbers of total nuclei and actively replicating nuclei were counted separately using NIS-elements advanced research microscope imaging software (Nikon). The proliferation rate was defined as the percentage of actively replicating nuclei in total nuclei. The proliferation experiment was repeated 4 times, each time using satellite cells isolated from a different steer.

2.2.4 Differentiation and fusion experiments

Bovine myoblasts were seeded in growth medium at approximately 80% confluency and cultured overnight. Myoblasts were then cultured in differentiation medium containing DMEM, 2% horse serum (**HS**; Atlanta Biologicals, Lawrenceville, GA), 2% L-glutamine, and 1% ABAM, as described before (Ge et al., 2013). The effects of OXT on bovine myoblasts differentiation were determined by including 10 nM and 100 nM OXT in the differentiation medium. Medium and treatment were refreshed every 24 h throughout differentiation. Myoblasts cultured for 48 h in differentiation medium were collected for total RNA isolation and mRNA expression analyses and were stained with Giemsa for fusion index quantification. This differentiation and fusion

experiment was repeated at least 6 times, each time using satellite cells isolated from a different animal.

2.2.5 Preparing myoblasts for phosphorylation detection

To determine the effects of OXT on phosphorylation of ERK proteins, bovine myoblasts were cultured in serum-free medium overnight. Myoblasts were first treated with 10 μM U0126, 1 μM BIM-1 or control (DMSO) in serum-free medium for 30 min, and then with 10 nM OXT or control for another 30 min before being lysed for total protein isolation.

2.2.6 RNA extraction, RT-PCR, and quantitative reverse transcription PCR (RT-qPCR)

TRI reagent was used for total mRNA extraction following the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Concentrations of total RNA samples were measured using NanoDrop 1000 (ThermoFisher Scientific). For reverse transcription, 1 μg total RNA was reverse transcribed into cDNA using a two-step method with ImProm-II reverse transcriptase and random primers in a final volume of 20 μL (Promega, Madison, WI), according to the manufacturer's instruction.

Traditional PCR was used to detect OXTR and OXT mRNA expression in various bovine tissues and cells. The PCR reaction was set up in a 25 μL system, containing 50 ng cDNA, 12.5 μL DreamTaq Green PCR Master Mix (2×) (ThermoFisher Scientific), and 1 μM gene-specific forward and reverse primers (**Table 2.1**) in PCR tubes (USA Scientific, Inc., Ocala, FL). The reactions were carried out in an Eppendorf 5331 MasterCycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) following the thermal cycling protocol: 3 min initial denaturation at 95℃ followed by 35 cycles of 30 s for denaturation at 95℃, 30 s for annealing at

60℃, and 1 min for extension at 72℃, and 10 min for the final extension at 72℃. Following the amplification, 25 µL of the PCR products were loaded to a 1.8% agarose gel containing 0.5 µg/mL Ethidium Bromide (ThermoFisher Scientific). DNA electrophoresis was conducted at 110V for 1 h using BioRad Model 3000Xi computer controlled electrophoresis power supply (Bio-Rad Laboratories, Hercules, CA). The images of agarose gels were taken using FluorChem SP gel imaging system (Alpha Innotech, San Leandro, CA).

Real-time PCR was used to quantify mRNAs of oxytocin (OXTR), myogenin (**MYOG**), myosin heavy chain 3 (**MYH3**), creatine kinase, muscle (**CKM**), and myoglobin (**MB**), using the SYBR Green method. The PCR reaction was set up in a 10 µl system, containing 10 ng cDNA, 5 µL iTaq Universal SYBR Green Supermix (2×) (Bio-Rad Laboratories), and 500 nM gene-specific forward and reverse primers (**Table 2.1**) in 96-well plates (Bio-Rad Laboratories). The reactions were carried out in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) following the standard thermal cycling protocol: 30 s polymerase activation and DNA denaturation at 95℃ followed by 40 cycles of 5 s for denaturation at 95℃ and 30 s for annealing, extension, and fluorescence read at 60℃. The relative abundance of target mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) by normalizing to the abundance of 18S ribosomal RNA (**18S**) (Thellin et al., 1999).

2.2.7 Giemsa staining and Fusion index

Myoblasts and myotubes at 48 h of differentiation were stained with Giemsa stain as described by Freshney (Freshney, 2015). In short, cells were washed with PBS, and then fixed in 50% methanol in PBS for 2 min, 100% methanol for 10 min, and rinsed with fresh 100% methanol for 2 min. The fixed cells were then incubated with Giemsa stain (RICCA Chemical Company,

Arlington, TX) for 1 min, and 1:10 diluted Giemsa stain in water for 1 min on a shaker. Cells were finally rinsed with tap water for 2 min. Stained cells were photographed with an inverted microscope. Nine randomly selected images were taken from each well at 10 × magnification. Numbers of total nuclei and fused nuclei were counted separately using ImageJ (U. S. National Institutes of Health, Bethesda, MD). At least 500 total cell nuclei were counted for each treatment in each experiment. The fusion index was defined as the percentage of total nuclei that were in myotubes containing at least 3 nuclei. The fusion experiment was repeated 8 times, each time using satellite cells isolated from a different animal.

2.2.8 Total protein isolation from bovine tissues and cells

Total protein from various bovine organs was isolated by homogenizing the tissue in the radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented with Halt protease inhibitor cocktail (100X) (ThermoFisher Scientific). Total protein from cells was isolated by lysing the cells in the RIPA buffer added with protease inhibitor cocktail and PhosSTOP phosphorylation inhibitor (Roche Holding AG, Basel, Switzerland). Tissue homogenates or total cellular lysates were centrifuged at 93,000 g for 15 min at 4℃. The supernatant was collected and protein concentration of the supernatant was determined with a BCA assay.

2.2.9 Western blotting

For western blotting, 15 μg of protein per sample were mixed with 4X Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 355 mM β-mercaptoethanol

(ThermoFisher Scientific) and heated at 95℃ for 5 min. After cooling to room temperature, protein samples were loaded into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**) gels placed in a Mini PROTEAN 3 Cell (Bio-Rad Laboratories). The proteins were separated by running the SDS-PAGE initially at 80 V for 30 min and then at 120 V for 2 h in tris-glycine-SDS running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) at 4℃. The separated proteins were transferred from gels onto 0.2 µm nitrocellulose membranes (Bio-Rad Laboratories) by electrophoresis at 90 V for 90 min at 4℃ in tris-glycine transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) in a Mini Trans-Blot Cell (Bio-Rad Laboratories).

For western blotting of oxytocin receptor (OXTR) protein, the membranes were blocked in 5% non-fat dry milk dissolved in tris-buffered saline-Tween 20 (TBS-T) buffer (20 mM Tris-HCl, 500 mM NaCl, and 0.05% Tween-20) for 1 h at room temperature. The intact membrane was first incubated with 1:500 diluted OXTR antibody (Abcam, Cambridge, United Kingdom), and then stripped and incubated with 0.02 µg/mL β-Tubulin antibody (Developmental Studies Hybridoma Bank).

For western blotting of phospho-ERK protein, the membranes were blocked in 5% BSA (ThermoFisher Scientific) dissolved in tris-buffered saline-Tween 20 (TBS-T) buffer (20 mM Tris-HCl, 500 mM NaCl, and 0.05% Tween-20) for 1 h at room temperature. Firstly, the membranes were incubated with 1:2000 diluted phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody (#9106, Cell Signaling Technology) and then the membranes were stripped and incubated with 1:1000 diluted p44/42 MAPK (ERK1/2) antibody (#4695, Cell Signaling Technology).

The stripping was done by incubating the membrane in restore fluorescent western blot stripping buffer (ThermoFisher Scientific) for 15 min. The stripped membrane was washed 3 times

with TBST and blocked for 1 h at room temperature before incubation with the beta tubulin antibody.

All incubations with primary antibodies were carried out at 4℃ overnight. Following incubation with a primary antibody, the membrane was washed 3 times with TBST, each time for 5 min, and then incubated with 1:25,000 diluted secondary antibody IRDye 800CW Goat anti-Mouse or Goat anti-Rabbit IgG (H + L) (LI-COR Biosciences, Lincoln, NE) in TBST for 1 h at room temperature. The membrane was washed 3 times with TBST, rinsed with PBS, and then scanned with a LI-COR Odyssey imaging system (LI-COR Biosciences).

The captured images were analyzed using the Image Studio Lite program (LI-COR Biosciences). The intensity of the phospho-p44/42 MAPK protein band was normalized to the intensity of the p44/42 MAPK band from the same membrane.

2.2.10 Statistics

Differences among different treatment groups at the same time points were analyzed using ANOVA followed by Tukey test in JMP. Edu staining data and fusion index data were analyzed after arcsine square root transformation. All data are expressed as mean ± SEM (standard error of means). A difference is considered significant if the P value is < 0.05.

2.3 Results

2.3.1 Expression of oxytocin receptor (OXTR) in bovine skeletal muscle, myoblasts, and myotubes

Based on RT-PCR, high-level expression of OXTR mRNA was detected in bovine mammary gland and uterus (**Figure 2.1.A**), tissues that are known as major targets of OXT.

Moderate expression of OXTR mRNA was detected in liver, spleen, kidney, lung, and small intestine. Moderate expression of OXTR mRNA was also detected in bovine skeletal muscle, myoblasts, and myotubes (**Figure 2.1.A**). The expression of OXTR mRNA in bovine skeletal muscle and primary bovine myoblasts and myotubes were confirmed in multiple samples (**Figure 2.1.B**). In the meanwhile, high-level OXT mRNA expression was detected in bovine myoblasts, myotubes, uterus, and small intestine (**Figure 2.1.A**). Moderate expression of OXT mRNA was seen in liver, pituitary, spleen, and fat (**Figure 2.1.A**).

Based on Western blotting, high-level expression of OXTR protein was detected in bovine mammary gland, myoblasts, myotubes, and pituitary (**Figure 2.1.C**). Low-level expression of OXTR protein was detected in bovine spleen, kidney, uterus, and skeletal muscle (**Figure 2.1.C**).

2.3.2 Oxytocin increased the proliferation rate of bovine myoblasts

The proliferation rates of bovine myoblasts treated with 10 nM and 100 nM OXT for 24 hours as well as untreated myoblasts (i.e., control myoblasts) were analyzed using EdU assay (**Figure 2.2.A**). Myoblasts treated with 10 nM and 100 nM OXT contained 7.2% and 10.1% more EdU-positive myoblasts (i.e., proliferating myoblasts) compared to control myoblasts ($P < 0.05$) (**Figure 2.2.B**). The percentages of Edu-positive cells in myoblasts treated with 10 nM and 100 nM OXT were not different ($P > 0.1$). Myoblasts treated with 200 ng/ml IGF-I for 24 hours had 23.0% more EdU-positive cells than control myoblasts (data not shown).

2.3.3 Oxytocin did not affect the differentiation and fusion of bovine myoblasts into myotubes

The differentiation status in myoblasts treated with 10 nM and 100 nM OXT and control myoblasts for 48 hours was assessed by quantifying mRNA expression levels of 4 myoblast

differentiation marker genes: MYOG (Londhe and Davie, 2011), MYH3 (Allen et al., 2001), CKM (Lyons et al., 1991), and MB (Weller et al., 1986; Devlin et al., 1989) (**Figure 2.3**). Myoblasts treated with 10 nM OXT and 100 nM OXT had increased expression of MB mRNA compared to control myoblasts ($P < 0.05$), but they were not different in expression of MYOG, CKM, or MYH3 mRNA ($P > 0.05$).

The fusion status in control myoblasts and myoblasts treated with 10 nM and 100 nM OXT for 48 hours was assessed by staining the cells with Giemsa and by calculating the fusion index. Representative images of Giemsa-stained myoblasts and myotubes are shown in **Figure 2.4.A**. The fusion index in myoblasts treated with 10 nM or 100 nM OXT was not different from that in control myoblasts ($P > 0.1$, **Figure 2.4.B**).

2.3.4 Oxytocin-stimulated proliferation in bovine myoblasts was blocked by the ERK1/2 inhibitor but not the PKC inhibitor

The possible involvements of ERK and PKC signaling pathways in OXT stimulation of bovine myoblasts proliferation were investigated by treating the cells with OXT in the presence of the MEK inhibitor U0126 or PKC inhibitor BIM-1. The proliferation rate of bovine myoblasts was assessed by EdU assay (**Figure 2.5.A**). Compared to control group (i.e., untreated myoblasts), OXT-treated myoblasts contained 23.0% more EdU-positive cells ($P < 0.05$, **Figure 2.5.B**). Myoblasts treated with both OXT and U0126 contained 48.2% and 36.3% less EdU-positive cells than myoblasts treated with OXT and control myoblasts, respectively ($P < 0.05$, **Figure 2.5.B**). Myoblasts treated with U0126 only contained 41.6% less EdU-positive cells than control cells ($P < 0.05$). Myoblasts treated with U0126 did not differ myoblasts treated with OXT and U0126 in the percentage of EdU-positive cells ($P > 0.1$, **Figure 2.5.B**). Myoblasts treated with both OXT

and BIM-1 were not different from myoblasts treated with OXT in the percentage of EdU-positive cells ($P > 0.1$, **Figure 2.5.B**). Myoblasts treated with BIM-1 only, however, had a greater percentage of EdU-positive cells than control myoblasts ($P < 0.05$, **Figure 2.5.B**)

2.3.5 Oxytocin treatment did not alter ERK1/2 phosphorylation in bovine myoblasts

To further determine whether OXT stimulates bovine myoblast proliferation through ERK signaling, we determined the effect of OXT on ERK1/2 phosphorylation in bovine myoblasts by western blotting (**Figure 2.6.A**). Compared to control, OXT did not alter the level of phosphorylated ERK1/2 proteins in bovine myoblasts ($P > 0.1$, **Figure 2.6.B**). Compared to control myoblasts, myoblasts treated with U0126 only or both U0126 and OXT had less phosphorylated ERK1/2 proteins ($P < 0.05$). Myoblasts treated with BIM-1 or both BIM-1 and OXT had more phosphorylated ERK1/2 proteins than control myoblasts ($P < 0.05$) (**Figure 2.6.B**).

2.4 Discussion

Oxytocin, a very abundant neurohypophysial hormone, shares highly conserved chemical structure among placental mammals. Mainly released from the pituitary gland, OXT acts both as a neuromodulator in the central nervous system and as a hormone in the circulation and peripheral systems through its receptors (Barberis and Tribollet, 1996; Ivell and Walther, 1999; Moberg, 2003). Oxytocin receptor expression has been detected in different brain regions where OXT exerts its effects in behavioral functions including specific maternal, sexual, and social behaviors (Pedersen et al., 1982; Andari et al., 2010; Insel, 2010). In the periphery, OXTR was originally thought to be present only in uterus, mammary gland, and heart, mediating the OXT effects on uterine contraction, milk ejection, and cardiovascular control (Wakerley and Lincoln, 1973; Fuchs

et al., 1984; Uvnäs‐Moberg and Eriksson, 1996; Petersson, 2002). OXTR has been found in various cancer cells (Cassoni et al., 2004).

Recent studies have also shown OXTR expression in stem cells of bone, adipose tissue, and striated muscle stem cells (Breton et al., 2002; Tamma et al., 2009; Noiseux et al., 2012; Beranger et al., 2014; Elabd et al., 2014). In this study, we found that OXTR mRNA and protein were expressed in bovine skeletal muscle, myoblasts and myotubes. This result suggests that OXT may play a role in skeletal muscle development, growth, or function in cattle.

OXT can stimulate, inhibit, or show no effects on cell proliferation depending on the cell system utilized. These different effects are mediated by various signaling pathways including increasing intracellular Ca^{2+} or cAMP and activation of MAP kinases (Bussolati and Cassoni, 2001; Rimoldi et al., 2003; Cassoni et al., 2004). In this study, we found that OXT stimulated proliferation of bovine myoblasts. A similar stimulatory effect has been reported on murine myoblasts (Elabd et al., 2014). MAP kinase signaling is one of the classic pathways that are essential for promoting the growth-related effects of growth factors and their respective tyrosine kinase receptors (Chang and Karin, 2001). In human myometrial cells or CHO cell line, OXTR can activate the p42/44 MAPK (i.e., ERK1/2) and stimulate the cell proliferation through Ras/ERK-dependent pathway (Ohmichi et al., 1995; Strakova et al., 1998; Hoare et al., 1999). In human myometrial cells or CHO cell line, OXTR can activate the p42/44 MAPK (i.e., ERK1/2) and stimulate the cell proliferation through the Ras/ERK-dependent pathway (Ohmichi et al., 1995; Strakova et al., 1998; Hoare et al., 1999). In aging mice, OXT can rapidly improve muscle regeneration ability by stimulating aged skeletal muscle stem cell activation and proliferation through the ERK signaling pathway (Elabd et al., 2014). In this study, we found that the OXT-increased proliferation in bovine myoblasts was dependent on the activity of MEK1/2. This result

supports the possibility that OXT stimulates bovine myoblast proliferation through the ERK signaling pathway. However, OXT did not change ERK1/2 phosphorylation in bovine myoblasts. Therefore, how ERK1/2 participate in oxytocin-stimulated proliferation of bovine myoblasts remains unclear.

In summary, the present study has shown that exogenous OXT promotes the proliferation but has no effect on the differentiation and fusion of bovine myoblasts in culture. The stimulatory effect of OXT on bovine myoblast proliferation may involve ERK1/2, but this involvement does not include ERK1/2 phosphorylation.

