THE EFFECT OF AGE AND NUTRIENT STATUS ON GROWTH CHARACTERISTICS OF TURKEY SATELLITE CELLS

THESIS

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ABSTRACT

Myogenic satellite cells are heterogeneous multipotential stem cells required for muscle repair, maintenance, and growth. The membrane-associated heparan sulfate proteoglycans syndecan-4 and glypican-1 differentially regulate satellite cell proliferation, differentiation, fibroblast growth factor 2 (FGF2) signal transduction, and expression of myogenic regulatory factors. The objective of Specific Aims 1 and 2 was to determine the effect of age on satellite cell proliferation and differentiation using cells isolated from the pectoralis major muscle of 1 d, 7 wk and 16 wk old turkeys. Proliferation was significantly reduced in the 16 wk satellite cells, while differentiation was decreased in the 7 wk and the 16 wk cells beginning at 48 h of differentiation. Fibroblast growth factor 2 responsiveness was highest in the 1 d and 7 wk cells during proliferation; during differentiation there was an age-dependent response to FGF2. Syndecan-4 and glypican-1 expressing satellite cell populations decreased with age. These data demonstrate that declining syndecan-4 and glypican-1 satellite cell subpopulations which may be associated with age-related changes in satellite cell proliferation, differentiation, and FGF2 responsiveness.

In specific aim 3, the effect of overexpressing syndecan-4 and glypican-1 was investigated. Syndecan-4 and glypican-1 overexpression did not have a significant effect on proliferation and differentiation in 1 d, 7 wk and 16 wk satellite cells. Overexpression of syndecan-4 and glypican-1 did not affect FGF2 responsiveness during proliferation.
During differentiation, overexpression of syndecan-4 and glypican-1 increased differentiation at 48 h of differentiation in 1 d, 7 wk and 16 wk cells treated with FGF2 and decreased differentiation in 16 wk cells not treated with FGF2. Expression of myogenic regulatory factors MyoD, myogenin, and MRF4 were also affected by overexpression of syndecan-4 and glypican-1. These data demonstrate that syndecan-4 and glypican-1 overexpression does not significantly impact proliferation and differentiation in 16 wk cells. Thus, syndecan-4 and glypican-1 are not responsible for the decline in proliferation, differentiation, and FGF2 responsiveness observed in 16 wk satellite cells.

In addition to the role of satellite cell age on growth characteristics, nutrient status also affects satellite cell function. The objective of Specific Aim 4 was to determine the effect of nutrition on proliferation, differentiation, myogenic transcriptional regulatory factors, and syndecan-4 and glypican-1 expression in satellite cells from 1 d through 16 wk of age. A nutrient restriction similar to an in vivo feed restriction was simulated in vitro by restricting the concentration of methionine (Met). Nutrient restriction decreased proliferation at all ages. Differentiation was increased in 1 d nutrient restricted cells; while treatment of 7 wk cells with 3 mg/L Met/9.6 mg/L Cysteine (Cys) medium resulted in decreased differentiation. Reduced Met and Cys medium did not significantly affect 16 wk cells at 72 h of differentiation, though the medium with no Met and Cys resulted in significantly decreased differentiation in all ages during differentiation. Methionine and Cys restriction also had differential effects on syndecan-4, glypican-1, MyoD, myogenin and MRF4 mRNA expression. These data demonstrate that nutrient restriction differentially affects turkey satellite cells in an age specific manner.
Dedicated To My Family
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ABSTRACTS


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CHAPTER 1

LITERATURE REVIEW

Muscle growth is important in animal agriculture because muscle is the main component of meat. Poultry meat production is especially important, with more than 8.6 billion broilers and 248 million turkeys produced in the United States in 2011 (USDA Poultry Production and Value Summary, 2012). Recently, selection for meat production birds has focused on breeding birds that have faster growth and increased muscle deposition, especially in the pectoralis major (p. major), the main muscle composing the breast muscle. The above-noted increases in growth rate and muscle mass have coincided with increases in meat quality problems, such as a condition called pale, soft, and exudative in turkey meat, which results in meat with a decreased water holding capacity.

Producers have also adopted feeding strategies to attempt to maximize growth while minimizing feed costs. Such feeding strategies include feed restriction or protein restriction during the immediate posthatch period. Moore et al. (2005) suggested that feeding growth promotants is most effective in young animals because satellite cells have the highest mitotic activity immediately post hatch. Satellite cells, the cells responsible for posthatch muscle growth, have been shown to be affected by age (Mozdziak et al., 1994; Barani et al., 2003; Li et al., 2007; Velleman et al., 2010b). At this time, it is not
well known if satellite cells are affected while animals are still growing, or if the changes to the satellite cell occur after animals have ceased to grow. It is also not well understood how these practices, coupled with changes related to age, affect satellite cells. A better understanding of the biology underlying satellite cell changes during muscle growth may allow for maximized growth potential and meat quality while still allowing producers to minimize their cost per kilogram of gain.

1.1 Muscle types

There are three types of muscle found in vertebrates: cardiac muscle, smooth muscle and skeletal muscle. Smooth muscle is muscle that is under involuntary nerve control, is found, for instance, in the digestive tract, uterus, and mammary gland, and is responsible for the involuntary muscle contractions that are required, for example, for peristalsis. Cardiac muscle is a striated muscle that is also under involuntary nerve regulation and is found in the heart. This muscle is responsible for pumping blood throughout the pulmonary system. Of great importance in the animal and meat sciences is skeletal muscle, the muscle type most commonly consumed as meat. Skeletal muscle is striated muscle that is under voluntary nerve control, and accounts for the muscles involved primarily in locomotion.

1.2 Skeletal muscle structure

Skeletal muscle is made up of many bundles of muscle fibers that are surrounded by a layer of connective tissue called the epimysium. Each bundle of muscle fibers is called a fasciculus, and each individual fasciculus is surrounded by its own layer of connective tissue called the perimysium. Inside each fasciculus are several muscle fibers. These muscle fibers are separated by yet another layer of connective tissue called the
endomysium. Muscle fibers are large multinucleated myocytes with a large cytoskeletal component that is responsible for the contractile ability of the muscle: myofibrils. Each myofibril is composed of thick and thin myofilaments. The arrangement of the thick filaments and the thin filaments compose multiple sarcomeres, the contractile unit of the muscle, per myofibril (Figure 1.1).

The thin filaments, composed primarily of actin, tropomyosin, troponins, tropomodulin and nebulin, are anchored at the Z-line, which contains desmin and α-actinin. The thick filaments, composed primarily of myosin and titin, are anchored together at the M-line, with M-lines and Z-lines alternating within the myofibril. One sarcomere is defined as the area between two Z-lines, and contains a central M-line. Extending outward from the M-line is the H zone, where the myosin filament exists without any overlap with the actin filament, followed by the A band, the area where the myosin and actin filaments overlap, and finally the I band, where the actin filaments exist without any overlap with the myosin filament (Figure 1.1).

The thin filament is a polymer of many actin molecules, as well as the associated molecules tropomyosin, troponin, nebulin and tropomodulin. The length of the thin filament is regulated by nebulin, which matches its multiple 35 amino acid actin binding motifs to an equal number of helical repeats of actin (Jin and Wang, 1991; Kruger et al., 1991; Labeit et al., 1991). The end of the thin filament closest to the M-line is capped with tropomodulin, which functions in stabilizing the actin filament (Fowler et al., 1993). The thin filament is anchored at the Z-line, which is composed of CapZ and α-actinin (Alberts et al., 2008).
Myosin is a protein dimer composed of two heavy chains and two copies of two different light chains. The globular domain of the N-terminus of the myosin heavy chain is the region that binds to actin, and is attached to a long sequence of amino acids that make up the α-helical myosin tail region. Attached near each globular myosin head domain is one copy of each of the two light chains. Two myosin heavy chains with attached light chains dimerize when the two α-helical tails coil around each other, becoming one coiled-coil tail with two myosin heads (Kerwin and Bandman, 1991). The tails of many myosin molecules intermingle, forming the structure called the thick filament, each of which can contain hundreds of myosin heads (Reviewed in Alberts et al., 2008). The myosin filaments are tethered to the Z-line by titin, which lends passive elasticity to the sarcomere via its Ig and Proline, Glutamic Acid, Valine, and Lysine-rich (PEVK) domains (Trombitás et al., 1998), preventing the misalignment of the thick and thin filaments (Horowits et al., 1986).

Muscle contraction is accomplished by the sliding of the thin filaments against the thick filaments, resulting in a shortening of the sarcomere. The prevention of constant muscle contractions is key in maintaining functionality of muscle. This is accomplished by preventing myosin heads from binding to actin except when initiated by an influx of calcium. The complex that regulates this is the troponin complex, which consists of troponin I, troponin T and troponin C (Reviewed in Farah and Reinach, 1995). When skeletal muscle is at rest, the troponin I-T complex causes tropomyosin to relocate from its typical position in the grooves of the actin α-helix to a position along the filament that prevents the myosin heads from binding to actin. Upon the influx of calcium ions, troponin C binds to the calcium, causing troponin I-T to release actin, allowing for
tropomyosin to return to its preferred place and for myosin heads to bind to the actin filament. The influx of calcium ions occurs through the sarcoplasmic reticulum, a specialized membrane system continuous with the sarcolemma that allows for the storage and subsequent release of calcium upon sufficient membrane depolarization. The sarcoplasmic reticulum has attached transverse tubules, or T-tubules, which connect the sarcoplasmic reticulum to the sarcolemma, allowing for rapid transmission of action potentials from the sarcolemma to the sarcoplasmic reticulum.

The actual mechanical sliding of the myosin filaments against the actin filaments occurs only when myosin is able to bind to actin. Myosin heads attached to the actin filament bind to adenosine triphosphate (ATP), which causes a change in the conformation of the myosin head, causing it to release the actin filament and to become “cocked.” The ATP is then hydrolyzed, with the resulting adenosine diphosphate (ADP) and inorganic phosphate (P_i) remaining tightly bound to the myosin head. The inorganic phosphate is released when the myosin head binds to the actin, which in turn allows the myosin head to bind more tightly to the actin filament. The release of P_i causes a conformational change in the myosin head, ratcheting back to the structure it held previous to binding with ATP. This movement is referred to as the “power stroke”, and results in the movement of the actin filament against the myosin filament. The myosin head remains tightly bound to the actin filament until it binds to ATP and the cycle starts over (Figure 1.2).
1.3 Muscle fiber types

Muscles fibers can be broken down into several different types, and individual muscles are made up of more than one type. Classically, fiber types are classified upon their physical appearance – whether they are predominantly “red” or “white” in color. This classification is the basis for dark versus white meat: breast meat is composed primarily of white muscle fibers, while thigh meat is composed primarily of red muscle fibers. Fibers can also be classified by how fast they contract when exposed to stimulation. Type I fibers contract more slowly than type II fibers. This is due to the differing metabolism between the two fiber types. Type I fibers generate energy via oxidative metabolism, which requires oxygen, and is therefore an aerobic reaction. In this type of metabolism, glucose is broken down into pyruvate, which is then shunted through the Krebs cycle. Oxidative metabolism yields approximately 36 molecules of ATP (Figure 1.3). Type II fibers, or fast twitch fibers, generate energy via glycolysis, a quicker, less efficient anaerobic reaction. Thus, fast twitch fibers yield 6 molecules of ATP for every molecule of glucose (Figure 1.3). Consequently, fast twitch fibers contract rapidly in short bursts and become fatigued quickly, while slow twitch fibers contract more slowly and for a longer period of time. Slow twitch fibers are important for posture and compose the strength muscle such as the legs, while fast twitch fibers are most often seen in muscles that are not used for long periods of time, such as the pectoralis major muscle. Not all muscles are composed of only one type of muscle fibers. Muscles that are composed primarily of slow-twitch fibers are referred to as type I, primarily fast-twitch are classified as type IIB, and intermediate fibers, composed of a
mix of fast-twitch and slow-twitch fibers are classified as type IIA (Brooke and Kaiser, 1970).