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Table 2.1 Nucleotide sequences of PCR primers used in this study

Gene	Direction	Primer sequence	GenBank accession Number
18S	Forward	5’-GTAACCCGTTGAACCCCATT-3’	DQ222453
	Reverse	5’-CCATCCAATCGGTAGTAGCG-3’	
OXTR	Forward	5’-GCGCCTTAAAGGACTCGAA-3’	NM_174134.2
	Reverse	5’-CCTATCAGTCACAGCGTGGA-3’	
OXT	Forward	5’-CCAAGAGGAGAACTACCTGCC-3’	NM_176855
	Reverse	5’-CTGCGCTCCGACGGTATC-3’	
MYOG	Forward	5’-TGGGCGTGTAAGGTGTGTAA-3’	NM_001111325
	Reverse	5’-TATGGGAGCTGCATTCACTG-3’	
MYH3	Forward	5’-CTGGAGGAAATGAGGGATGA-3’	NM_001101835
	Reverse	5’-CACTCTTGAGAAGGGGCTTG-3’	
CKM	Forward	5’-TGGAGATGATCTGGACCCCA-3’	NM_174773.4
	Reverse	5’-TTTCCCCTTGAACTCACCCG-3’	
MB	Forward	5’-AGTCACATGCCAACAAGCAC-3’	NM_173881.2
	Reverse	5’-CATCAGCACCGAAGTCTGAA-3’	

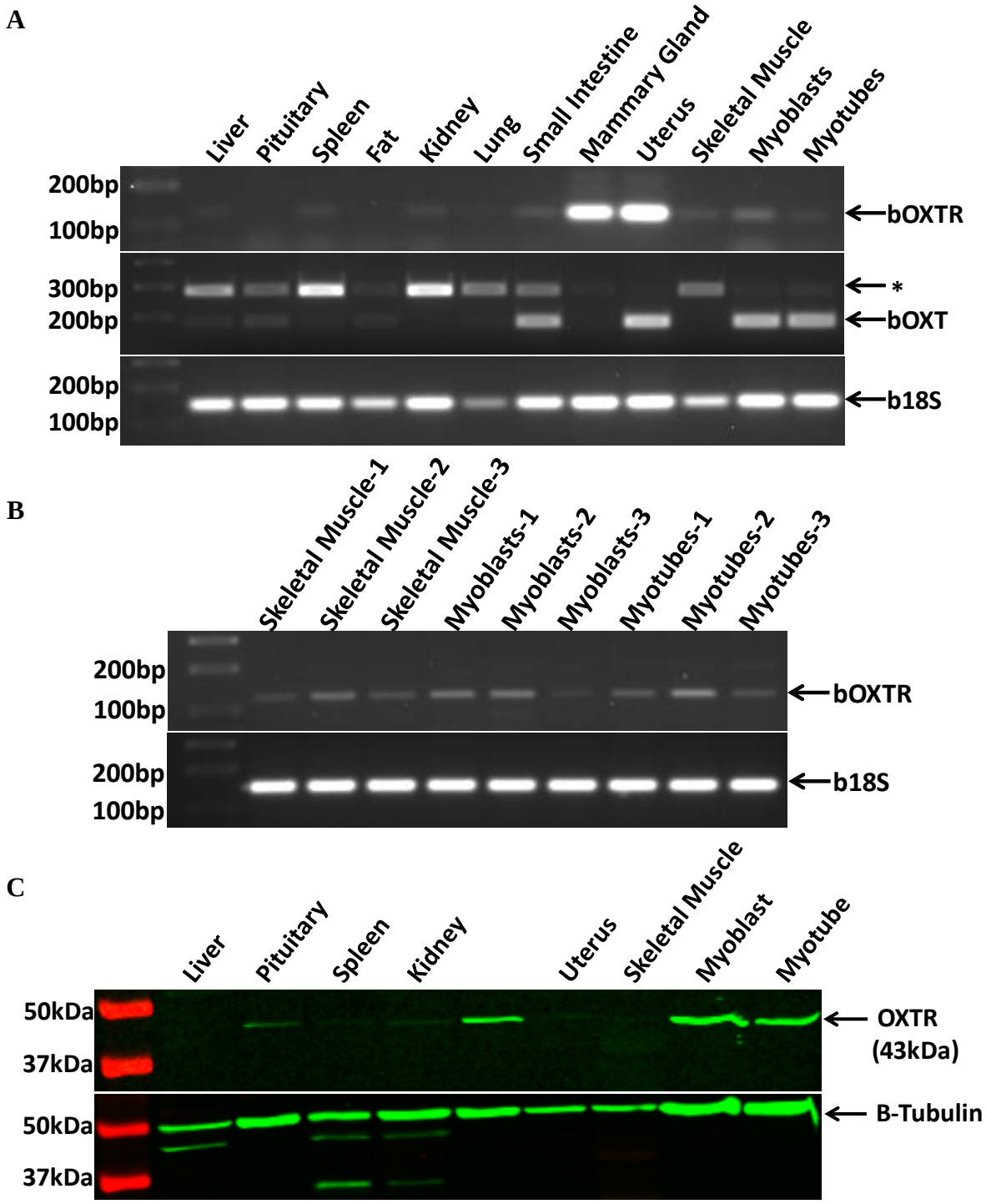


Figure 2.1 mRNA and protein expression of oxytocin receptor (OXTR) in bovine tissues, myoblasts, and myotubes. Tissues except skeletal muscle were collected from cows. Skeletal muscles were collected from steers. Myoblasts were isolated from skeletal muscles from steers. Myoblasts were cultured in growth medium. Myotubes were formed by culturing myoblasts in differentiation medium for 48 h. A, B). Pictures of RT-PCR analyses of OXTR and OXT mRNA (18S RNA was amplified as a loading control). *, amplified from genomic DNA and differed from the amplified OXT cDNA by a 90 bp intron between the two primers. C) Western blot analyses of OXTR protein. Beta-tubulin was detected as a loading control.

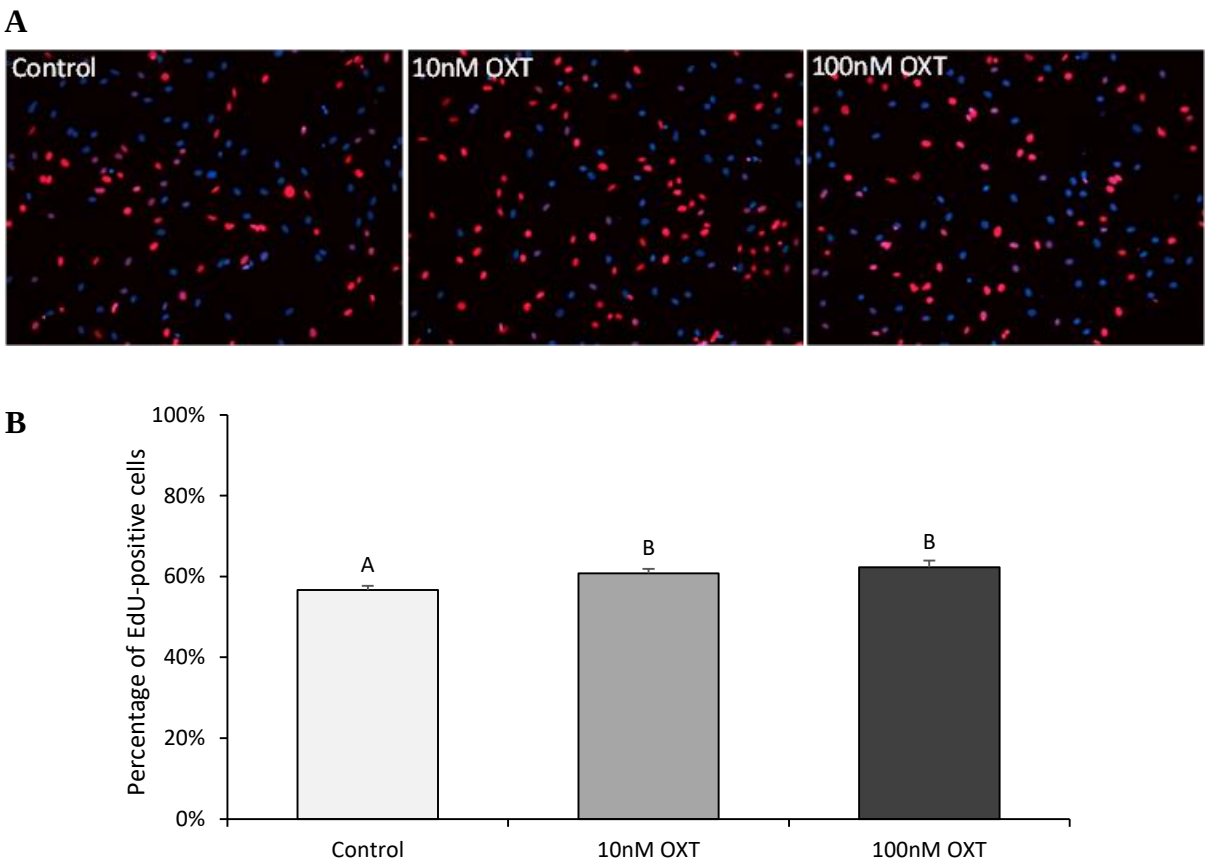


Figure 2.2 Effects of oxytocin on bovine myoblasts proliferation. Myoblasts were cultured in the presence of 10 nM or 100 nM oxytocin or control for 24 h. Proliferating cells were detected using an EdU assay. A). Representative pictures of cells labeled with EdU (red) and DAPI (blue). B). Average percentages of EdU-positive cells. The experimental unit was animal. Bars not sharing the same letter label are different (n = 4, $P < 0.05$).

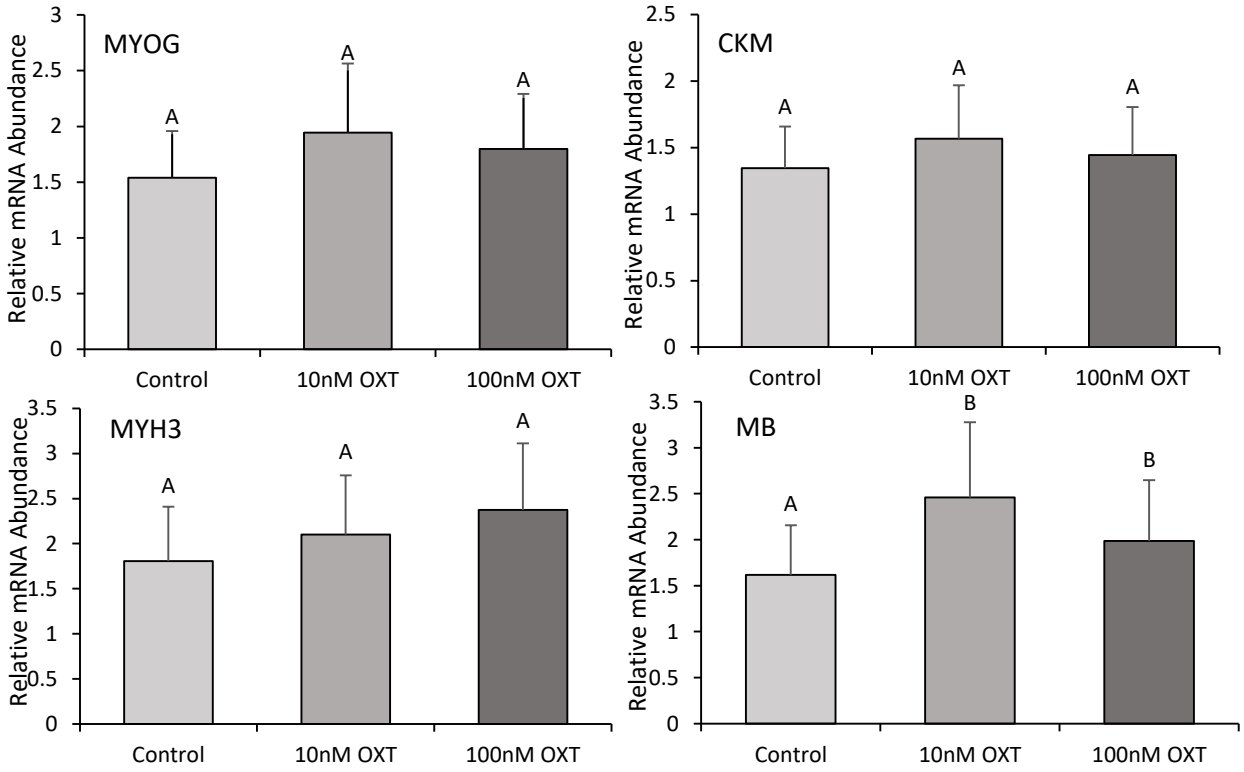
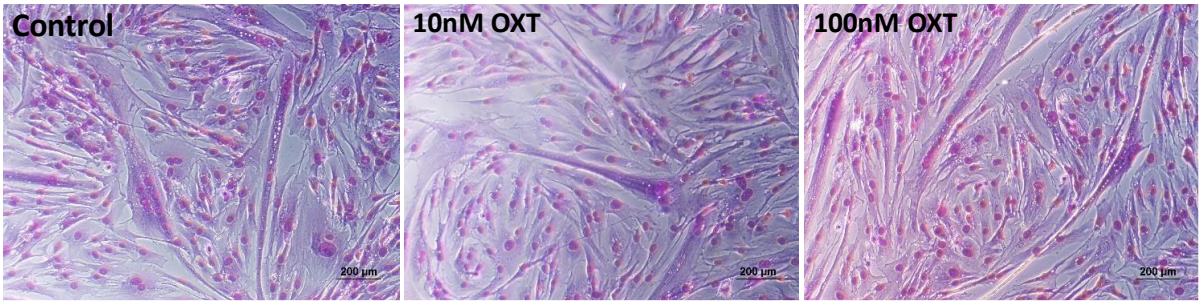


Figure 2.3 Effect of oxytocin on MYOG, CKM, MYH3, and MB mRNA expression in bovine myoblasts. Myoblasts isolated from steers were cultured in differentiation medium (DM) in the presence of 10 nM or 100 nM oxytocin, or control for 48 h. mRNA expression was quantified by RT-qPCR. Bars not sharing the same letter label are different within time (n = 6, $P < 0.05$). Experimental unit was animal.

A



B

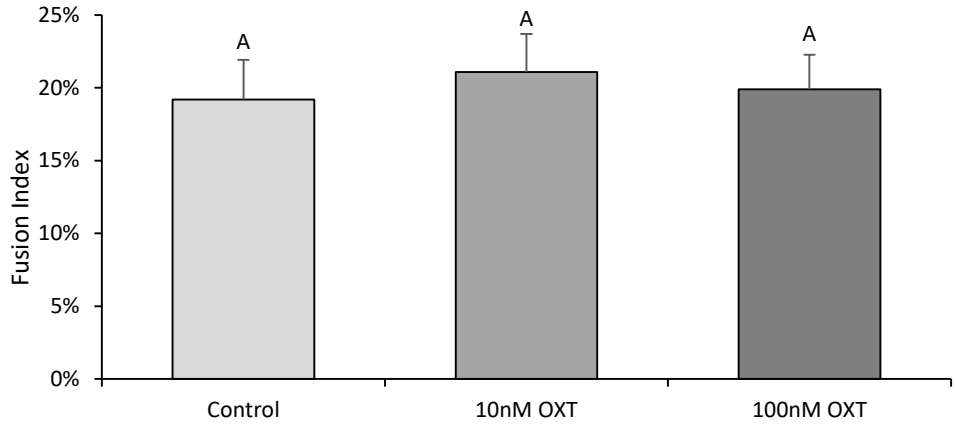
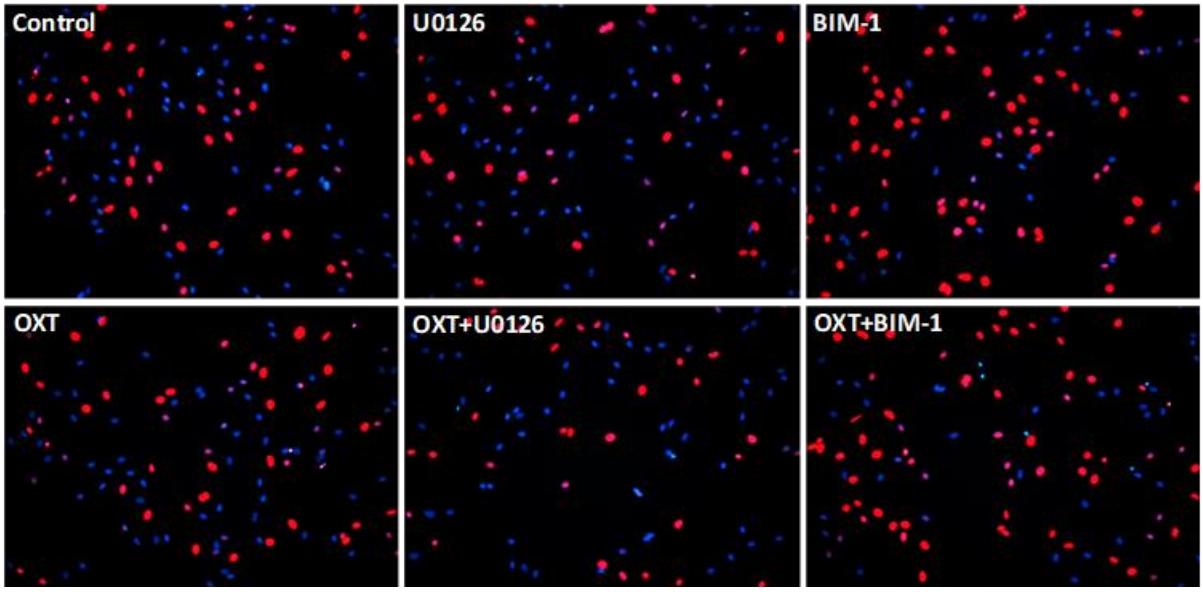


Figure 2.4 Effect of oxytocin on fusion of bovine myoblasts into myotubes. Myoblasts were cultured in differentiation medium (DM) in the presence of 10 nM or 100 nM OXT, or control for 48 h and stained with Giemsa. A). Representative micrographs (10 × magnification) of stained cells. B). Quantification of fusion index. Fusion index is defined as the percentage of nuclei located in myotubes containing 3 or more nuclei. Bars sharing the same uppercase letter label are not different within time (n = 8, $P > 0.05$). Experimental unit is animal.

A



B

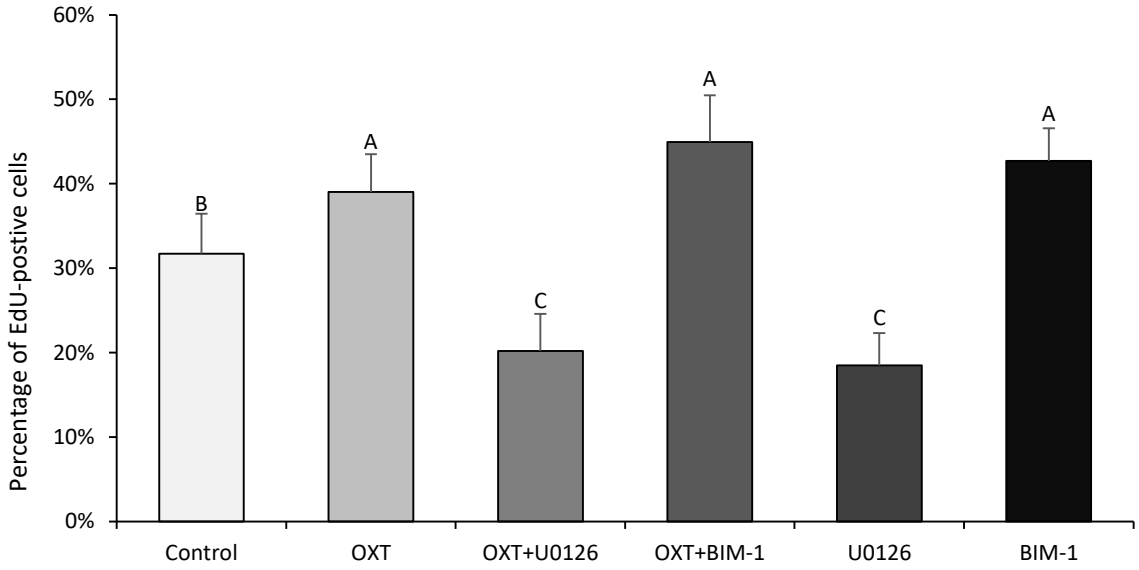


Figure 2.5 Effects of ERK1/2 inhibitor U0126 and PKC inhibitor BIM-1 on oxytocin-stimulated proliferation bovine myoblasts. Myoblasts were cultured in proliferation medium in the presence of control or 10 nM oxytocin with or without 10 μM U0126 or 1 μM BIM-1 for 24 h. Proliferating cells were identified by EdU labeling (Red). All nuclei were labeled by DAPI (blue). A). Representative pictures of EdU-labeled cells. B). Percentage of EdU-positive cells. Experimental unit was animal. Bars not sharing the same letter label are different (n = 6, $P < 0.05$).

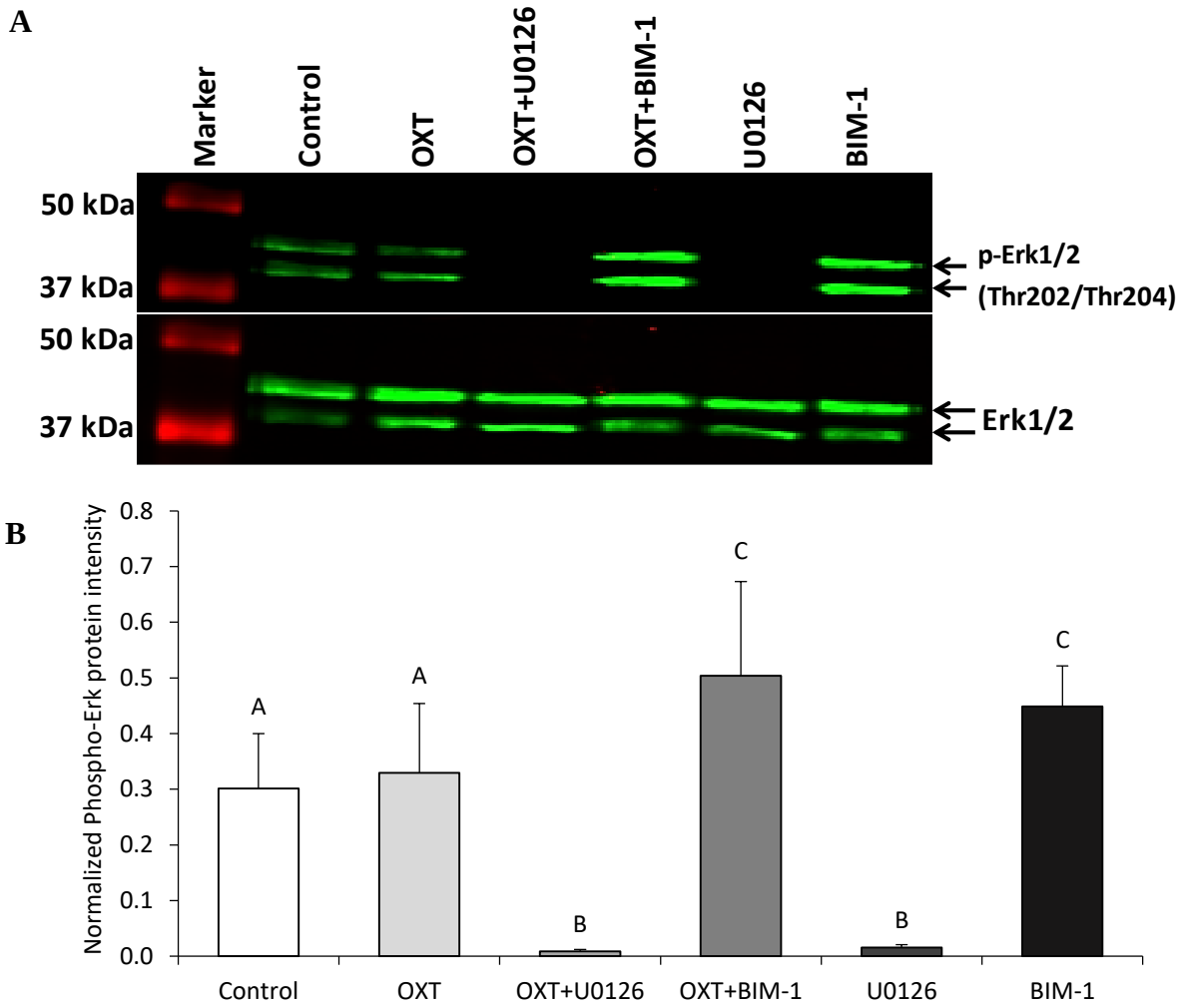


Figure 2.6 Effects of oxytocin on phosphorylation of ERK1/2 proteins in bovine myoblasts. Myoblasts from steers were treated in serum-free medium with 10 nM oxytocin, 10 μM U0126, and 1 μM BIM-1, separately or in combination for 30 min. Phospho- ERK and total ERK proteins were detected by western blotting. A). Representative western blots. B). Quantification of band intensities. The phospho- ERK protein level on y-axis was normalized to that of total ERK protein. Bars not sharing the same uppercase letter label are different between different treatment group (n = 8, $P < 0.05$). Experimental unit was animal.

Chapter 3 Effects of arachidonic acid on bovine myoblast proliferation, differentiation, and fusion

Abstract

Arachidonic acid (AA) is not only a major lipid component of the plasma membrane but also the precursor of prostaglandins (PG). The objective of this study was to determine the effects of AA and its major prostaglandin derivatives PGE_2, PGF_{2a}, and PGI_2 on the proliferation, differentiation, and fusion of bovine myoblasts in vitro. Satellite cells were isolated from Angus or Angus crossbred steers and expanded as myoblasts in growth medium for approximately 10 days before being used in the proliferation or differentiation tests. In the proliferation test, myoblasts were cultured in proliferation medium with 10 μM AA, 1 μM PGE_2, 1 μM $PGF_{2\alpha}$, 1 μM PGI_2, or vehicle control for 24 h, and the proliferating cells were identified by EdU labeling. This test revealed that AA, PGE_2, PGF_{2a}, and PGI_2 each increased the number of proliferating cells by 13%, 24%, 16%, and 16%, respectively, compared to the control (n = 7, $P < 0.05$). In the differentiation and fusion test, myoblasts were induced to differentiate and fuse into myotubes in the presence of the aforementioned treatments for 0, 24 and 48 h. The differentiation status was assessed by reverse transcription-quantitative PCR of myogenin (MYOG), myosin heavy chain 3 (MYH3), and muscle creatine kinase (CKM), myomaker (MYMK) mRNAs, which are markers of differentiated myoblasts and fused myotubes. The fusion level was estimated by calculating the percentage of nuclei located in myotubes, i.e., fusion index at 72 h differentiation. Compared to the control, AA increased MYOG and MYH3 mRNA expression at 24 and 48 h, and CKM and MYMK expression at 48 h of differentiation ($P < 0.05$); PGE_2 increased MYOG, MYH3, CKM, and MYMK mRNA expression at 24 and 48 h ($P < 0.05$); PGF_{2a} increased MYOG, MYH3, and CKM mRNA expression at 24 and 48 h of differentiation ($P < 0.05$); PGI_2 had no effect on mRNA expression

of any of the four markers. Compared to the control, PGE_2 increased the fusion index at 72 h of differentiation by 14% ($P < 0.05$) while the other treatments had no effect on this. Co-treatment of AA and either the cyclooxygenase (COX)-1 and COX-2 inhibitor indomethacin or the COX-2-specific inhibitor NS-398 reversed the stimulatory effect of AA on myoblast proliferation (N = 4, $P < 0.05$). Overall, this study demonstrates that AA, PGE_2, PGF_{2a}, and PGI_2 stimulate the proliferation, AA and PGE_2 stimulate the differentiation, and PGE_2 stimulates the fusion of bovine myoblasts *in vitro*. These results support the possibilities that AA stimulates myoblast proliferation through its metabolites PGE_2, PGF_{2a}, or PGI_2 and that AA stimulates bovine myoblast differentiation through PGE_2.

3.1 Introduction

Arachidonic acid (AA) is a type of esterified tetra-unsaturated fatty acid that is present in all mammalian cells, including skeletal muscle cells. Arachidonic acid is a major component of cell membrane phospholipids (PLs), and it is important for the maintenance of normal cellular membrane fluidity.

Arachidonic acid also serves as a precursor to a category of signaling molecules called eicosanoids, including prostaglandins, leukotrienes, thromboxanes, and prostacyclins (Brash, 2001). Upon liberation from membrane phospholipids by cytosolic phospholipase A_2 (PLA_2), free AA in cytoplasm is transformed by the rate-limiting enzymes cyclooxygenase-1 and -2 (COX-1/2; also known as prostaglandin G/H synthase-1/2) into intermediate product prostaglandin G_2 (PGG_2) and more stable prostaglandin H_2 (PGH_2) (Kulmacz, 1998; Smith et al., 2000). COX-1 is considered a constitutively expressed enzyme to keep basal prostaglandin synthesis while COX-2 is the inducible enzyme form found at the sites of inflammation (Crofford, 1997; Seibert et al., 1997). Specific prostaglandin synthases convert PGH_2 into different prostanoids (Berlin et al., 1979; Nowak et al., 1983; Bondesen et al., 2004). Among all the catalytic products from AA, prostaglandin E_2 (PGE_2), prostaglandin F_{2a} (PGF_{2a}), prostaglandin D_2 (PGD_2) and prostacyclin (PGI_2) are known as major prostaglandins (Funk, 2001).

Prostaglandins are released during exercise, inflammation, bone fractures, and muscle injuries (Herbaczynska-Cedro and Staszewska-Barczak, 1974; Herbaczynska-Cedro et al., 1976; Dekel et al., 1981; Prisk and Huard, 2003). A number of studies have demonstrated that exercise influences the concentration of AA in skeletal muscle (Andersson et al., 2000; Ramsay et al., 2001; Terpstra, 2004; Corcoran et al., 2007). Prostaglandins are involved in different physiological processes such as vasodilation and protein turnover, and in pathological processes such as

inflammation and cancer (Kilbom and Wennmalm, 1976; Rodemann and Goldberg, 1982; Goldberg et al., 1984; Chulada et al., 2000; Prisk and Huard, 2003). Since the discovery that prostaglandin E_1 (PGE_1) boosted the fusion of chick myoblasts into myotubes (Zalin, 1977), all major prostaglandins have been found to play a role in myogenesis (Horsley and Pavlath, 2003; Otis et al., 2005; Shen et al., 2006; Velica and Bunce, 2008; Veliça et al., 2010; Markworth and Cameron-Smith, 2011; Liu et al., 2015; Mo et al., 2015). Administration of COX inhibitors inhibits the proliferation and differentiation of satellite cells (Cossu and Biressi, 2005; Persson, 2015).

The potential role of AA and prostaglandins in bovine skeletal muscle growth and development did not appear to have been studied. Therefore, the objective of this study was to determine the effects of AA and major prostaglandins on the proliferation, differentiation, and fusion of bovine primary myoblasts in vitro.

3.2 Materials and methods

3.2.1 Isolation of bovine satellite cells

Bovine satellite cells were isolated from Angus or Angus crossbred steers as previously published (Ge et al., 2012). Briefly, steer extensor carpi radius muscle was taken at slaughter and transported to lab in sterile phosphate-buffered saline (**PBS**) with 1% antibiotic-antimycotic solution (**ABAM**; CORNING, Corning, NY) on ice. Muscle samples were then decontaminated by rinsing them with 70% ethanol. Muscle samples were cut into small pieces while connective and adipose tissues were removed. Muscle tissues were ground through a meat grinder. Ground muscle was digested in a PBS solution containing 1% ABAM and 1 mg/mL PRONASE protease, *Streptomyces griseus* (Calbiochem, San Diego, CA), at 37℃ for 1 h in a water bath. Following the digestion, the muscle was centrifuged at 1,500 g for 10 min. The pellet was resuspended in PBS.

The mixture was centrifuged at 400 g at room temperature for 5 min, and the supernatant was collected. This two-speed (first 1,500 g and then 400 g) centrifugation cycle was repeated 3 more times to enrich satellite cells. A last 1,500 g centrifugation was performed to collect the enriched satellite cells. The isolated satellite cells were resuspended in growth medium containing Dulbecco's Modified Eagle Medium (**DMEM**; CORNING, Corning, NY), 10% fetal bovine serum (**FBS**; Atlanta Biologicals, Lawrenceville, GA), 2% L-glutamine (CORNING), and 1% ABAM. Bovine satellite cells were cultured in growth medium at 37℃ with 5% CO_2 for approximately 10 days before being used for proliferation or differentiation experiments.

3.2.2 Proliferation experiments

For proliferation assays, bovine myoblasts were seeded in growth medium at a density of approximately 2×10^4 cells/well in 24-well plates. Approximately 4 hours after seeding, when most myoblasts would have attached to the plates, growth medium was removed. Cells were washed twice with warm (37℃) PBS, and then cultured in proliferation medium containing 2.5% FBS, 2% L-glutamine, and 1% ABAM overnight.

To determine the effects of arachidonic acid and prostaglandins on bovine myoblasts proliferation, 10 μM arachidonic acid (AA), 1 μM Prostaglandin E_2 (PGE_2), 1 μM Prostaglandin F_{2a} (PGF_{2a}), 1 μM Prostaglandin I_2 (PGI_2) (Cayman chemical, Ann Arbor, MI), or 10 μM arachidonic acid and 100 μM indomethacin or 50 μM NS-398 (Cayman chemical, Ann Arbor, MI) dissolved in ethanol were added to the proliferation medium. Indomethacin is a nonselective inhibitor of both COX-1 and COX-2 while NS-398 is a type of COX-2 specific inhibitor (Whittle, 1981; Futaki et al., 1994).

3.2.3 EdU Proliferation Assay

Myoblast proliferation rate was determined through EdU assay (Salic and Mitchison, 2008). When myoblasts had been cultured in proliferation medium overnight, half of the culture medium was replaced by freshly made proliferation medium containing treatment and 20 μM EdU (ThermoFisher Scientific, Waltham, MA). Following a 24 h incubation in the CO_2 incubator, myoblasts were fixed in 4% paraformaldehyde (PFA) (ThermoFisher Scientific) for 15 min and then permeabilized in 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for 20 min. Cells were then incubated in Click-iT reaction cocktail with fluorescence dye Alexa flour 594 (ThermoFisher Scientific) for 30 min at room temperature. Finally, cells were incubated with 5 μg/ml Hoechst 33342 (FisherThermo Scientific) in PBS for 30 min at room temperature to stain DNA.