1.4 Muscle growth and development

Three germ layers are formed during embryogenesis: the endoderm, the mesoderm and the ectoderm. The endoderm gives rise to the gastrointestinal tract, respiratory tract, endocrine glands and other organs; the ectoderm gives rise to the skin and the nervous system, and the mesoderm forms bone, connective tissue and skeletal muscle. Myogenic precursor cells originate from the dermomyotome of somites, embryonic structures that flank the notochord and give rise to the skeletal muscle of the limbs and trunk (Reviewed in Christ and Brand-Saberi, 2002). During embryonic development, myogenic precursor cells differentiate into embryonic myoblasts (Figure 1.4). Muscle is formed from these embryonic myoblasts, which proliferate, align and fuse to form multinucleated myotubes, which will become myofibers. This type of growth is referred to as hyperplasia, an increase in myofiber number. Myofiber numbers are determined by birth (Smith, 1963), and embryonic myoblasts withdraw from the cell cycle around the time of birth or hatch. Because these myoblasts withdraw from the cell cycle, they are no longer able to proliferate and fuse to form new myotubes. Therefore, post-natal/posthatch muscle growth is instead caused by an increase in myofiber size. This mechanism of growth, hypertrophy, is dependent upon a type of multipotential stem cell called an adult myoblast, or satellite cell. The exact origin of satellite cells is not fully known, though it is thought that they also arise from the dermomyotome and share a common origin with embryonic myogenic precursor cells (Gros et al., 2005). Satellite
cells are located between the sarcolemma and the basement membrane (Mauro, 1961) and contribute to myofibrillar growth by donating their nuclei to existing myofibers (Moss and LeBlond, 1970). The location of satellite cells – between the basement membrane and the sarcolemma – is referred to as the satellite cell niche.

Satellite cells are typically quiescent in adult muscle (Schultz et al., 1978), and express Paired box transcription factor 7 (Pax7) (Seale et al., 2000), but do not express myogenic regulatory factors until they become activated (Cornelison and Wold, 1997). Paired box transcription factor 3 (Pax3) is a paralog of Pax7 that is required for the organization and formation of the dermomyotome (Tremblay et al., 1998). The myogenic regulatory factor myogenic differentiation 1 (MyoD) is required for proliferation (Davis et al., 1987), while myogenin expression is required for differentiation (Hasty et al., 1993) (Figure 1.5). In MyoD null mice, myoblasts exhibit delayed (3 d) myotube formation compared to those of wild type mice (White et al., 2000), indicating that MyoD expression increases the ability of myoblasts to withdraw from the cell cycle and to differentiate into myotubes, but is not explicitly required for myoblasts to differentiate. Yablonka-Reuveni et al. (1999) also noted that very few MyoD null satellite cells transitioned to myogenin expressing cells and that myogenic factor 5 (Myf5) expression was higher in MyoD -/- cells versus wild-type cells, leading to the conclusion that Myf5 may be responsible for the maintenance of the quiescent satellite cell population. The requirement for MyoD expression prior to myogenin expression was supported by the findings of Sabourin et al. (1999). However, Kuang et al. (2007) found that Pax7+/Myf5- satellite cells give rise to Pax7+/Myf5+ satellite cells, and that Pax7+/Myf5- satellite cells contribute to the satellite cell reserve while those that
are Pax7+/Myf5+ become differentiated. Expression of myogenic regulatory factor 4 (MRF4) is thought to act upstream of MyoD and be capable of activating expression of myogenin (Kassar-Duchossoy et al., 2004). Expression of MRF4 is high in differentiated skeletal muscle of adult animals (Hinterberger et al., 1991; Bober et al., 1991), indicating that it plays a role in fiber formation, though high expression has been reported in satellite cells during proliferation (Shin et al., 2012a). Rawls et al. (1998) used knockout mice to determine that there is some overlap in the function of MyoD and MRF4, and that a knockout of both genes results in severe muscle deficiency.

Satellite cells are a heterogeneous cell population. This means that they vary in their growth properties, origin, and expression of myogenic regulatory factors, cell surface proteins, and other cell characteristics. For example, satellite cells that display one myogenic regulatory factor profile are able to give rise to daughter cells with a completely different myogenic regulatory profile (Kuang et al., 2007). Because heterogeneity results in variable activation, proliferation, differentiation and ability to respond to growth factors, it might play a key role in the ability of satellite cells to proliferate and differentiate throughout the life of an animal. The exact functional significance of satellite cell heterogeneity is not completely determined.

1.5 Extracellular matrix

Satellite cells respond (e.g. activation, proliferation and differentiation) to the extrinsic or extracellular matrix (ECM) environment surrounding them in the satellite cell niche (Bischoff, 1990). The ECM is a complex network of macromolecules, including collagens, proteoglycans, and non-collagenous glycoproteins, secreted by the cells and anchored in the extracellular environment. The ECM serves several purposes, including
providing a substrate for cell adhesion and migration, facilitating signaling between cells and the ECM, facilitating signaling between cells, providing the architecture that allows for tissues to maintain their physical properties, and is required for growth and development. The composition of the ECM is always changing: It is affected by age and developmental stage, and is regulated by the cells that it surrounds. Thus, the cells generate their own specific extracellular environment uniquely suited for their own needs. The ECM found in skeletal muscle differs, for example, from that in the vascular system, which in turn differs from that of the liver, which differs from any number of other tissues.

1.6 Collagen

Collagens are the major protein component of the ECM and account for an estimated 25% of the total protein mass in mammals (Alberts et al., 2008). The defining feature of a collagen molecule is a helical structure composed of three polypeptide strands (α-strands) that are wound together in a right-handed triple-helix. The polypeptides are unique in that glycine is expressed every 3rd amino acid, resulting in a repeating Gly-X-Y motif, where X and Y are often proline and lysine. Repeats of this motif allows for the formation of hydrogen bonds between the α-strands, which helps to stabilize the collagen (Bella et al., 1995). Currently, there are 28 different collagens (Gordon et al., 2010), which are divided into several subgroups, including fibril-forming collagen, fibril-associated collagen with interrupted triple-helix collagens (FACIT), multiplexin collagen, transmembrane collagens, network forming collagens, beaded-filament forming collagens, and anchoring collagens (Table 1.1) (Reviewed in Bonod-Bidaud and Ruggiero, 2013).
1.7 Fibrillar collagen