Stained cells were photographed with a fluorescence microscope (Nikon, Tokyo, Japan). Ten randomly selected images were taken from each well at 10× magnification. Numbers of total nuclei and actively replicating nuclei were counted separately using NIS-elements advanced research microscope imaging software (Nikon). The proliferation rate was defined as the percentage of actively replicating nuclei in total nuclei. The proliferation experiment was repeated at least 4 times, each time using satellite cells isolated from a different steer.

3.2.4 Differentiation and fusion experiments

Bovine myoblasts were seeded in growth medium at approximately 80% confluency and cultured overnight. Myoblasts were then cultured in differentiation medium containing DMEM, 2% horse serum (**HS**; Atlanta Biologicals, Lawrenceville, GA), 2% L-glutamine, and 1% ABAM, as described before (Ge et al., 2013). The effects of arachidonic acid, prostaglandins, or COX

inhibitors on bovine myoblasts differentiation were determined by including 10 μM AA, 1 μM PGE_2, 1 μM PGF_{2a}, 1 μM PGI_2, or 10 μM arachidonic acid and 100 μM indomethacin or 50 μM NS-398 in the differentiation medium. Medium and treatment were refreshed every 24 h.

Myoblasts cultured for 0, 24, and 48 h in differentiation medium were collected for total RNA isolation and mRNA expression analyses. Myoblasts cultured for 72 h in differentiation medium were stained with Giemsa for fusion index quantification. This differentiation and fusion experiment was repeated 6 times, each time using satellite cells isolated from a different steer.

3.2.5 RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

TRI reagent was used for total mRNA extraction following the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Concentrations of total RNA samples were measured using NanoDrop 1000 (ThermoFisher Scientific). For reverse transcription, 1 μg total RNA was reverse transcribed into cDNA using a two-step method with ImProm-II reverse transcriptase and random primers in a final volume of 20 μL (Promega, Madison, WI), according to the manufacturer's instruction.

Quantitative PCR of mRNAs of myogenin (**MYOG**), myosin heavy chain 3 (**MYH3**), creatine kinase, muscle (**CKM**), and myomaker (**MYMK,** also known as transmembrane protein 8C or TMEM8C) was performed using the SYBR Green method. The PCR reaction was set up in a 10 μl system, containing 10 ng cDNA, 5 μL iTaq Universal SYBR Green Supermix (2×) (Bio-Rad Laboratories, Hercules, CA), and 500 nM gene-specific forward and reverse primers (**Table 3.1**) in 96-well plates (Bio-Rad Laboratories). The reactions were carried out in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) following the standard thermal cycling protocol: 30 s polymerase activation and DNA denaturation at 95℃ followed by 40 cycles of 5 s

for denaturation at 95℃ and 30 s for annealing, extension, and fluorescence read at 60℃. The relative abundance of target mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) by normalizing to the average abundance of reference genes 18S ribosomal RNA (**18S**) (Thellin et al., 1999), hydroxymethylbilane synthase (**HMBS**) mRNA (Vandesompele et al., 2002), and splicing factor 3a subunit 1(**SF3A1**) mRNA (Cui et al., 2011). 18S is the classical reference gene, while HMBS and SF3A1 genes were selected as reference genes because of their stability in measurement through different statistical algorithms (Cui et al., 2011).

3.2.6 Giemsa staining and fusion index

Giemsa staining of myoblasts and myotubes was performed as previously described (Freshney, 2015). Cells were washed with PBS, fixed in 50% methanol in PBS for 2 min and 100% methanol for 10 min, and rinsed with fresh 100% methanol for 2 min. The fixed cells were then incubated with Giemsa stain (RICCA Chemical Company, Arlington, TX) for 1 min, and 1:10 diluted Giemsa stain in water for 1 min on a shaker. Cells were rinsed with tap water for 2 min. Stained cells were photographed with an inverted microscope. Nine randomly selected images were taken from each well at 10× magnification. Numbers of total nuclei and fused nuclei were counted. Among the fused nuclei, numbers of myotubes with 2 to 4 fused nuclei and with 5 or more fused nuclei were counted separately. ImageJ (U. S. National Institutes of Health, Bethesda, MD) was used for cell counting. At least 3000 total cell nuclei were counted for each treatment in each experiment. The fusion index was defined as the percentage of total nuclei being in myotubes containing at least 2 nuclei. The fusion experiment was repeated 6 times, each time using satellite cells isolated from a different animal.

3.2.7 Statistics

Data from different treatment groups were analyzed using ANOVA followed by Tukey test in JMP. Edu staining data and fusion index data were analyzed after arcsine square root transformation. All data are expressed as mean ± SEM (standard error of means). A difference is considered significant if the associated *P* value is < 0.05.

3.3 Results

3.3.1 Arachidonic acid, prostaglandin E_2, F_{2a}, and I_2 stimulated proliferation in bovine myoblasts

The proliferation rates of bovine myoblasts treated with 10 µM AA, 1 µM PGE_2, 1 µM PGF_{2a}, and 1 µM PGI_2 were compared to the proliferation rate of untreated myoblasts (i.e., control myoblasts) using EdU assay (**Figure 3.1.A**). The myoblasts treated with AA, PGE_2, PGF_{2a}, and PGI_2 each had 13%, 24%, 16%, and 16%, respectively, more Edu-positive, i.e., proliferating cells, than control cells at the end of 24 h-treatment ($P < 0.05$) (**Figure 3.1.B**). The percentages of proliferating cells were not different among cells treated with AA, PGE_2, PGF_{2a}, and PGI_2 ($P > 0.05$) (**Figure 3.1.B**).

3.3.2 Arachidonic acid, prostaglandin E_2, and F_{2a} promoted differentiation in bovine myoblasts

The effects of AA, PGE_2, PGF_{2a}, and PGI_2 on bovine myoblasts differentiation were assessed by quantification of myoblast differentiation marker genes MYOG (Londhe and Davie, 2011), MYH3 (Allen et al., 2001), CKM (Lyons et al., 1991), and MYMK (Millay et al., 2013) at 0, 24, and 48 h after differentiation induction (**Figure 3.2**). In control myoblasts, mRNA expression of MYOG, MYH3, CKM, and MYMK increased during differentiation ($P < 0.05$,

Figure 3.2). Myoblasts treated with AA had greater expression of MYOG and MYH3 mRNAs at 24 h of differentiation, greater expression of MYOG, MYH3, CKM, and MYMK mRNAs at 48 h of differentiation, than control myoblasts ($P < 0.05$). PGE_2-treated myoblasts had higher expression levels of all four marker mRNAs than control myoblasts at 24 h and 48 h of differentiation ($P < 0.05$). PGF_{2a}-treated myoblasts had higher expression of all four marker mRNAs at 24 h and higher expression of MYOG, MYH3, and CKM mRNAs at 48 h of differentiation than control myoblasts ($P < 0.05$). mRNA expression of none of the four markers was different between PGI_2-treated myoblasts and control myoblasts at either 24 or 48 h of differentiation ($P > 0.05$).

3.3.3 Prostaglandin E_2 but not arachidonic acid increased myoblasts fusion into myotubes

The effects of AA, PGE_2, F_{2a}, and I_2 on myoblasts fusion into myotubes were determined by calculating fusion index. Representative images of Giemsa-stained myoblasts and myotubes at 72 h of differentiation are shown in **Figure 3.3.A**. Compared to control myoblasts, PGE_2-treated myoblasts formed 13.7% more myotubes containing 5 or more nuclei ($P < 0.05$). Percentages of myotubes containing 5 or more nuclei were not different between control myoblasts and myoblasts treated with AA, PGF_{2a}, or PGI_2 ($P > 0.05$) (**Figure 3.3.B**). No difference was observed in the percentage of myotubes containing 2 to 4 fused nuclei between in any of the four groups ($P > 0.05$).

3.3.4 Indomethacin and NS-398 reversed the stimulatory effects of arachidonic acid on bovine myoblasts proliferation

The stimulatory effect of AA on bovine myoblasts proliferation was further determined by adding AA with the COX enzyme inhibitor indomethacin or NS-398 to myoblast proliferation medium. Representative photographs of EdU-stained myoblasts are shown in **Figure 3.4.A**. The proliferation rate of myoblasts treated with AA and indomethacin was not only lower than that of myoblasts treated with AA only but also lower than the proliferation rate of control myoblasts ($P < 0.05$) (**Figure 3.4.B**). The proliferation rate of myoblasts treated with AA and NS-398 was lower than that of myoblasts treated with AA ($P < 0.05$) but was not different from that of control myoblasts ($P > 0.05$).

3.4 Discussion

Arachidonic acid is a 20-carbon carboxylic acid with four *cis*-double bonds. Almost all the endogenous arachidonic acid is esterified to phospholipids in cell membrane (Tallima and El Ridi, 2017). However, AA can be released from membrane phospholipids upon PLA_2 hydrolysis. Free AA can be metabolized to hormones including different types of prostaglandins by cyclooxygenases and other enzymes (Brash, 2001). AA has been widely included in human diets as supplements or in baby formula (Yoshizawa et al., 2013; Tateishi et al., 2015). This study was designed to determine the impact of AA on proliferation and differentiation of primary bovine myoblasts in vitro and to determine if the effect could be exerted through its biologically active metabolites prostaglandins.

Both PGE_2 and PGF_{2a} are critical for stretch-induced myoblast proliferation in mice (Otis et al., 2005). PGF_{2a} treatment enhances the proliferation of fetal human myoblasts (Zalin, 1987). PGE_2 increases the proliferation rate of primary mouse myoblasts through EP4 receptor and downstream mediator Nurr1 (Mo et al., 2015; Ho et al., 2017). The presence of PGE_2 in damaged

muscle tissues accelerates satellite cell proliferation (Ho et al., 2017). In this study, we found that not only PGE_2 and PGF_{2a} but also PGI_2 and AA stimulated the proliferation of primary bovine myoblasts in culture. To the best of our knowledge, no report is available with regard to the effect of AA or PGI_2 on proliferation of myoblasts from other species.

In this study, we observed that AA, PGE_2, and PGF_{2a} increased the terminal differentiation in bovine myoblasts and that PGE_2 also had a stimulatory effect on bovine myoblasts fusion into myotubes. These results are not completely consistent with findings in other myoblast models. In C2C12 myoblasts, AA enhances the formation of myotubes with more than 5 nuclei but reduces the number of myotubes with 2 to 4 nuclei (Markworth and Cameron-Smith, 2012). In C2C12 myoblasts, PGE_2 induces differentiation (Mo et al., 2012; Ho et al., 2017), while PGF_{2a} facilitates the secondary fusion (Horsley and Pavlath, 2003; Shen et al., 2006; Markworth and Cameron-Smith, 2011). In primary mouse myoblasts, PGI_2 stimulates the primary fusion (Bondesen et al., 2007).

In this study, we found that similar to AA, PGE_2, PGF_{2a}, and PGI_2 stimulated bovine myoblast proliferation and that similar to AA, PGE_2 and PGF_{2a} stimulated bovine myoblast differentiation. Because AA can be metabolized to produce prostaglandins, having similar effects supports the possibility that the stimulatory effect of AA on bovine myoblast proliferation is mediated through PGE_2, PGF_{2a}, or PGI_2 and that the stimulatory effect of AA on bovine myoblast differentiation is mediated through PGE_2 or PGF_{2a}.

COX-1 and COX-2 are the rate-limiting enzymes catalyzing the generation of prostaglandins from AA (Chakraborty et al., 1996). Both COX-1 and COX-2 have been found to play important roles in skeletal muscle formation and growth. In healthy skeletal muscle, physical exercise-induced upregulation of PGE_2, PGF_{2a}, and 6-keto-PGF_{1a} can be ablated by ibuprofen, a

common over-the-counter non-selective COX inhibitor (Trappe et al., 2001). In vitro, cyclical stretch of primary mouse myoblasts increased PGE_2 and PGF_{2a} production, and this increase was not observed in COX-2-inhibited or COX-2-deficient cells (Otis et al., 2005). Inhibition of COX-2 expression decreased the proliferation rate of satellite cells, while inhibition of COX-1 and -2 reduced the fusion of satellite cells into myotubes in rats (Mendias et al., 2004). Muscle hypertrophy in rats is attenuated by ibuprofen (Soltow et al., 2006). Muscle healing after injury is delayed by COX-2 knockout or COX-2-specific non-steroidal anti-inflammatory drugs (NSAIDs) in mice (Prisk and Huard, 2003; Shen et al., 2006; Velica and Bunce, 2008). In the meanwhile, COX-2-specific inhibitor does not influence intramuscular PGE_2 level, satellite cell activity, or increased protein synthesis after resistance exercise (Burd et al., 2010; Paulsen et al., 2010). In our study, treating bovine myoblasts with AA and the non-selective COX-1 and COX-2 inhibitor indomethacin or COX-2-specific inhibitor NS-398 completely blocked the stimulatory effect of AA on bovine myoblast proliferation. This result further supports the possibility that the effect of AA on bovine myoblast proliferation is mediated through PGE_2, PGF_{2a}, or PGI_2. In this study, we found that the addition of indomethacin not only blocked the AA-induced proliferation but also reduced the basal proliferation of bovine myoblasts. This result suggests that basal proliferation of bovine myoblasts in vitro is stimulated by COX-1-mediated production of prostaglandins from AA.

Other than the AA-COX-PGs cascade, AA may affect myoblasts through metabolites such as leukotrienes catalyzed by the lipoxygenase (LOX). Leukotriene B4 (LTB_4), the product of 5-lipoxygenase (5-LOX) and leukotriene A4 (LTA_4) hydrolase, is found to promote rat primary myoblasts proliferation and fusion (Sun et al., 2009). AA can directly activate MAPK signaling (Alexander et al., 2001) and replenish intracellular Ca^{2+} concentration in other types of cells

(Gailly, 1998; Woolcott et al., 2006). Therefore, we suspect that AA might also affect bovine myoblast proliferation or differentiation independent of COX or LOX.

In conclusion, the present study has demonstrated that exogenous AA, PGE_2, and PGF_{2a} stimulate the proliferation and differentiation of bovine myoblasts in culture. The results of the present study support the possibility that the effects of AA on bovine myoblast proliferation and differentiation are executed through COX-medicated production of PGE_2 and PGF_{2a}.

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Table 3.1 Nucleotide sequences of qPCR primers used in this study

Gene	Direction	Primer sequence	GenBank accession Number
18S	Forward	5’-GTAACCCGTTGAACCCCATT-3’	DQ222453
	Reverse	5’-CCATCCAATCGGTAGTAGCG-3’	
HMBS	Forward	5’-CTTTGGAGAGGAATGAAGTGG-3’	NM_001046207.1
	Reverse	5’-AATGGTGAAGCCAGGAGGAA-3’	
SF3A1	Forward	5’-GCGGGAGGAAGAAGTAGGAG-3’	NM_001081510.1
	Reverse	5’-TCAGCAAGAGGGACACAAA-3’	
MYOG	Forward	5’-TGGGCGTGTAAGGTGTGTAA-3’	NM_001111325
	Reverse	5’-TATGGGAGCTGCATTCACTG-3’	
MYH3	Forward	5’-CTGGAGGAAATGAGGGATGA-3’	NM_001101835
	Reverse	5’-CACTCTTGAGAAGGGGCTTG-3’	
CKM	Forward	5’-TGGAGATGATCTGGACCCCA-3’	NM_174773.4
	Reverse	5’-TTTCCCCTTGAACTCACCCG-3’	
MYMK	Forward	5’-GCTCGGCCATCCTCATCATT-3’	NM_001193046.1
	Reverse	5’-GTCCCAGTCCTCGAAGAAGAA-3’	

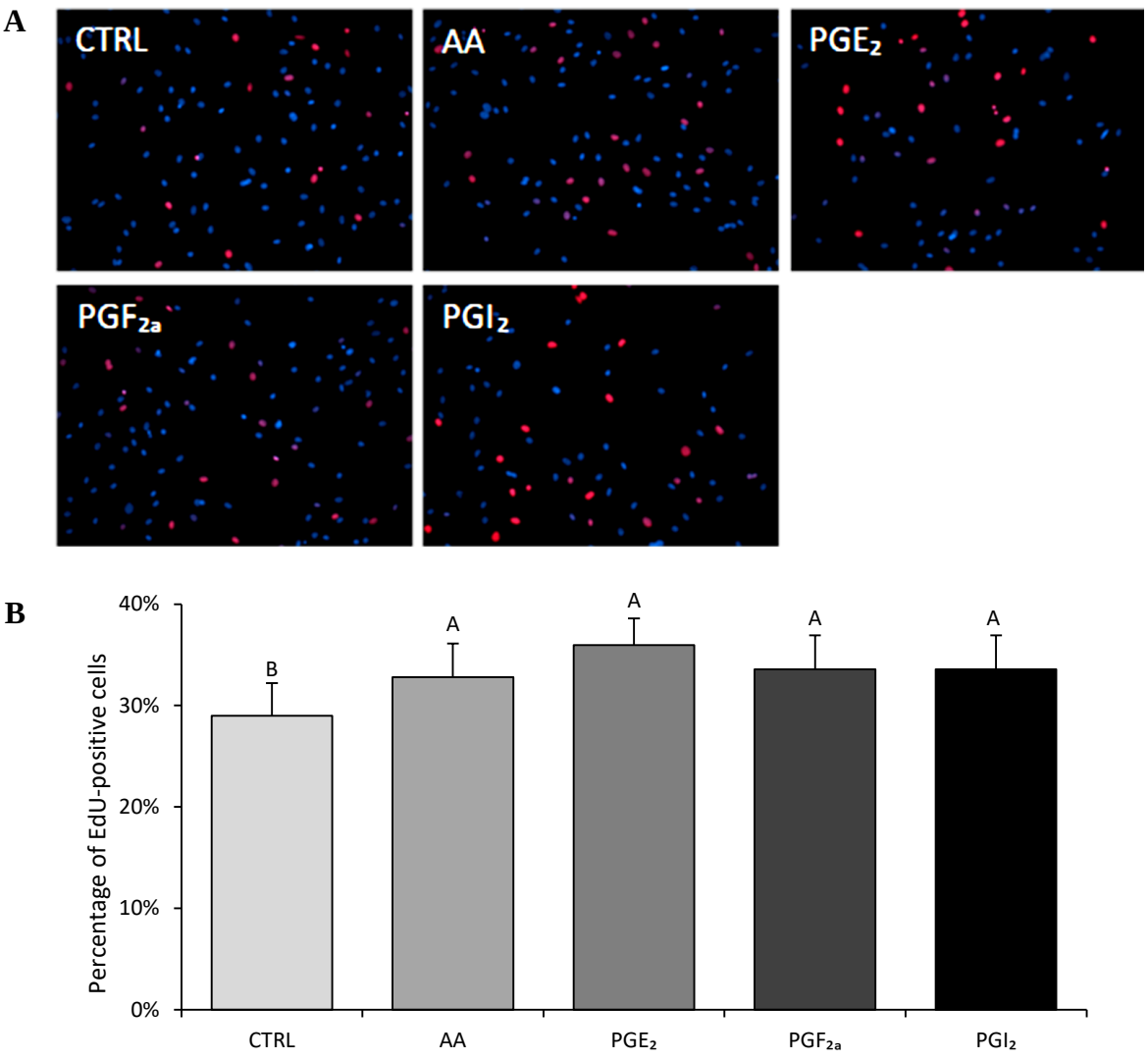

Figure 3.1 Effects of arachidonic acid, prostaglandin E_2, F_{2a}, and I_2 on bovine myoblasts proliferation. Bovine myoblasts were cultured in proliferation medium in the presence of arachidonic acid (AA), prostaglandin E_2 (PGE_2), PGF_{2a}, PGI_2, or control (CTRL) for 24 hours. Proliferating myoblasts were detected using an EdU assay kit. (A) Representative images of Edu-stained cells. Edu-positive nuclei are indicated in red; all nuclei are indicated in blue. (B) Percentages of Edu-positive nuclei. Experimental unit was animal (n = 7). Bars not sharing the same letter label are different ($P < 0.05$).

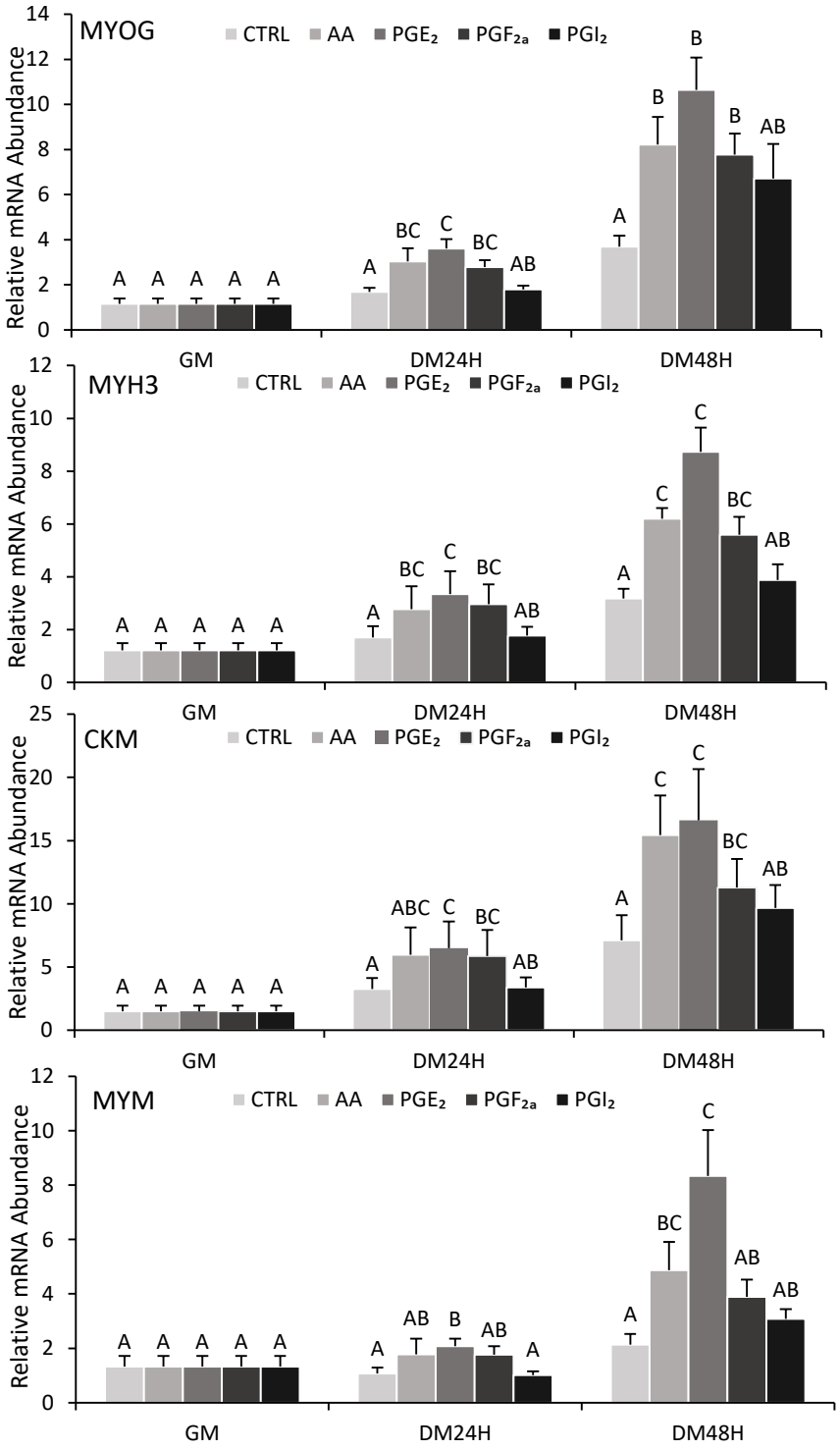

Figure 3.2 Relative expression levels of MYOG, MYH3, CKM, and MYMK mRNAs in bovine myoblasts before and during differentiation. Myoblasts isolated from 6 steers were cultured in differentiation medium (DM) in the presence of arachidonic acid (AA), prostaglandin E_2 (PGE_2), PGF_{2a}, PGI_2 or control (CTRL) for 24 and 48 h. Myoblasts cultured in growth medium(GM) before differentiation were used as undifferentiated myoblasts. mRNA expression of four differentiation marker genes was quantified by real-time qPCR. Bars not sharing the same letter label are different within time (n = 6, $P < 0.05$).

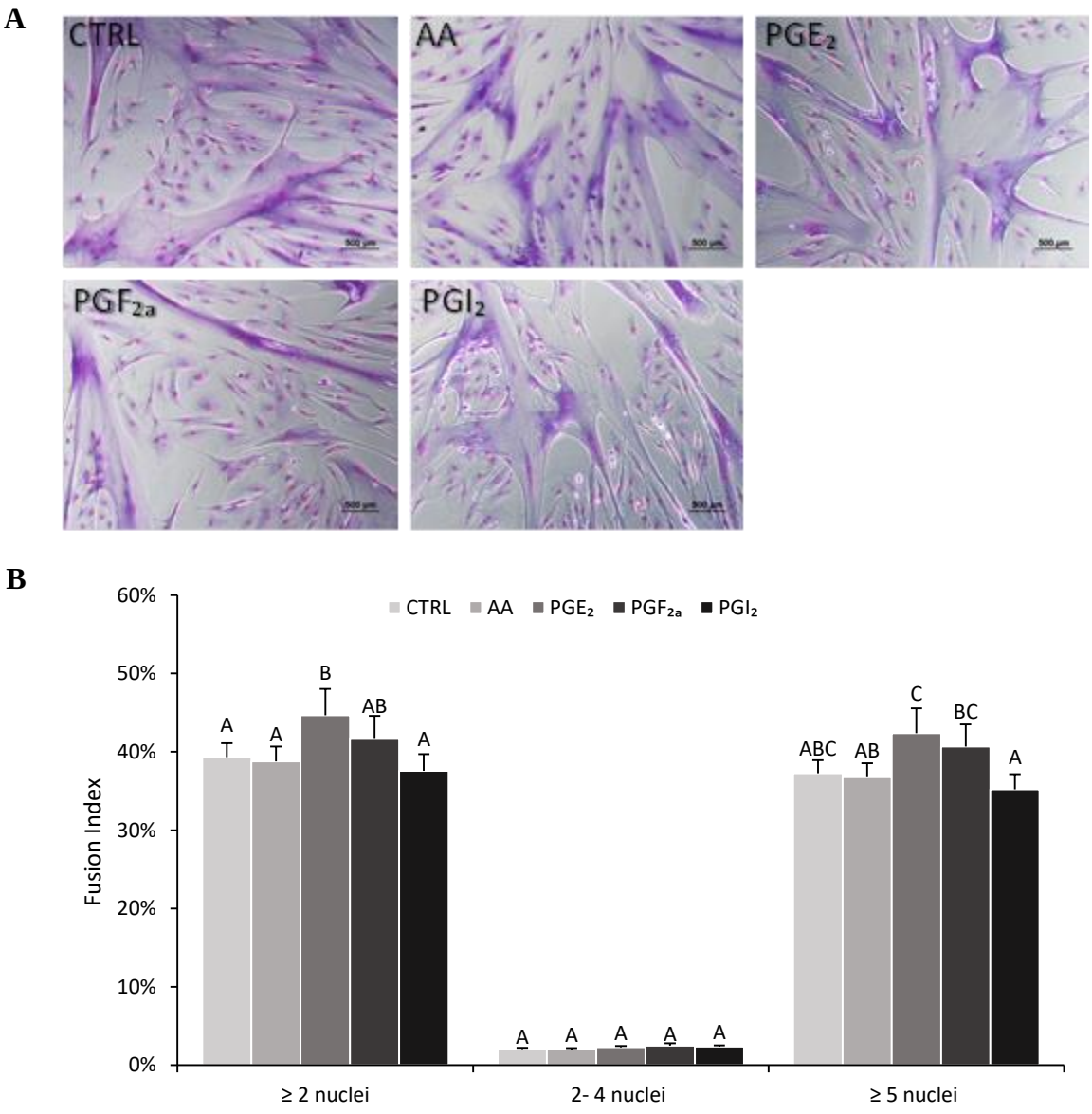

Figure 3.3 Effect of arachidonic acid, prostagladin E2, F2a, and I2 on fusion of bovine myoblasts into myotubes. Myoblasts from 6 steers were cultured in differentiation medium (DM) in the presence of arachidonic acid (AA), prostaglandin E_2 (PGE_2), PGF_{2a}, PGI_2 or control (CTRL) for 72 hours. Myoblasts were then fixed and stained with Giemsa. (A) Shown are representative micrographs (10 × magnification) of stained cells. (B) Quantification of fusion index. Nuclei in myotubes containing 2 or more nuclei, 2 to 4 nuclei, or 5 or more nuclei and total nuclei were counted from randomly taken images. Bars not sharing the same uppercase letter label are different within time (n = 6, $P < 0.05$).

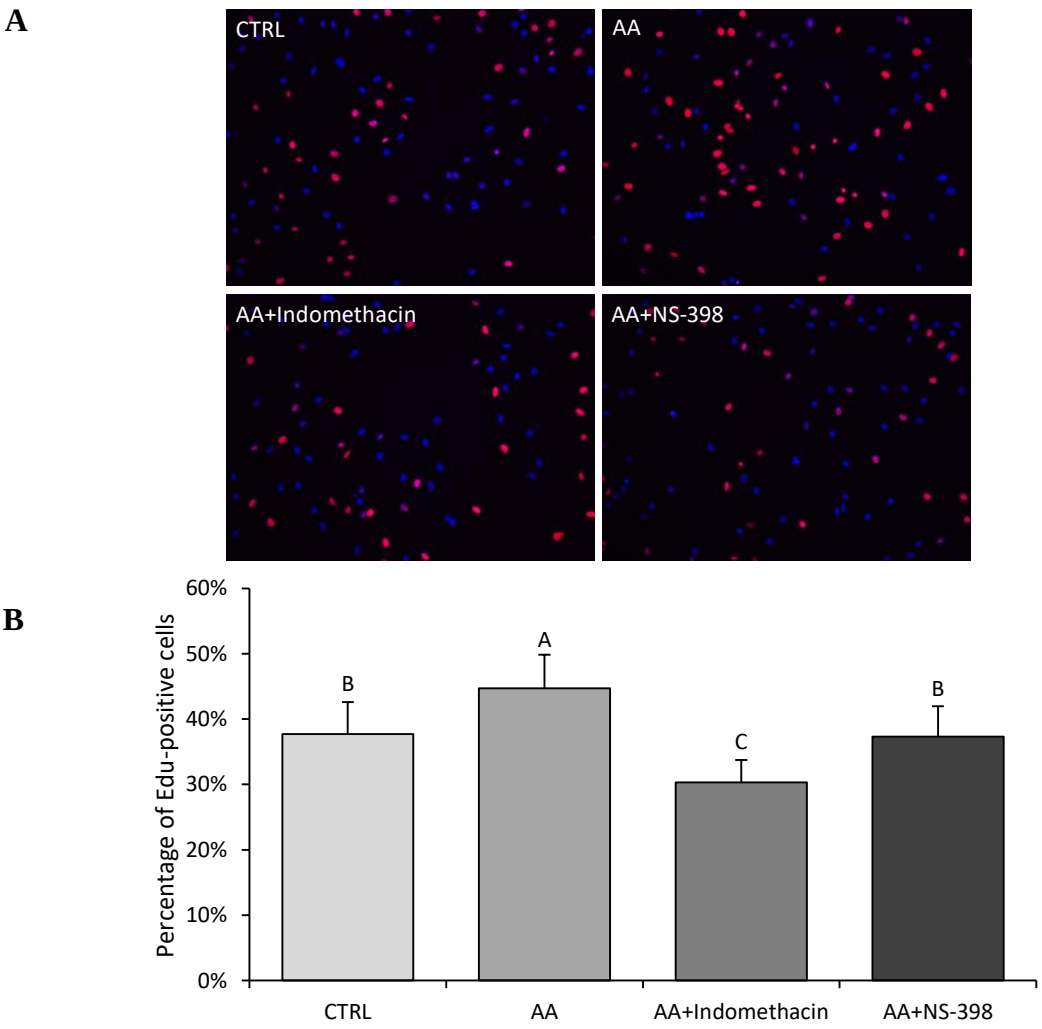

Figure 3.4 Effects of arachidonic acid and COX inhibitors on bovine myoblasts proliferation. Bovine myoblasts were cultured in proliferation medium in the presence of arachidonic acid (AA), AA with indomethacin, AA with NS-398 or control (CTRL) for 24 hours. Proliferating myoblasts were detected using an EdU assay kit. (A) Representative micrographs of Edu-stained cells. Edu-stained nuclei are indicated in pink; all nuclei are indicated in blue. (B) Percentages of Edu-positive nuclei. Experimental unit was animal. Bars not sharing the same letter label are different ($n = 4$, $P < 0.05$).

Chapter 4 Association of increased proteasome activity with differentiation and fusion in bovine myoblasts

Abstract

The objective of this study was to determine the role of the proteasome in the differentiation and fusion of bovine myoblasts into myotubes. This objective stemmed from an earlier, unexpected observation that expression of many genes encoding the ubiquitin-proteasome pathway (UPP) was upregulated during myoblast differentiation and fusion into myotubes. This objective was achieved by conducting a series of cell culture experiments with satellite cells isolated from 5 Angus or Angus crossbred steers (experimental unit). Satellite cells were initially expanded as myoblasts in growth medium and were then induced to differentiate and fuse into myotubes in differentiation medium. An assay of the 20S proteasome activity in myoblasts at 0, 24, 48, and 72 h of differentiation confirmed that the proteasome activity increased during myoblast differentiation and fusion. Myoblast differentiation and fusion into myotubes was nearly completely blocked by adding 5 μM lactacystin, a specific inhibitor of the 20S proteasome, to the differentiation medium, as indicated by the mRNA expression levels of 4 markers of differentiated myoblasts and fused myotubes: myogenin (MYOG), myosin heavy chain 3 (MYH3), muscle creatine kinase (CKM), and Myomaker (MYMK). A western blot analysis of poly-ubiquitinated protein confirmed the inhibition of lactacystin on the proteasome in bovine myoblasts. Inhibitor of DNA-binding 1 (ID1) is one of the proteins that inhibit myoblast differentiation and fusion and that is known to be degraded by the proteasome in several cell types. Both ID1 protein and mRNA expression in bovine myoblasts decreased during differentiation and fusion into myotubes, and the decrease in ID1 protein, not ID1 mRNA, was reversed ($P < 0.05$) by including lactacystin in the differentiation medium. However, this reversal was no longer observed when the mRNA translation inhibitor

cycloheximide was also included in the differentiation medium ($P < 0.05$). In summary, this study reveals that the proteasome activity in bovine myoblasts increases during differentiation and fusion, and that the increased proteasome activity may benefit myoblast differentiation and fusion in part by reducing the accumulation of the ID1 protein.