Fibrillar collagens have very long triple-helical structures that assemble into collagen polymers called collagen fibrils (molecular weight of about 300 kDa). The triple-helical collagen molecules align and form cross-links joining together many collagen molecules to form one collagen fiber. The cross-linking arrangement that the collagen helices form is called a quarter stagger array, the overlap of which results in the striated appearance of collagen fibrils (Figure 1.6). Collagen fibrils are the functional form of collagen. The collagen cross-links are either divalent or trivalent (reviewed in McCormick, 1994). Divalent, or ketoamine cross-links, are non-permanent cross-links that occur between two collagen molecules within the same fibril. Trivalent cross-links, or hydroxypyridinium (HP) cross-links, are permanent cross-links between three collagen molecules, only two of which need to be on the same fibril. The number of trivalent HP cross-linkages increase with age, and is the cause for decreased meat tenderness with increasing age (Iqbal et al., 1999). Fibrillar collagens include type I, II, III, V, XI, XXIV and XXVII (Reviewed in Ricard-Blum and Ruggiero, 2005).

Collagen types have a distribution that is specific to tissue type. The two main fibrillar collagens found in muscle tissue are collagen types I and III. Collagen type I is found in most tissues, but is the main structural component of bone. Type I collagen is a heterotrimer that contains two identical alpha chains and one alpha chain that differs from the others. Collagen type III, a homotrimer with three identical alpha chains, is considered embryonic collagen, and while fetal tissues contain a large amount of type III collagen, as animals age, the proportion of tissue shifts away from type III collagen and towards type I collagen (McCormick, 1994). Some type V collagen is also found in
skeletal muscle, and is associated with the myofibrils. Fibrillar collagen is especially important in skeletal muscle, as it provides the structural support needed to facilitate muscle contraction without destroying the muscle fibers. Collagen fibers themselves can be heterotypic and can contain collagen molecules of different fiber types. For example, collagens type I and III occur in the same fibril, as can type II.

1.8 Non-fibrillar collagen

The non-fibrillar collagens contain helical domains with Gly-X-Y repeats that are interrupted by non-helical regions, resulting in molecules that are able to bind to other identical molecules or associate with other collagens. Non-fibrillar collagens include the FACIT collagens (types IX, X, XII, XIV, XVI, XIX, XX, XXI, and XXII); network forming collagens types IV and VIII; basement membrane multiplexin collagen types XV and XVIII; transmembrane collagen types XXV, XIII, XVII and XXIII; bead-forming collagen type VI; and anchoring collagen VII (Brown and Timpl, 1995; Ricard-Blum and Ruggiero, 2005; Gordon and Hahn, 2010). Collagen type IV is important in skeletal muscle because it forms the basement membrane that surrounds muscle fibers and under which satellite cells are located. Collagen type XV is thought to be required for proper muscle support and capillary structure (Eklund et al., 2001). Type IX is a fibril-associated collagen that may function to stabilize the ECM (Müller-Glauser et al., 1986). Collagens are also involved in bone development. For example, type X collagen, a fibril-associated collagen produced by hypertrophic chondrocytes, is essential for the mineralization of developing bone through its ability to bind calcium (Table 1.1) (Schmid and Linsenmeyer, 1985).
Glycoproteins are proteins that have been post-translationally glycosylated, meaning that they have a core protein with oligosaccharides attached by either oxygen or nitrogen. These adhesive proteins can be located in the ECM, with one of the most prevalent being fibronectin. Fibronectin has specific sites for the binding of cell surface proteins as well as other ECM molecules. Fibronectin is a dimer linked by two disulfide bonds (Figure 1.7) that contains binding sites for heparin, fibrin, and collagen and is critical in the formation of focal adhesions, or points of attachment between cells and the ECM. Integrins bind to fibronectin at the Arginine-Glycine-Aspartic Acid sequence, or RGD sequence. Integrins are transmembrane heterodimeric cell surface proteins composed of an alpha and a beta subunit that are involved in the formation of focal adhesions, or points of contact between cells and the ECM that connect the ECM with the cellular cytoskeleton necessary in cell adhesion. In skeletal muscle, integrins with one α5 and one β1 subunit are required for migration (Menko and Boettiger, 1987). The formation of focal adhesions is necessary for long-term cell survival and cell migration because loss of contact with the matrix triggers apoptosis (Frisch and Ruoslahti, 1997).

Proteoglycans are composed of a central polypeptide core with one or more attached glycosaminoglycan (GAG) chains, and can vary in size from small proteoglycans with a core protein size of about 20 kDa to very large proteoglycans with a core protein of approximately 450 kDa (reviewed in Couchman and Pataki, 2012). Glycosaminoglycans are unbranched polysaccharides with repeating disaccharide units.

Proteoglycans are often classified based on their attached GAG chains (Figure
1.8), resulting in four main types of proteoglycans: heparan sulfate, keratan sulfate, dermatan sulfate and chondroitin sulfate. It is important to note that not all proteoglycans are limited to just one type of GAG chain. Hyaluronic acid, another GAG chain, is a polymer of N-acetylglucosamine and glucuronic acid with a 1-3 and 1-4 linkage, and is the only GAG chain that does not covalently link to a core protein and is not sulfated. Chondroitin sulfate is composed of a 1-4 and 1-3 linkage of N-acetylglucosamine and glucuronic acid; dermatan sulfate is composed of a 1-4 and 1-3 linkage of N-acetylglucosamine and iduronic acid; keratan sulfate a 1-3 and 1-4 linkage of N-acetyl glucosamine and galactose; and heparan sulfate is a 1-4 linkage of N-acetylglucosamine and glucuronic acid. Chondroitin sulfate, dermatan sulfate and heparan sulfate are linked to the oxygen on a serine or threonine of the core protein (O-linked), while keratan sulfate is linked via the amide group of an asparagine (N-linked).

Proteoglycans can be categorized by their function as aggregating, non-aggregating or cell surface associated proteoglycans (Couchman and Pataki, 2012) (Table 1.2). Aggregating proteoglycans are very large proteoglycans that typically have over 100 GAG chains, and are the only proteoglycans to ionically bind to hyaluronic acid. Because these GAG chains are hydrophobic and inflexible, aggregating proteoglycans tend to occupy a relatively large volume for their mass. Carboxyl and sulfate groups located on the GAG chains result in the GAG chains having a highly negative charge, which helps to recruit water to the ECM by ionically binding to the water. This attraction of water to the ECM enables the ECM to withstand compression, an example being load-bearing joints. Examples of aggregating proteoglycans include versican and aggrecan.
Versican is thought to be crucial in regulating the spacing of muscle fibers (Snow et al., 2005).