4.1 Introduction

Skeletal muscle is a dynamic tissue important for locomotion, heat generation, and homeostasis. Skeletal muscle is a major product of meat animals like cattle. Myoblast differentiation is a key event in the process of skeletal muscle development and is characterized by the expression of muscle-specific transcriptome and fusion of mononuclear cells into multi-nucleated myotubes. Myoblast determination protein (MyoD), myogenin (MyoG), and myogenic regulatory factor 4 (MRF4; also known as Myf6), which are basic helix loop helix (bHLH) transcription factors, are the key players in orchestrating myoblast differentiation and fusion (Olson, 1992).

In general, a bHLH transcription factor has a conserved motif containing two amphipathic α-helices separated by a loop and a positively charged basic region that facilitates DNA binding. During DNA transcription, dimerized bHLH proteins recognize and bind to a hexanucleotide sequence CANNTG (where N can be any nucleotide), also called an E box. The bHLH family has a large number of transcription factors that can be categorized into various classes based on tissue specification, dimerization capabilities, and DNA-binding specificities. Class I HLH proteins, also known as enhancer (E) proteins, are expressed in many types of tissues, are capable of forming homo- or hetero-dimers, and are only binding to E box sites. Class II HLH proteins are tissue-specifically expressed and contain members of skeletal muscle myogenic regulatory factors (MRFs) MyoD, Myf5, MyoG, and MRF4. Class II HLH factors only efficiently form heterodimers with the constitutively expressed E proteins such as E12, E47 (products of E2A gene), and HEB (Murre et al., 1989; Conway et al., 2004). These heterodimers bind to E boxes in the promoters and enhancers of muscle-specific genes. Class V HLH members such as inhibitor of DNA binding (ID) proteins ID1, 2, 3, and 4, are negative regulators of Class I and II bHLH transcription factors

(Benezra et al., 1990). Although containing the conserved HLH motif, ID proteins and other Class V HLH proteins lack the basic DNA binding domain and hence cannot bind directly to DNA. However, ID proteins bind to E proteins with high affinity, inhibiting the formation of E protein/tissue-specific bHLH heterodimers and binding of tissue-specific bHLH to DNA (Langlands et al., 1997).

Activation and continuous expression of MyoG is essential for myoblasts differentiation and fusion (Hasty et al., 1993; Knapp et al., 2006). At the initial stage of myoblast differentiation, the MyoD/E protein heterodimer binds to the E box of the MyoG promoter, resulting in transcriptional activation of MyoG in myoblasts. In late myoblast differentiation, the E protein HEB binds to MyoG, not MyoD, to form heterodimers that can activate the promoters of muscle-specific genes, leading to terminal differentiation (Parker et al., 2006). In proliferating myoblasts, most of the detectable E2A protein forms complexes with ID proteins. Overexpression of ID1 protein in mouse myoblast cell line C2C12 has negative effects on their differentiation and fusion into myotubes (Jen et al., 1992).

The ubiquitin-proteasome pathway (UPP) is the major pathway degrading short-living proteins such as MyoD (Lingbeck et al., 2003; Geng et al., 2012) and ID1 (Bounpheng et al., 1999; Sun et al., 2005). In this proteolytic system, ubiquitin is activated by ubiquitin-activating enzyme E1 and transferred to ubiquitin-conjugating enzyme E2. E2 shuttles the ubiquitin to substrate-specific ubiquitin ligase E3, which in turn tags ubiquitin onto protein substrates to be degraded. The ubiquitinated protein is recognized by the 19S regulatory subunit of the proteasome in an ATP-dependent manner and enters the barrel-shaped 20S catalytic core of the proteasome. The degradation process is completed in the assembled 26S proteasome. Both 20S and 26S proteasomes are also able to degrade cellular proteins independent of ubiquitination (Grune et al.,

2003; Orlowski and Wilk, 2003; Hoyt and Coffino, 2004; Baugh et al., 2009). On the other hand, other than tagging proteins for degradation, ubiquitination can post-translationally modify cellular proteins to alter their stability, activity, and subcellular localization (Mukhopadhyay and Riezman, 2007).

Previous RNA-seq data from our lab showed that expression of many genes encoding the UPP was upregulated during bovine myoblast differentiation and fusion into myotubes (data not shown). Therefore, the objective of this study was to determine the role of the upregulated UPP in the differentiation and fusion of bovine myoblasts into myotubes. We hypothesized that the upregulated UPP is to degrade the ID proteins, thereby allowing MyoD and MyoG to promote myoblasts differentiation and fusion into myotubes.

4.2 Materials and methods

4.2.1 Isolation of bovine satellite cells

Bovine satellite cells were isolated from Angus or Angus crossbred steers following protocols adapted from previous publications (Johnson et al., 1998; Ge et al., 2012). Briefly, extensor carpi radius muscles were collected from steer forelimbs and transported to lab in sterile phosphate-buffered saline (**PBS**) with 1% antibiotic-antimycotic solution (**ABAM**; CORNING, Corning, NY) on ice. Muscle samples were then soaked in 70% ethanol for 1 minute to remove potential contamination. Surrounding connective tissues were removed with scissors. Muscles were cut into small pieces and all visible intermuscular connective tissue was trimmed. Muscles were ground through a meat grinder. Grounded muscle was digested in a PBS solution (at 1 g muscle /1 ml solution) containing 1% ABAM and 1 mg/mL PRONASE protease, *Streptomyces griseus* (Calbiochem, San Diego, CA), at 37°C for 1 h. The digestion was shaken to mix every 10

min. The digested muscle was centrifuged at 1,500g at room temperature for 10 min. The pellet was collected and resuspended in PBS. The mixture was centrifuged at 400g at room temperature for 5 min, and the supernatant, which contained satellite cells, was collected. The supernatant was centrifuged at 1,500g for 10 min at room temperature and the pellet was collected. This two-speed (first 400g and then 1,500g) centrifugation cycle was repeated 3 more times to purify satellite cells. Following the last centrifugation at 1,500g, satellite cells were resuspended in growth medium containing Dulbecco's Modified Eagle Medium (**DMEM**; CORNING, Corning, NY), 10% fetal bovine serum (**FBS**; Atlanta Biologicals, Lawrenceville, GA), 2% L-glutamine (CORNING), and 1% ABAM, as described before (Ge et al., 2013). At least 90% of satellite cells isolated this way were $Pax7^+$ cells determined by an immunocytochemistry method.

4.2.2 Culture of bovine satellite cells

Bovine satellite cells were expanded in growth medium described above at 37°C and 5% CO_2 for approximately 10 days before being used for experiments. To induce differentiation and fusion, bovine myoblasts were reseeded in growth medium at approximately 80% confluency and cultured overnight. Growth medium was replaced with differentiation medium containing DMEM, 2% horse serum (**HS**; Atlanta Biologicals, Lawrenceville, GA), 2% L-glutamine, and 1% ABAM, as described previously (Ge et al., 2013). Lactacystin is a 20S proteasome-specific inhibitor derived from the metabolite of bacteria *Streptomyces.* Specifically, the hydrolysis product of lactacystin, clasto-lactacystin β-lactone, can covalently bind to Thr-1 of 20S proteasome β-type subunits and inhibit the proteolytic activity of 20S proteasome (Kisselev and Goldberg, 2001). Cycloheximide blocks protein synthesis by binding the ribosome and inhibiting eEF2-mediated translocation of tRNA and mRNA, thus inhibiting the translational elongation (Schneider-Poetsch

et al., 2010). To determine the effect of lactacystin or cycloheximide on myoblast differentiation, myoblasts were cultured in the presence of 5 μM lactacystin (Cayman Chemical, Ann Arbor, MI) or 10 μg/mL cycloheximide (Cayman Chemical), or both, for 6, 12, 24, 48, and 72 h. Medium and treatment were refreshed every 24 h.

4.2.3 20S proteasome activity assay

Proteasomes were isolated as described (Hoffman et al., 1992). Briefly, cells cultured in 6-well plates were washed with PBS and then scraped in 70 μL/well lysis buffer consisting of 50 mM HEPES pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 2 mM ATP (ATP added prior to use). Cells were lysed for 30 min on ice with vortexing every 10 min. The lysates were centrifuged at 15,700 g for 20 min at 4℃. The supernatant was collected and stored at -80℃.

The protein concentrations of proteasome lysates were measured using a BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA). The 20S proteasome activity was assayed using a 20S Proteasome Assay Kit (EMD Millipore, Billerica, MA). The principle of this kit is that the 20S proteasome recognizes the labeled substrate LLVY-7-amino-4-methylcoumrin (AMC) and cleaves the fluorophore AMC from LLVY-AMC. The free fluorophore was quantified using a fluorometer. The assay was set up in 96-well flat black plates following the manufacturer's instruction. Each sample was assayed in duplicate using 20 μL of cell lysates. Background fluorescence was controlled by including buffer blank and substrate blank. Proteasome positive control (1:16 diluted, from the kit) was assayed in the presence or absence of lactacystin (250 nM). The standard curve was prepared by measuring the fluorescence from a serial dilution of AMC standards ranging from 0.05 μM to 12.5 μM. The plate was incubated at 37℃ for 1 h. The fluorescence was measured with a Tecan Infinite 200 Pro plate reader (Tecan, Männedorf,

Switzerland) using fluorescence filter setting at 380 nm excitation wavelength /460 nm emission wavelength. The 20S proteasome activity in each sample is normalized to the protein concentration in each sample and is expressed as relative fluorescence units (RFU).

4.2.4 RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted using TRI reagent based on the manufacturer's method (Molecular Research Center, Cincinnati, OH). Extracted RNA was dissolved in diethyl pyrocarbonate (**DEPC**)-treated water and stored at -80℃. Concentrations of total RNA samples were measured using NanoDrop 1000 (ThermoFisher Scientific). For reverse transcription, 1 µg of total RNA was reverse transcribed into cDNA using ImProm-II reverse transcriptase and random primers in a final volume of 20 µL (Promega, Madison, WI), according to the manufacturer's protocol. Quantitative PCR was performed using the SYBR Green method or the TaqMan method.

A. SYBR Green Method. Quantitative PCR of myogenin (**MYOG**), myosin heavy chain 3 (**MYH3**), creatine kinase, muscle (**CKM**), and myomaker (**MYMK,** also known as transmembrane protein 8C or TMEM8C) mRNAs was performed using the SYBR Green chemistry. The PCR reaction was set up in a total volume of 10 µl, containing 10 ng cDNA, 5 µL iTaq Universal SYBR Green Supermix (2X) (Bio-Rad Laboratories, Hercules, CA), and 500 nM gene-specific forward and reverse primers (**Table 4.1**.) in 96-well plates (Bio-Rad Laboratories). The PCR reactions were carried out in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) following the standard thermal cycling protocol: 30 s for polymerase activation and DNA denaturation at 95℃ followed by 40 cycles of 5 s for denaturation at 95℃ and 30 s for annealing, extension, and fluorescence read at 60℃. The relative abundance of target mRNAs was

calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) by normalizing to the average abundance of 18S ribosomal RNA (**18S**), hydroxymethylbilane synthase (**HMBS**) mRNA, and splicing factor 3a subunit 1(**SF3A1**) mRNA. These genes were selected as reference genes because they were found to be stably expressed in different conditions (Cui et al., 2011). (Thellin et al., 1999), (Vandesompele et al., 2002), (Cui et al., 2011).

B. TaqMan Method. TaqMan real-time PCR was applied to quantify inhibitor of DNA binding 1 (ID1) mRNA, which has a high GC content. TaqMan assay primers for ID1 and HMBS (as reference gene) were purchased from ThermoFisher Scientific. The TaqMan PCR reaction was set up in a total volume of 20 µL containing 10 µL TaqMan Fast Advanced Master Mix (2X) (ThermoFisher Scientific), 1 µL TaqMan assay primer (20X), 2 µL cDNA (10 ng/µL), and 7 µL nuclease-free water in 96-well plates. The TaqMan PCR reaction was run in a Bio-Rad CFX96 Real-Time PCR detection system thermal cycler under the following program: 2 min Uracil-DNA glycosylase (UNG) activation at 50℃, 20 s polymerase activation at 95℃, and 40 cycles of 3 s denaturation at 95℃ and 30 s annealing/extension at 60℃. The relative abundance of ID1 mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method as described above.

4.2.5 Total cellular protein isolation and Western blotting

Total cellular protein from cultured cells was isolated by lysing the cells in the radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented with Halt protease inhibitor cocktail (100X) (ThermoFisher Scientific). Following incubation on ice for 10 min, cells were scraped, vortexed for 1 min, and centrifuged at 10,000

rpm for 15 min at 4℃. The supernatant was collected and protein concentration of the supernatant was determined with a BCA assay.

For Western blotting, 45 μg of protein per sample were mixed with 4X Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 355 mM β-mercaptoethanol (ThermoFisher Scientific) and heated at 95℃ for 5 min. After cooling to room temperature, protein samples were loaded into 10% or 12% (for target protein at molecular weight < 30 kDa) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**) gels placed in a Mini PROTEAN 3 Cell (Bio-Rad Laboratories). The proteins were separated by running SDS-PAGE at 80 V for 30 min followed by 120 V for 2 h in tris-glycine-SDS running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) at 4℃. Separated proteins were transferred from gels onto 0.2 μm nitrocellulose membranes (Bio-Rad Laboratories) in tris-glycine transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) in a Mini Trans-Blot Cell (Bio-Rad Laboratories) by running at 90 V for 1 h at 4℃. The membranes were blocked with 5% non-fat dry milk dissolved in tris-buffered saline-Tween 20 (TBS-T) buffer (20 mM Tris-HCl, 500 mM NaCl, and 0.05% Tween-20) for 1 h at room temperature.

For ID1 protein Western blotting, the membrane was cut into two pieces in between 25 kD and 37 kD protein bands. The lower piece of the membrane was incubated with 1:500 diluted ID1 antibody (sc-133104, Santa Cruz Biotechnology, Dallas, TX), while the upper piece was incubated with 0.02 μg/mL β-tubulin antibody (Developmental Studies Hybridoma Bank, Iowa City, Iowa). For Western blotting of ubiquitinated proteins, the intact membrane was first incubated with 1:500 diluted ubiquitin antibody (sc-8017, Santa Cruz Biotechnology), and then stripped and incubated with 0.02 μg/mL β-Tubulin antibody (Developmental Studies Hybridoma Bank). The stripping was done by incubating the membrane in restore fluorescent Western blot stripping buffer

(ThermoFisher Scientific) for 15 min at room temperature. The stripped membrane was washed 3 times with TBST and blocked for 1 h at room temperature before incubation with the β-tubulin antibody.

All primary antibodies were diluted in TBST containing 5% non-fat dry milk. All incubations with primary antibodies were carried out at 4℃ overnight. Following incubation with a primary antibody, the membrane was washed 3 times with TBST, each time for 5 min, and then incubated with 1:25,000 diluted secondary antibody IRDye 800CW Goat anti-Mouse IgG (H + L) (LI-COR Biosciences, Lincoln, NE) in TBST containing 5% non-fat dry milk for 1 h at room temperature. The membrane was washed 3 times with TBST, rinsed with PBS, and then scanned with a LI-COR Odyssey imaging system (LI-COR Biosciences).

The captured images were analyzed using the Image Studio Lite program (LI-COR Biosciences). Intensity of poly-ubiquitinated protein bands was measured from bands above the > 37 kD marker on the Western blots. The intensity of the ID1 protein band or ubiquitinated protein band was normalized to the intensity of the β-Tubulin band from the same sample.

4.2.6 Giemsa staining

Myoblasts and myotubes were stained with Giemsa stain as described (Freshney, 2015). Briefly, cells were first washed with PBS, and then fixed by incubation with 50:50 PBS/methanol solution for 2 min, 100% methanol for 10 min, and 100% methanol for 2 min. The fixed cells were first incubated with Giemsa stain (RICCA Chemical Company, Arlington, TX) for 1 min, and then 1:10 diluted Giemsa stain for 1 min on a shaker. The stained cells were rinsed with tap water for 2 min, and photographed with an inverted microscope. Images were taken from randomly selected areas of each well at 10X magnification.

4.2.7 Statistics

For the proteasome activity data, the main effect of time was tested. For the other data, main effects of treatments at different time points were evaluated. ANOVA was used to evaluate the differences among different time points within the same treatment group. Mixed Model was utilized to analyze the effects of time, treatment, and time × treatment under repeated measure with animals being considered as random effects. Tukey test following either ANOVA or Mixed Model was used to compare multiple differences. All analyses were performed in JMP. All data are expressed as mean ± SEM (standard error of means). A difference is considered significant if the associated P value is < 0.05.

4.3 Results

4.3.1 Differentiation and fusion of bovine myoblasts into myotubes was associated with increased 20S proteasome activity

We compared the proteolytic activity of 20S proteasome in bovine myoblasts at 24, 48, and 72 h of differentiation as well as in growth medium immediately before differentiation (**Figure 4.1**). Proteasome activity at 24 h of differentiation was not different ($P > 0.05$) from that in growth medium, i.e., before differentiation. Proteasome activity at 48 h of differentiation was 45% greater than ($P < 0.05$) that in growth medium. Proteasome activity in bovine myoblasts continued to increase from 48 h to 72 h of differentiation ($P < 0.05$).