Non-aggregating proteoglycans can function in regulating collagen cross-linking, collagen fibrillogenesis, cell growth, and regulation of growth factors. Decorin, for example, is a small arch-shaped proteoglycan (Weber et al., 1996) with a core protein size of 36 kDa (Couchman and Pataki, 2012). Decorin is involved in the formation of collagen cross-links and is essential in proper collagen fibril formation (Danielson et al., 1997), cell growth (Moscatello et al., 1998; Iozzo et al., 1999; Schönherr et al., 2005), and the regulation of myostatin and transforming growth factor beta (TGFβ) (Schönherr et al., 1998; Kishioka et al., 2008). A member of the small leucine-rich proteoglycans, decorin has one attached dermatan sulfate or chondroitin sulfate GAG chain.

Decorin has been shown to impact proliferation and differentiation through its interaction with various growth factors. Myostatin and TGFβ have been shown to decrease satellite cell proliferation and differentiation (Allen and Boxhorn, 1987; McFarland et al., 2006). Decorin has been shown to facilitate proliferation and differentiation by binding to and inactivating TGFβ (Schönherr et al., 1998) and myostatin, a member of the TGFβ family (Kishioka et al., 2008). Schönherr et al. (2005) found that decorin can also stimulate proliferation and differentiation by binding to the insulin like growth factor I receptor.

In addition to having a stimulatory effect, decorin can also decrease cell proliferation and differentiation by activating the epidermal growth factor receptor (Moscatello et al., 1998; Iozzo et al., 1999). Platelet-derived growth factor (PDGF) isoform BB also stimulates proliferation in mouse myogenic cells (Yablonka-Reuveni,
The short isoform of PDGF-BB has been shown capable of binding to dermatan sulfate and chondroitin sulfate proteoglycans such as decorin or biglycan (García-Olivas et al., 2003; Koźma et al., 2009), which may affect the availability of PDGF in tissue.

Cell-surface associated growth factor regulating proteoglycans include the heparan sulfate proteoglycans in the syndecan and glypican families, which differentially regulate fibroblast growth factor 2 (FGF2). Fibroblast growth factor 2 stimulates proliferation and inhibits differentiation (Dollenmeier et al., 1981) through binding to its tyrosine kinase receptor located on the cell membrane. In order for FGF2 to bind to this receptor, it must first bind with heparan sulfate chains (Yayon et al., 1991). The ECM can either function to help present FGF2 to its tyrosine kinase receptor, or it can sequester FGF2 from its receptor, as is the case with how glypican-1 functions to inhibit proliferation and permit differentiation (Brandan et al, 1996; Gutiérrez and Brandan, 2010; Velleman et al., 2013). Fibroblast growth factor 2 is a potent inhibitor of differentiation (Dollenmeier, 1981), and the addition of FGF2 to culture media has been shown to increase satellite cell activation in rat myofibers (Yablonka-Reuveni et al., 1999), and to increase proliferation of aged satellite cells in mouse myofibers (Shefer et al., 2006).

1.11 Syndecans in skeletal muscle

Syndecans are a family of 4 transmembrane heparan sulfate proteoglycans involved in cell signaling, all of which are found in skeletal muscle. Syndecans contain a core protein containing a conserved cytoplasmic domain, transmembrane domain and extracellular domain (Figure 1.9). Syndecan-1 is expressed most highly in epithelial cells, syndecan-2 in fibroblasts, and syndecan-3 in neural tissue. Syndecan-4 is
expressed in the majority of adult tissue, and expressed at all developmental stages (Kim et al., 1994). Attached to the extracellular domain of syndecan-4 are three heparan sulfate chains attached at Ser\(^{38}\), Ser\(^{65}\), Ser\(^{67}\) (Zhang et al., 2008) and two N-linked glycosylation chains attached at Asn\(^{124}\) and Asn\(^{139}\) (Song et al., 2011) in turkey skeletal muscle.

Syndecan-4 has been shown to initiate intracellular signaling via its cytoplasmic domain upon binding to FGF2 (Volk et al., 1999; Horowitz et al., 2002; Zhang et al., 2003), though it may also function in an FGF2 independent manner during satellite cell proliferation in turkeys (Velleman et al., 2007). Fibroblast growth factor 2 is important in the activation of satellite cells (Bischoff, 1986). The cytoplasmic domain of syndecan-4 has two highly conserved regions, C1 and C2, as well as one variable region. This variable region has been shown to interact with other syndecan-4 cytoplasmic domain variable regions to form homodimers (Lee et al., 1998; Shin et al., 2001), forming a structure that is stabilized by the binding of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) to the variable region. Upon the formation of dimers, syndecan-4 forms oligomers which activate protein kinase C alpha (PKC\(\alpha\)) via the binding of the catalytic region of PKC\(\alpha\) to the variable region of syndecan-4 (Oh et al., 1997; Horowitz et al., 1999; Couchman et al., 2002; Keum et al., 2004). In skeletal muscle, syndecan-4 has been shown to form oligomers of syndecan-4 (Shin et al., 2012b), and that the cytoplasmic domain of syndecan-4 is involved in the regulation of PKC\(\alpha\) regulation and FGF2 responsiveness (Song et al., 2012a, 2012b). Both the transmembrane and the cytoplasmic domain are involved in the formation of oligomers, though the mutation of the oligomerization region of the transmembrane domain results in a more significant decrease in ras
homolog gene family member A (RhoA) activity than do mutations of the cytoplasmic domain (Choi et al., 2012). The cytoplasmic domain is critical in mediating satellite cell migration through the activation of RhoA (Shin et al., 2013). Migration is essential for muscle formation and repair: without migration, satellite cells would be unable to align to form myotubes, and would be unable to migrate to the site of muscle damage.

The attached GAG and N-chains on syndecan-4 also play a role in its function. Zhang et al. (2008) found that turkey satellite cells transfected with syndecan-4 GAG chain mutants, which have their heparan sulfate chains removed, had no effect on satellite cell proliferation versus the wild type, and no effect on FGF2 responsiveness, and a limited effect on differentiation. This further supported the conclusion that syndecan-4 can function in an FGF2-independent manner, and that the regulation of proliferation by syndecan-4 is not dependent upon the attachment of GAG chains.

Syndecan-4 N-chain mutants, which are missing their N-linked glycosylation chains, had no effect on satellite cell proliferation, though the deletion of the N-chain at Asn^{124} resulted in increased FGF2 responsiveness compared to those transfected with wild-type syndecan-4 (Song et al., 2011). The N-chains may affect the ability of satellite cells to form dimers needed for the successful formation of focal adhesions.

The deletion of both the GAG chains and the N-chains resulted in no difference during differentiation, though there is an increase in proliferation and FGF2 responsiveness during proliferation (Song et al., 2011). The overexpression of wild type syndecan-4 was hypothesized to decrease proliferation by increasing focal adhesion formation to the point where satellite cells are unable to migrate because they are too
tightly bound to the ECM. Thus, the interaction between the GAG and the N-chains may be crucial in the formation of focal adhesions (Song et al., 2011).

In addition to regulating satellite cells via FGF2 and the activation of the RhoA pathway, syndecan-4 has also been shown to affect the expression of the myogenic regulatory factors MyoD, myogenin and MRF4. Shin et al. (2012a) found that a knockdown of syndecan-4 in turkey satellite cells resulted in an increase in the expression of MyoD, myogenin, and MRF4 at 72 h post-transfection, and a decrease in MyoD and MRF4 expression at 24 h of differentiation. Thus, the knockdown of syndecan-4 is likely to cause a decrease in the ability of satellite cells to proliferate and differentiate by decreasing the expression of the myogenic regulatory factors necessary for proliferation and differentiation.

1.12 Glypican in skeletal muscle

Glypicans are a family of 6 heparan sulfate cell surface proteoglycans. Only glypican-1 has been found in skeletal muscle (Campos et al., 1993). Glypican-1 is an extracellular proteoglycan, attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor, with a core protein that contains a globular, cysteine-rich region (Figure 1.10). Glypican-1 also has three heparan sulfate chains attached at Ser$^{483}$, Ser$^{485}$, and Ser$^{487}$ (Zhang et al., 2007), and three N-linked glycosylation chains attached at Asn$^{76}$, Asn$^{113}$, and Asn$^{382}$ (Song et al., 2010) in turkey skeletal muscle.

Glypican-1 promotes differentiation by sequestering FGF2 from its receptor by binding to FGF2 and being shed into the extracellular matrix via the cleavage of the GPI anchor (Velleman et al., 2013). Glypican-1 can also promote differentiation by its localization in lipid rafts in the cell membrane while the FGF2 receptor is located in non-
raft portions of the cell membrane, as well as by the internalization of glypican-1 upon binding to FGF2 (Gutiérrez and Brandan, 2010).

The GAG and N-chains that are attached to glypican-1 also play a role in the regulation of satellite cell activity. Song et al. (2010) showed that turkey satellite cells transfected with glypican-1 without GAG chains or N-chains had significantly increased proliferation, suggesting that the GAG and N-chain deletion may affect the folding of glypican-1, resulting in a conformation that does not function to bind FGF2. Song et al. (2010) also demonstrated that glypican-1 constructs with no N-chains were more responsive to FGF2 during proliferation, which might be caused by a structural change to glypican-1 that increases FGF2 binding to its receptor. Transfection of turkey satellite cells with glypican-1 constructs without GAG chains resulted in decreased differentiation compared to the wild-type glypican-1 (Zhang et al., 2007). Taken together, these data indicated that FGF2 responsiveness is glypican-1 dependent.

Glypican-1 has also been implicated in the regulation of the myogenic regulatory factors MyoD, myogenin, MRF4 and Pax7. Shin et al. (2012) found that the knockdown of glypican-1 in turkey satellite cells resulted in decreased expression of MyoD, myogenin, MRF4 and Pax7, although the expression of MyoD was not affected at 72 h of differentiation. Thus, glypican-1 also regulates the proliferation and differentiation through the modulation of the myogenic growth factors required for proliferation and differentiation of satellite cells.

1.13 Effect of nutrition on satellite cells

Industry practices in poultry production are to feed restrict or nutrient restrict birds in the first 2 wk of life (Arce et al., 1992; Acar et al. 1995). This is done in attempt
to minimize the occurrence of conditions such as tibial dyschondroplasia and metabolic conditions such as ascites by allowing skeletal growth to catch up with muscle growth (Hester et al., 1990; Arce et al., 1992; Elliot and Edwards, 1994; Acar et al. 1995). Restricting feed in birds is also practiced in order to decrease the cost per kilogram of gain by capitalizing on compensatory gain when the birds are returned to full feed intake (Reviewed in Sahraei, 2012). Current handling procedures also make it likely that newly hatched chicks and poults will be without food or water for up to 3 d immediately posthatch while being shipped from the hatchery to the growing facility. These industry practices have been shown to have a negative effect on satellite cell function.

Halevy et al. (2000) reported that broiler chicks that had been feed deprived for 48 h posthatch had decreased and delayed satellite cell proliferation and body weight along with altered breast muscle morphology when compared to their full-fed counterparts. Feed depriving chicks had the most drastic effect on muscle growth during the first 2 d posthatch, and the least effect when it occurred during d 4 to 6 posthatch (Halevy et al., 2000). These data indicated that satellite cells are more sensitive to feed restriction immediately after hatch. Feed deprivation for the first 3 d posthatch also decreased heparan sulfate proteoglycan expression in broilers (Velleman and Mozdziak, 2005) and decreased satellite cell mitotic activity in broilers and turkeys (Mozdziak et al., 2002; Halevy et al., 2003). Because satellite cells are maximally active immediately posthatch, the negative effects on breast muscle growth and muscle morphology are likely due to negative effects of feed restriction on satellite cell function.