4.3.2 Lactacystin inhibited the differentiation and fusion of bovine myoblasts into myotubes

The role of increased proteasome activity in differentiation and fusion of myoblasts into myotubes was determined by adding the proteasome inhibitor lactacystin to myoblast differentiation medium. The differentiation status of myoblasts was first assessed by quantifying mRNA expression of myoblast differentiation markers MYOG (Londhe and Davie, 2011), MYH3 (Allen et al., 2001), CKM (Lyons et al., 1991), and MYMK (Millay et al., 2013). In control myoblasts, expression of MYOG, MYH3, CKM, and MYMK mRNAs increased during differentiation (**Figure 4.2**). By 48 h of differentiation, all four markers were expressed at higher levels than before differentiation ($P < 0.05$). Expression of CKM and MYH3 mRNAs continued to increase from 48 to 72 h of differentiation ($P < 0.05$). In lactacystin-treated myoblasts, none of the four marker genes increased expression during the 72 h differentiation (**Figure 4.2**). All four marker mRNAs were expressed at lower levels in lactacystin-treated myoblasts than in control myoblasts at 24, 48, and 72 h of differentiation ($P < 0.05$). Among the four markers, MYOG mRNA expression in lactacystin-treated myoblasts was less than in control myoblasts at as early as 12 h of differentiation ($P < 0.05$).

Representative images of Giemsa-stained myoblasts at 0, 6, 12, 24, 48, and 72 h of differentiation are shown in **Figure 4.3**. It was obvious that fewer and smaller myotubes were formed in lactacystin-treated myoblasts than in control myoblasts at 48 and 72h of differentiation.

4.3.3 Lactacystin increased the amount of total ubiquitinated protein in bovine myoblasts

As a 20S proteasome-specific inhibitor, lactacystin blocks the degradation of ubiquitinated proteins by the proteasome without altering protein ubiquitination (Kisselev and Goldberg, 2001). To determine if the lactacystin treatment was effective in blocking the activity of the proteasome in bovine myoblasts, total protein lysates from control myoblasts and lactacystin-treated myoblasts

were analyzed by Western blotting using an anti-ubiquitin antibody (**Figure 4.4.A**). In control myoblasts, total amount of ubiquitinated proteins was not changed throughout the 72 h differentiation ($P > 0.05$). Compared to control myoblasts, lactacystin-treated myoblasts had more ubiquitinated protein at 6, 12, 24, and 72 h of differentiation ($P < 0.05$, **Figure 4.4.B**).

4.3.4 Lactacystin increased the amount of inhibitor of differentiation 1 (ID1) protein in bovine myoblasts

ID proteins compete with myogenic specific transcription factors like MyoG for binding to E proteins and thereby negatively regulate myoblast differentiation (Gundersen et al., 1995). To determine the possibility that lactacystin inhibits myoblast differentiation by blocking proteasome degradation of ID proteins, the expression levels of ID1 protein, the most studied ID protein (Traasch-Azar et al., 2004; Lingbeck et al., 2005; Sun et al., 2005), in lactacystin-treated and control myoblasts were analyzed by Western blotting (**Figure 4.5.A**).

In control myoblasts, ID1 protein expression decreased at 24 h of differentiation compared to before differentiation and remained so at 48 and 72 h of differentiation ($P < 0.05$) (**Figure 4.5.B**). The expression levels of ID1 protein were higher in lactacystin-treated than in control myoblasts at 6 and 12 h of differentiation ($P < 0.05$). In control myoblasts, ID1 mRNA expression level started to decrease at 12 h of differentiation compared to before differentiation ($P < 0.05$) (**Figure 4.5.C**), decreased further at 24 h of differentiation ($P < 0.05$), and remained low at 48 and 72 h of differentiation ($P < 0.05$). However, the ID1 mRNA expression levels in lactacystin-treated myoblasts were lower than in control myoblasts at 6 and 12 h of differentiation ($P < 0.05$), which indicated that lactacystin-caused increases in ID1 protein expression in bovine myoblasts at 6 and 12 h of differentiation were not mediated at the ID1 mRNA level.

4.3.5 Lactacystin-caused increase in ID1 protein level was blocked by the protein synthesis inhibitor cycloheximide

To determine if the lactacystin-caused increase in ID1 protein abundance was a result of increased ID1 protein synthesis or reduced ID1 protein degradation, the protein synthesis inhibitor cycloheximide was added to myoblast culture with or without lactacystin. Western blotting (**Figure 4.6.A**) showed that ID1 protein level in myoblasts treated with both lactacystin and cycloheximide was not different from that in control myoblasts or in myoblasts treated with cycloheximide at any of the three differentiation times examined ($P > 0.05$) (**Figure 4.6.B**). At 6 and 12 h differentiation, ID1 protein expression level in myoblasts treated with both lactacystin and cycloheximide was lower than that in myoblast treated with lactacystin only ($P < 0.05$).

4.4 Discussion

Earlier RNA-seq data from our lab showed the up-regulation of genes involved in the UPP pathway during differentiation and fusion of bovine myoblasts into myotubes (data not shown). This finding triggered the conduct of this study to look into the function of the proteasome during bovine myoblast differentiation. By quantifying the proteolytic activity of the proteasome, we verified the upregulation of the proteasome during myoblast differentiation and fusion into myotubes. In muscles of chick embryos, the casein-degrading activity of the proteasome increased from embryonic days 11 to 17, when myoblast differentiation and fusion was most active (Ahn et al., 1991). In mice, overload-induced muscle hypertrophy was associated with increased proteasome activity (Baehr et al., 2014). These associations suggest that the increased proteasome activity might have a positive effect on myoblast differentiation or muscle hypertrophy.

We determined the role of the proteasome in myoblast differentiation by including the proteasome-specific inhibitor lactacystin in the differentiation medium. By analyzing the mRNA expression levels of MYOG, MYH3, CKM, and MYMK4, which are markers of myoblast differentiation (Lyons et al., 1991; Weintraub, 1993; Allen et al., 2001; Parker et al., 2006; Londhe and Davie, 2011; Millay et al., 2014) , we found that myoblast differentiation was markedly inhibited in the presence of lactacystin. Giemsa staining of myoblasts showed that myotube formation was apparently inhibited by lactacystin. We also confirmed that lactacystin caused accumulation of ubiquitinated protein in bovine myoblasts. These results, consistent with earlier observations in primary rabbit myoblasts (Gardrat et al., 1999) and rat L6 myoblast cell line (Kim et al., 1998), further support a positive role of the proteasome-mediated protein degradation in myoblast differentiation and fusion (Bell et al., 2016).

ID proteins, members of the bHLH family, are known to be the negative regulators of myoblast differentiation. ID proteins inhibit myoblast differentiation by competing with MyoD or MyoG for binding to E protein and hence for the formation of E protein/MyoD or E protein/MyoG complex (Benezra et al., 1990). In C2C12 cell line, overexpression of ID1 protein led to differentiation inhibition and reduced myotube formation (Jen et al., 1992). In C2C12 cells, ID1 mRNA and protein expression levels decrease once differentiation initiates (Sun et al., 2005). In bovine myoblasts, ID1 mRNA and protein expression levels were also observed to decrease during differentiation. Because ID1 functions as an inhibitor of myoblast differentiation, reducing ID1 mRNA and protein expression could be a mechanism that promotes myoblast differentiation and myotube formation. When bovine myoblasts were differentiated in the presence of the proteasome inhibitor lactacystin, the decrease in ID1 protein was reversed while the decrease in ID1 mRNA expression was not affected. These results together with the results that the proteasome activity

increased during myoblast differentiation suggest that the decrease in ID1 protein expression during myoblast differentiation is mediated in part by the increased proteasome activity. Myoblast differentiation was nearly completely inhibited by including lactacystin in the culture medium. It is likely that lactacystin causes ID1 accumulation, and that excessive ID1 competitively binds to E protein and thereby limits the formation of E protein/MyoD or E protein/MyoG dimer.

ID1 protein is degraded in cells such as HEK293 and HeLa cells through the UPP (Bounpheng et al., 1999; Traasch-Azar et al., 2004). Therefore, we initially thought the increased ID1 protein expression in lactacystin-treated myoblasts resulted from reduced degradation of ID1 protein by the proteasome. However, when bovine myoblasts were treated with lactacystin in the presence of the protein synthesis inhibitor cycloheximide, ID1 protein level was not increased compared to its expression level in untreated cells or in cells treated with cycloheximide only. These results indicate that the lactacystin-induced increase in ID1 protein abundance in differentiating bovine myoblasts is dependent on protein synthesis. Because lactacystin is known as a specific inhibitor of the proteasome, the observation that lactacystin increased ID1 protein abundance in bovine myoblasts in a protein synthesis-dependent manner suggests that the proteasome degrades newly synthesized ID1 protein or that the proteasome degrades a protein(s) that regulates ID1 protein synthesis. This latter possibility is supported by the finding that ID1 protein synthesis, not ID1 protein degradation, decreases with differentiation in C2C12 cells (Sun et al., 2005). In eukaryotes, the proteasome can modulate translation of mRNAs by targeting and cleaving the eIF4F subunit elF4G and the eIF3 subunit eIF3a (Kisselev and Goldberg, 2001; Baugh and Pilipenko, 2004). Whether the proteasome inhibits ID1 protein synthesis in bovine myoblasts by targeting and degrading the subunits of eIF4F and eIF3 remains to be determined in future studies.

In summary, the present study demonstrates that the proteasome activity in bovine myoblasts is upregulated during differentiation and fusion into myotubes and that the upregulated proteasome has a positive effect on myoblast differentiation and fusion into myotubes. The proteasome might promote differentiation and fusion in bovine myoblasts by reducing the accumulation of ID1 protein.

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Table 4.1 Nucleotide sequences of qPCR primers used in this study

Gene	Direction	Primer sequence	GenBank accession Number
18S	Forward	5'-GTAACCCGTTGAACCCCATT-3'	DQ222453
	Reverse	5'-CCATCCAATCGGTAGTAGCG-3'	
HMBS	Forward	5'-CTTTGGAGAGGAATGAAGTGG-3'	NM_001046207.1
	Reverse	5'-AATGGTGAAGCCAGGAGGAA-3'	
SF3A1	Forward	5'-GCGGGAGGAAGAAGTAGGAG-3'	NM_001081510.1
	Reverse	5'-TCAGCAAGAGGGACACAAA-3'	
MYOG	Forward	5'-TGGGCGTGTAAGGTGTGTAA-3'	NM_001111325
	Reverse	5'-TATGGGAGCTGCATTCACTG-3'	
MYH3	Forward	5'-CTGGAGGAAATGAGGGATGA-3'	NM_001101835
	Reverse	5'-CACTCTTGAGAAGGGGCTTG-3'	
CKM	Forward	5'-TGGAGATGATCTGGACCCCA-3'	NM_174773.4
	Reverse	5'-TTTCCCCTTGAACTCACCCG-3'	
MYMK	Forward	5'-GCTCGGCCATCCTCATCATT-3'	NM_001193046.1
	Reverse	5'-GTCCCAGTCCTCGAAGAAGAA-3'	

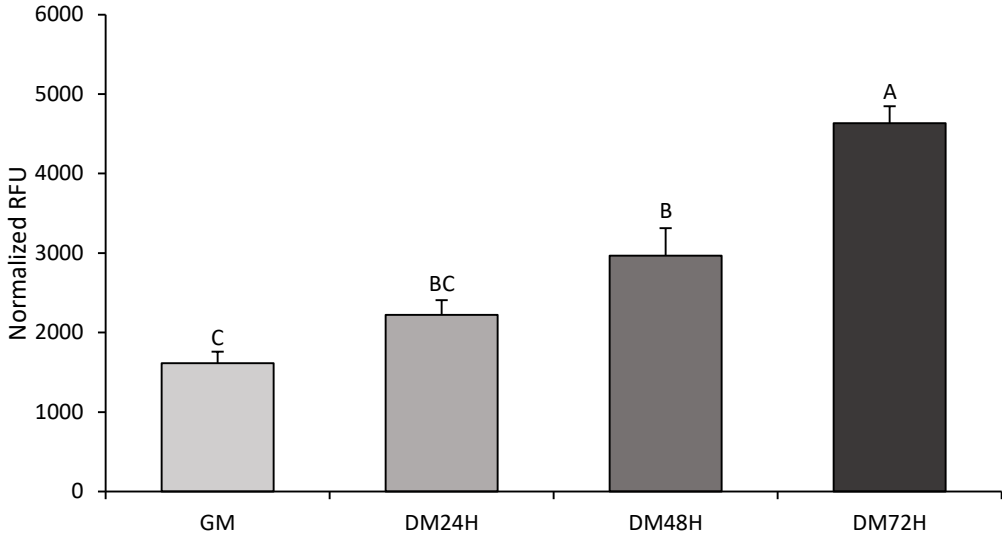


Figure 4.1 The proteasome activity in bovine myoblasts increases during differentiation. Proteasome activity in bovine myoblasts cultured in growth medium (GM) or differentiation medium (DM) for 24, 48, and 72 h was measured using a 20S proteasome activity assay kit. The proteasome activity was presented as relative fluorescence unit (RFU) in the graph. The experimental unit was animal. Bars not sharing the same letter label are different (n = 4, $P < 0.05$).

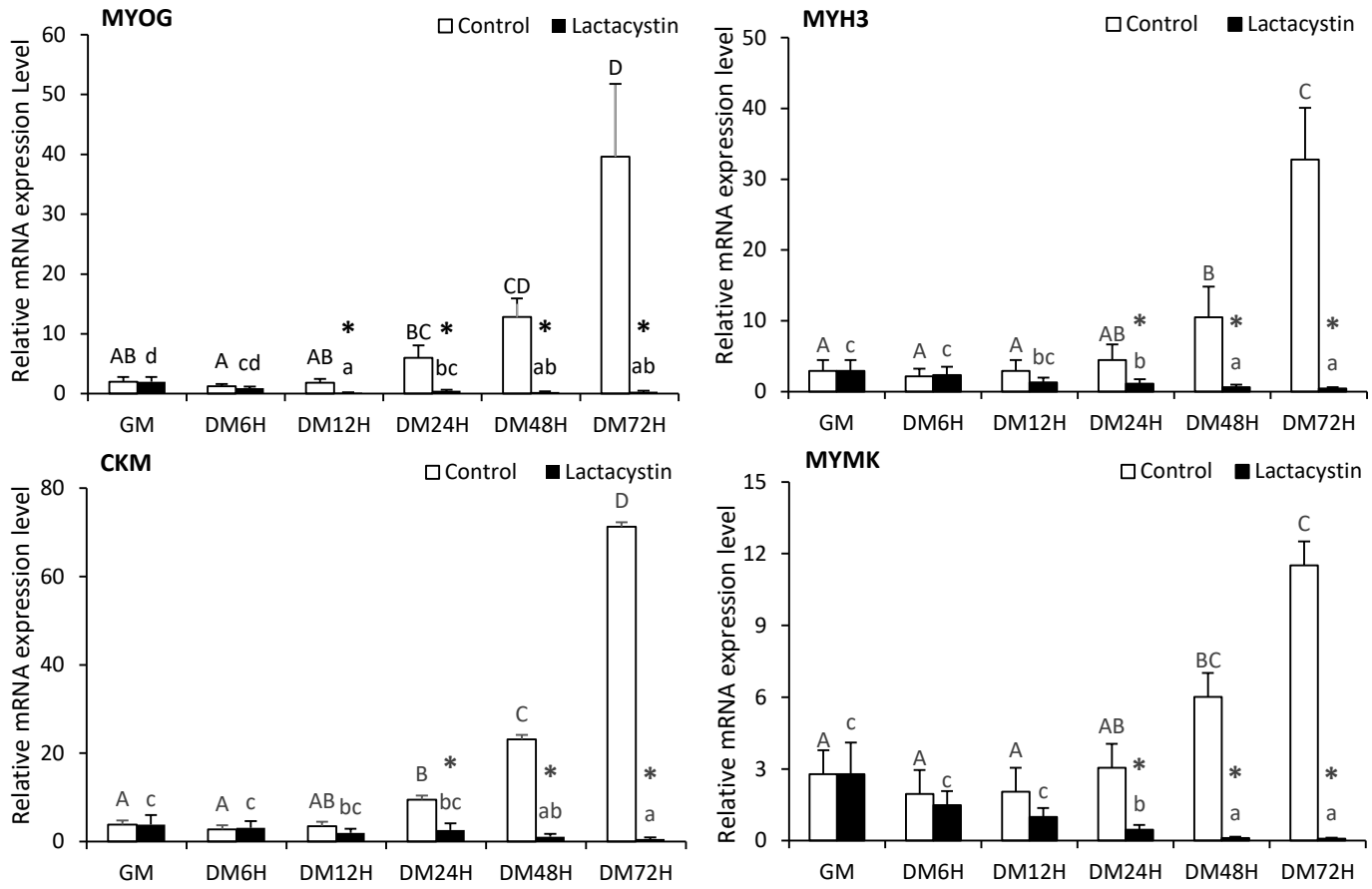


Figure 4.2 Relative expression levels of MYOG, MYH3, CKM, and MYMK mRNAs in bovine myoblasts before and during differentiation. Myoblasts isolated from 5 steers were cultured in differentiation medium (DM) in the presence of lactacystin or vehicle (control) for 6, 12, 24, 48, and 72 h. Myoblasts cultured in growth medium (GM) immediately before differentiation were used as undifferentiated myoblasts. mRNA expression of 4 differentiation marker genes was quantified by real-time qPCR. Bars not sharing the same uppercase letter label are different at different time points within control group (n = 5, $P < 0.05$). Bars not sharing the same lowercase letter label are different at different time points within lactacystin group (n = 5, $P < 0.05$). Bars labeled as * are different within time (n = 5, $P < 0.05$).