Increased fat deposition and necrosis, coupled with altered muscle morphology, were observed in broilers that were feed restricted by 20% for the first 2 wk posthatch.
(Velleman et al., 2010a). The cause of increased fat deposition in restricted broilers is not yet fully understood, but may be due to transdifferentiation of satellite cells into adipocytes, as this has been demonstrated to occur when satellite cells are exposed to the adipogenic inducers methyl-iso-butylxanthine, dexamethasone, indomethacin and insulin (Asakura et al., 2001).

### 1.15 Extracellular matrix changes with age

The ECM is marked by several changes as animals age. During skeletal muscle growth, versican is present surrounding developing myotubes, and declines as development progresses (Carrino et al., 1999). Conversely, decorin is localized to the epimysium early in development, but gradually spreads to the perimysium and endomysium (Carrino et al., 1999). Increasing age also results in changes to the cross-linkages found in collagen fibrils. As animals age, divalent collagen cross-linking decreases and trivalent collagen cross-linking increases (Eyre et al., 1984), which results in connective tissue that is stronger but has decreased stretch. Practically, this means that meat harvested from older animals will be less tender than meat harvested from younger animals. In addition to the changes in the ECM that result in decreased meat tenderness, alterations to the ECM also result in an evolving satellite cell niche.

Changes in the satellite cell niche are not well documented or understood, but are known to be of great importance. Muscle grafts transplanted from old to young and young to old rats exhibited growth characteristics fitting that of the host animal (Carlson and Faulkner, 1989). This indicates that growth characteristics of muscle are heavily determined by the extracellular environment. Age-related decreases in satellite cell activation, migration, and ability to differentiate are likely the cause for altered skeletal
muscle growth characteristics. Because satellite cell proliferation can be induced by the exogenous addition of FGF2 (Shefer et al., 2006), it is likely that FGF2 and its regulation are important changes with age. Thus, components of the ECM such as syndecan-4 and glypican-1 that are involved in activation, migration, and the regulation of FGF2 are likely altered with age.

1.16 Satellite cell changes with age

Alterations to the satellite cell population have been observed with age. Proliferation was found to be decreased in primary satellite cells isolated from the p. major of turkeys by 8 wk of age, while differentiation was significantly decreased by 4 wk of age (Velleman et al., 2010b). Similar changes with age have been reported in other animals (Barani et al., 2003; Li et al., 2011). Mitotic activity has also been shown to decrease with increasing age (Mozdziak et al., 1994), which would account for some of the loss of proliferative ability. Shefer et al. (2006) showed that satellite cell proliferation can be rescued by the addition of exogenous FGF2. Expression of syndecan-4 and glypican-1 also decreases in muscle tissue isolated from 16 wk old versus 1 d old turkey muscle versus (Sporer et al., 2011).

Collins et al. (2007) reported that there is a small population of satellite cells that appears to be unaffected by any age-related changes, and that this population of satellite cells appears to remain constant throughout the life of mice. This indicates that while there is a small subpopulation of satellite cells that are unaffected by age, the majority of satellite cells are affected by age. The identification of satellite cell subpopulations that vary in their expression of myogenic regulatory factors and in their sensitivity to age-related changes indicates that the satellite cell population is heterogeneous. It is likely
that age affects the heterogeneity of satellite cells. It is also likely that as age increases, different populations of satellite cells exist within a satellite cell niche that is also changing.

1.18 Purpose of experiments

The purpose of this study is to evaluate the effect of age on satellite cell behavior and heterogeneity. Because age has negative effects on satellite cell activity leading to decreased muscle growth and decreased muscle mass, there are likely functional changes occurring in the satellite cells. Nutrient status is also likely to affect satellite cells differentially during development. This leads to the overall hypothesis that: Satellite cell function is affected by age. Specifically, it is hypothesized that satellite cell function is affected by age-related changes: 1, in the expression of the cell surface proteoglycans syndecan-4 and glypican-1; 2, in myogenic regulatory factor profile; 3, in response to varying nutrient status.

To investigate age related changes, satellite cells isolated from 1 d, 7 wk and 16 wk randombred control line 2 (RBC2) male turkeys and expanded in culture will be used. These represent poults that are newly hatched and undergoing maximal satellite cell activity, a market age bird (16 wk) and a poult approximately halfway through the growing period. To determine the changes in the satellite cells, important characteristics will be measured, including the effect on the expression of syndecan-4, glypican-1 and myogenic regulatory factors as well as the effect of overexpressing syndecan-4, glypican-1 and both syndecan-4 and glypican-1 on satellite cell proliferation, differentiation, FGF2 responsiveness, and myogenic regulatory factor profile. The effect of nutrition, approximated in vitro by a methionine restriction that will limit protein accretion, with
age will also be determined. To investigate these hypotheses, the following specific aims will be pursued:

**Specific Aim 1** will determine the effect of age on syndecan-4 and glypican-1 expressing satellite cells, proliferation, differentiation, and fibroblast growth factor-2 (FGF2) responsiveness. The hypothesis for this aim is that the age of the satellite cells and the percentage of syndecan-4 and glypican-1 expressing cells affect satellite cell proliferation, differentiation and FGF2 responsiveness.

**Specific Aim 2** will determine the effect of age on myogenic regulatory factor expression in satellite cells. This aim will test the hypothesis that the age of the satellite cells affects the profile of the myogenic transcriptional regulatory factors expressed by satellite cells.

**Specific Aim 3** will determine the effect of transflecting turkey satellite cells at different ages with syndecan-4 and glypican-1 on proliferation, differentiation and FGF2 responsiveness. This aim will test the hypothesis that increasing syndecan-4 and glypican-1 expression will affect the proliferation, differentiation, FGF2 responsiveness and myogenic transcriptional regulatory factor profile of satellite cells by rescuing the growth characteristics of the cells.