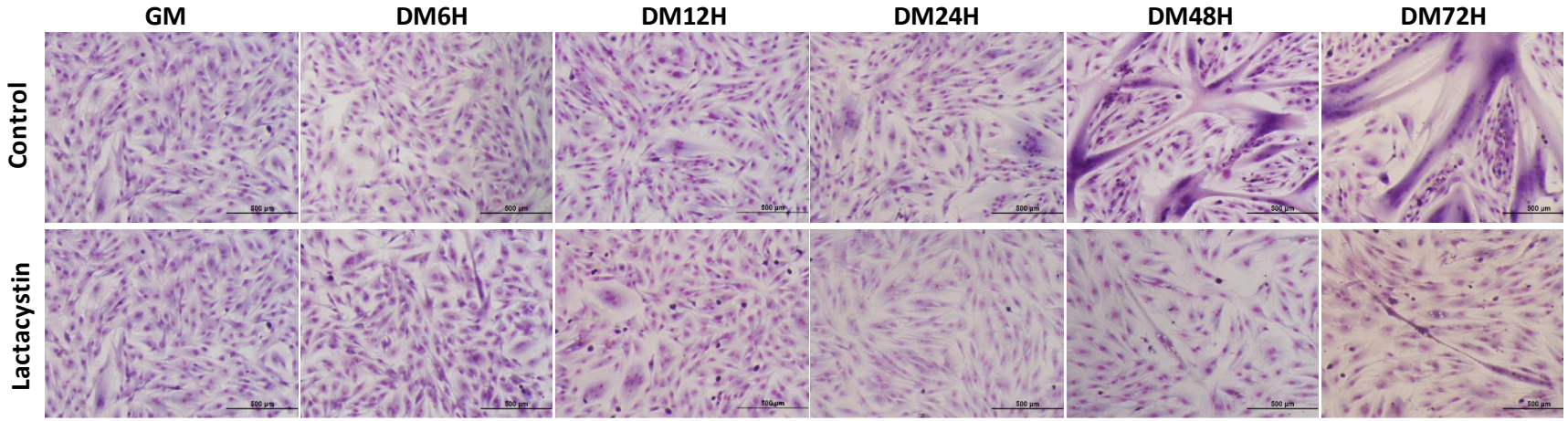
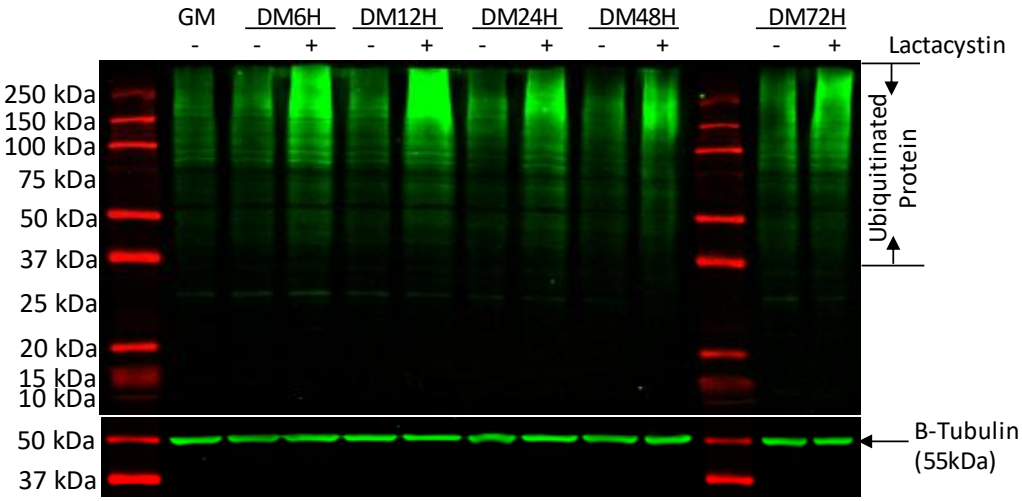


Figure 4.3 Effect of lactacystin on fusion of bovine myoblasts into myotubes. Myoblasts from steers were cultured in differentiation medium (DM) in the presence or absence (control) of lactacystin for 6, 12, 24, 48, and 72 h. These myoblasts as well as myoblasts cultured in growth medium (GM) were fixed and stained with Giemsa. Shown are representative micrographs (10× magnification) of stained cells.

A



B

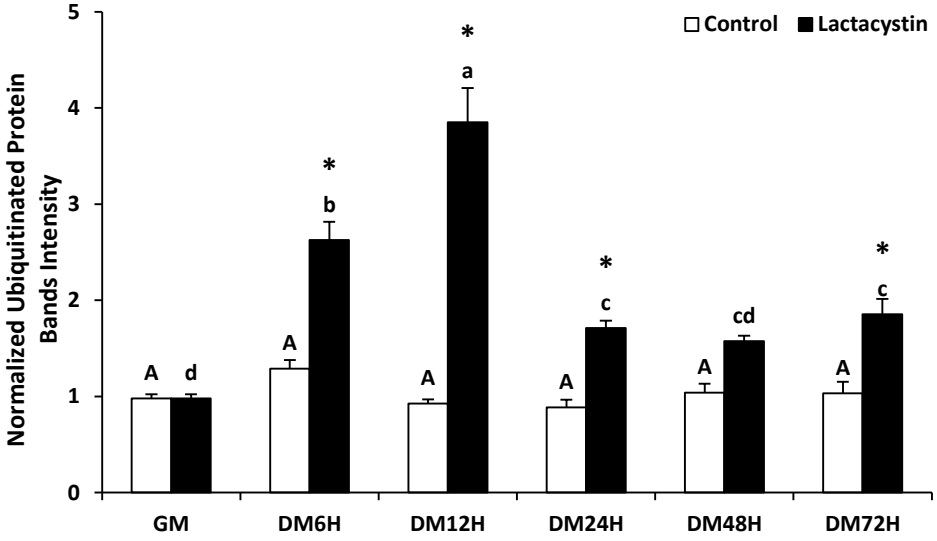


Figure 4.4 Effect of lactacysin on the accumulation of ubiquitinated protein in bovine myoblasts during differentiation. Myoblasts from 5 steers were differentiated in the presence or absence of lactacystin for 0 (GM), 6, 12, 24, 48, and 72 h. Ubiquitinated protein was detected by by western blotting using an antibody specific for ubiquitin. Beta tubulin was detected from the stripped membrane as a loading control. (A). Representative images of western blots. (B) Quantification of ubiquitinated protein. Intensity of ubiquitin-immunoreactive bands above the 37 KDa molecular weight marker was measured from each sample and normalized to the intensity of beta tubulin band in the same sample. Bars not sharing the same uppercase letter label are different between different time points within control group (n = 5, $P < 0.05$). Bars not sharing the same lowercase letter label are different between different time points within lactacystin group (n = 5, $P < 0.05$). Bars labeled as * are different within time (n = 5, $P < 0.05$).

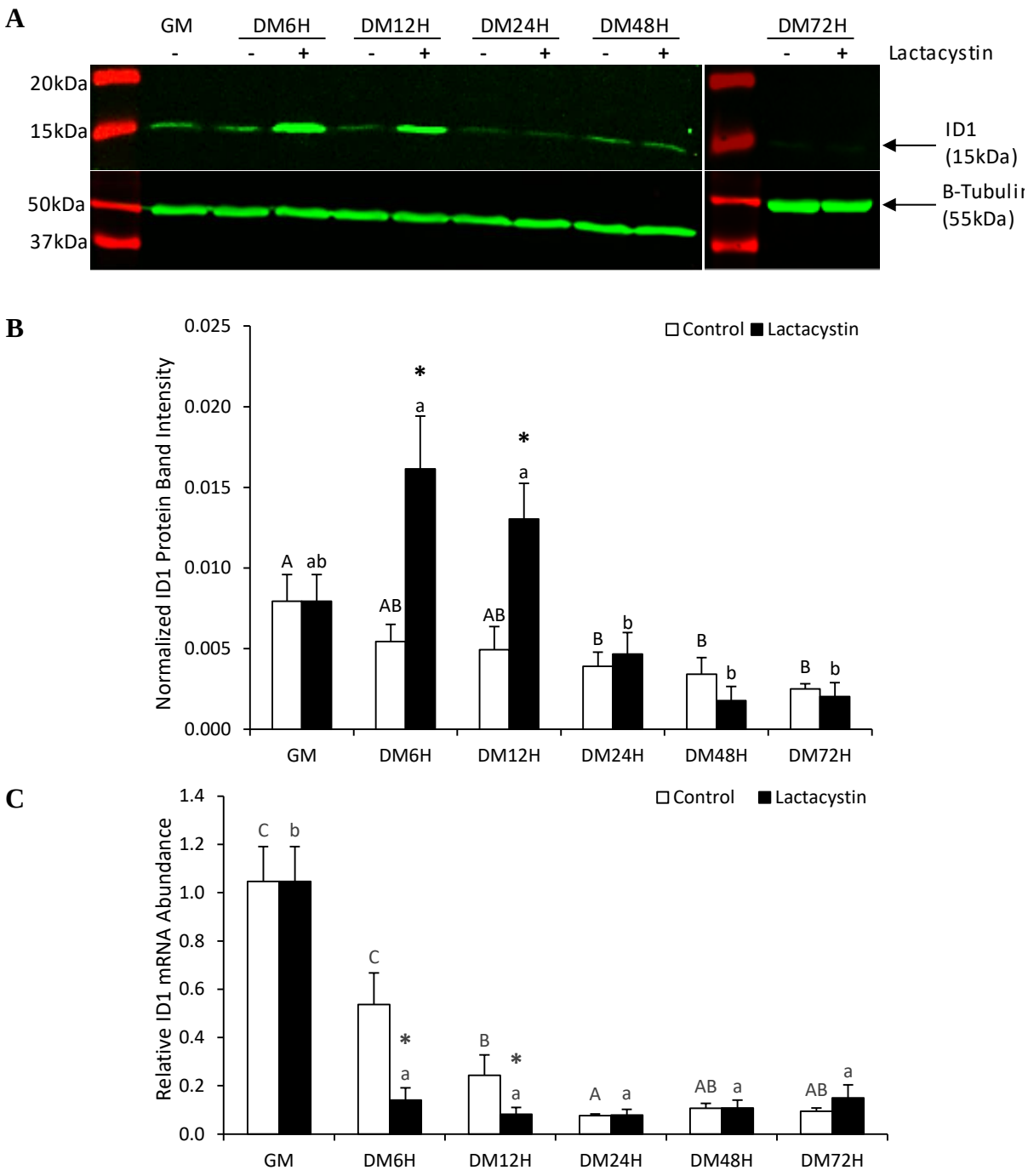


Figure 4.5 Effects of lactacystin on ID1 protein and mRNA epression in bovine myoblasts. Myoblasts from 5 steers were cultured in differentiation medium (DM) with or without lactacystin for 6, 12, 24, 48, and 72 h. Myoblasts cultured in growth medium (GM) immediately before differentiation were used as a baseline reference. ID1 protein was quantified by western blotting. ID1 mRNA was quantified by RT-qPCR. (A) Representative western blots. (B) Quantification of bands intensities. The ID1 protein level on y-axis was normalized to that of β-tubulin. (C) Expression level of ID1 mRNA. Bars not sharing the same uppercase letter label are different between different time points within control group (n = 5, $P < 0.05$). Bars not sharing the same lowercase letter label are different between different time points within lactacystin group (n = 5, $P < 0.05$). Bars labeled as * are different within time (n = 5, $P < 0.05$).

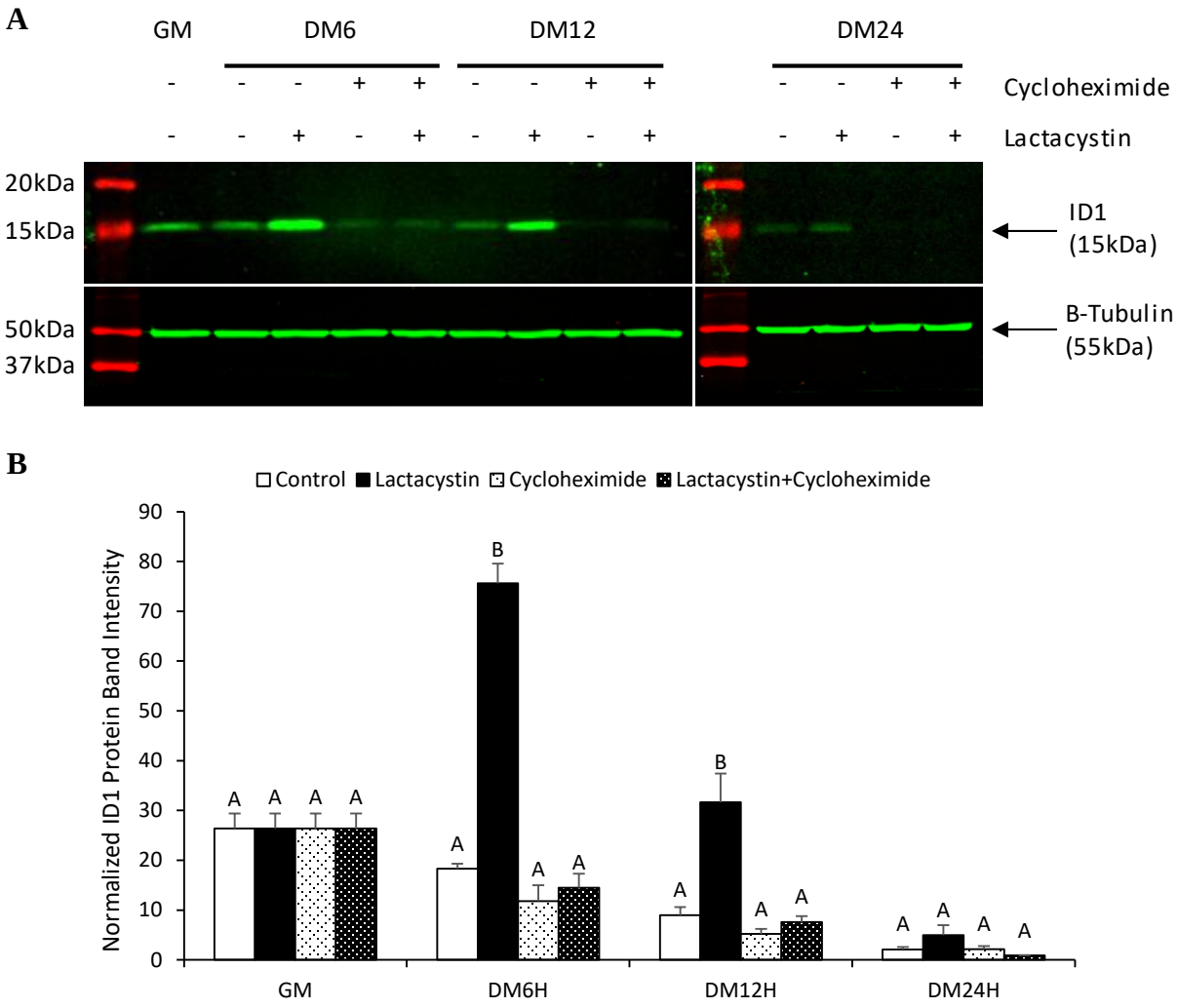


Figure 4.6 Effect of lactacystin on ID1 protein expression in bovine myoblasts in the presence or absence of cycloheximide. Myoblasts from 5 steers were cultured in differentiation medium (DM) lactacystin or cycloheximide or both for 0 (GM), 6, 12, and 24 h. ID1 protein was quantified by western blotting. (A). Representative wesern blots. (B) Quantification of ID1 protein bands. The ID1 band intensity in a sample was normalized to β-tubulin band intensity in the same sample. Bars not sharing the same letter label are different within time ($n = 5$, $P < 0.05$).

Chapter 5 Conclusions and implications

The process of myoblast proliferation, differentiation, and fusion is under the regulation of various extrinsic and intrinsic factors and physiological mechanisms. In this dissertation research, I studied the roles of oxytocin (OXT) and arachidonic acid (AA), as well as the proteasome in the proliferation, differentiation, and fusion of primary myoblasts isolated from adult cattle. Based on this dissertation research, oxytocin stimulates bovine myoblast proliferation; however, oxytocin has no effects on bovine myoblast differentiation or fusion. Arachidonic acid and its derivatives prostaglandins (PG) E_2, F_{2a}, and I_2 are found to be positive regulators of bovine myoblast proliferation. In addition, arachidonic acid, PGE2, and PGF2a promote bovine myoblast differentiation, and PGE_2 stimulates the fusion of bovine myoblasts into myotubes. The effects of arachidonic acid on myoblast proliferation and differentiation are likely mediated through its major metabolites, prostaglandin E_2 and F_{2a}. This dissertation research also shows that the activity of the proteasome increases during bovine myoblast differentiation and fusion. This increase is found to be essential to bovine myoblast differentiation and fusion into myotubes.

These results imply that administration of oxytocin, prostaglandin E2, F2a or I2, or dietary supplementation of arachidonic acid may improve muscle growth, i.e., meat production, in cattle. Before these applications are possible, in vivo cattle studies should be conducted to validate the in vitro effects of OXT, AA and prostaglandins on myoblast proliferation, differentiation, and fusion.