**Specific Aim 4** will determine the effect of nutrient status on syndecan-4 and glypican-1 expression in satellite cells isolated from turkeys of different ages, as well as the effect on proliferation, differentiation, the expression of syndecan-4 and glypican-1, and the profile of myogenic transcriptional regulatory factors. This aim will test the hypothesis that the nutritional status of satellite cells will affect the proliferation, differentiation, and the myogenic transcriptional regulatory factors expressed by the satellite cells in an age-dependent manner.
To determine the effect of age on satellite cells, previously isolated cells from the p. major of 1 d, 7 wk, and 16 wk old turkeys will be grown in culture. Cells from 1 d birds represent satellite cells at the peak of mitotic activity, 16 wk old cells represent cells form an approximately market-age bird, and 7 wk cells are representative of a bird that is mid-way through its growth to market weight. To eliminate any effects of growth selection, cells will be isolated from RBC2 turkeys, which have been maintained without any selection pressure and are representative of a 1966 turkey (Nestor et al., 1969), and all male cells will be used to eliminate any sex effects (Velleman et al., 2000; Song et al., 2013). Using isolated satellite cells will allow for the determination of the effect of age on just the satellite cells, although the disadvantage is that the satellite cells are removed from their in vivo environment and are no longer being impacted by the other cell types present in muscle.

In the study investigating the effect of age on satellite cell function, the experiments will focus on determining the ability of satellite cells to proliferate, differentiate and respond to FGF2, as well as the percentage of cells expressing the heparan sulfate proteoglycans syndecan-4 and glypican-1 and the mRNA expression of syndecan-4 and glypican-1 and the myogenic regulatory factors. It is expected that with increased age will come decreased satellite cell proliferation, differentiation and FGF2 responsiveness. It is also expected that the percentage of cells that express syndecan-4 and glypican-1 will decrease with increasing age, and that the myogenic regulatory factor profile will change due to the effect of altered syndecan-4 and glypican-1 expression (Shin et al., 2012). Because syndecan-4 is involved in satellite cell activation, proliferation (Cornelison et al., 2004), and migration (Shin et al., 2013) and glypican-1 is
critical in promoting differentiation through the sequestration of FGF2 from its receptor (Brandan et al., 1996; Gutiérrez and Brandan, 2010; Velleman et al., 2013), this change in the satellite cell heterogeneity may account for the expected decrease in proliferation, differentiation and FGF2 responsiveness.

Specific aim 3 will determine the ability to rescue the effects of age on satellite cell function by increasing the expression of syndecan-4 and glypican-1. This will be accomplished by transfecting cells of different ages with either or both syndecan-4 and glypican-1 expression plasmids to increase expression. The effect of these transfections will then be determined by measuring proliferation, differentiation, FGF2 responsiveness, and myogenic regulatory factor expression. The expected decrease in syndecan-4 and glypican-1 expression is thought to cause a decrease in satellite cell proliferation, differentiation, FGF2 responsiveness and an altered myogenic regulatory factor profile. Thus, transfecting satellite cells to express syndecan-4 and glypican-1 at higher levels is expected to cause an increase in proliferation, differentiation and FGF2 responsiveness, as well as altering the myogenic regulatory factor profile. The presence of exogenous FGF2 has been shown to increase proliferation of aged satellite cells (Shefer et al., 2006). Because of this, it is hypothesized that increasing the expression of syndecan-4 and glypican-1 will increase satellite cell proliferation and differentiation in the 16 wk satellite cells due to the involvement of syndecan-4 and glypican-1 in the mediation of FGF2 responsiveness.

In specific aim 4, examination of the effect of nutrition on satellite cell function, varying concentrations of methionine will be used to replicate different levels of feed restriction in vitro. Methionine restriction will be used to approximate a nutrient
deficiency because it is the first limiting amino acid in protein synthesis, and because simply diluting the media would cause osmotic imbalances that could cause the cells to lyse. Limiting methionine will cause amino acids to be degraded to pyruvate or intermediates of the Krebs cycle, and shuttling those carbon molecules to fatty acid synthesis, ultimately resulting in an increase in acetyl-coA, the substrate for lipid formation. This increase in acetyl-coA will likely cause increased fat synthesis, resulting in the satellite cells to transdifferentiate into adipocytes. This study aims to determine how the effect of nutrient status changes with age. Proliferation, differentiation and FGF2 responsiveness are expected to decrease with decreasing levels of methionine. Because fatty acid synthesis is expected to be upregulated with decreasing methionine concentration, myogenic regulatory factor expression is expected to decrease at the same time that adipogenic regulatory factor expression is expected to increase. It is also expected that satellite cells will be less responsive to decreased methionine concentration as age increases.

The overall results from this research have the potential to have broad implications for both the poultry industry as well as the biomedical sciences. Understanding when age-related changes in satellite cells occur will allow for the development of management and handling practices that maximize the growth potential of the birds without compromising the functionality of satellite cells. A working knowledge of how the effect of nutrient restriction on satellite cell growth changes with age could lead to the development of feeding strategies and diets that maximize satellite cell growth by allowing for feed restriction at ages that are less critical for satellite cell function. This could result in increased profitability for poultry producers.
Biomedically, this study could aid in the understanding of how and when age related changes occur during growth, which could lead to treatments and prevention strategies for conditions such as sarcopenia, the age-related loss of muscle mass, as well as other muscle growth disorders and myopathies such as muscular dystrophy.


Song, Y., McFarland, D.C., and Velleman, S.G. (2012a). Fibroblast growth factor 2 and protein kinase C alpha are involved in syndecan-4 cytoplasmic domain modulation of


Table 1.1. The 28 known collagen types, supramolecular assembly, and main functions. Adapted from Ricard-Blum and Ruggiero (2004) and Brown and Timpl (1995).

<table>
<thead>
<tr>
<th>Collagen type</th>
<th>Supramolecular assembly</th>
<th>Major function</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Striated fibrils</td>
<td>Tensile strength</td>
</tr>
<tr>
<td>II</td>
<td>Striated fibrils</td>
<td>Tensile strength</td>
</tr>
<tr>
<td>III</td>
<td>Striated fibrils</td>
<td>Tensile strength</td>
</tr>
<tr>
<td>IV</td>
<td>Network</td>
<td>Basement membrane structural component</td>
</tr>
<tr>
<td>V</td>
<td>Core of striated fibrils</td>
<td>Tensile strength</td>
</tr>
<tr>
<td>VI</td>
<td>Beaded-filaments, microfibrils</td>
<td>Bridging between cells and ECM</td>
</tr>
<tr>
<td>VII</td>
<td>Anchoring fibrils</td>
<td>Strengthens dermal-epidermal junction</td>
</tr>
<tr>
<td>VIII</td>
<td>Hexagonal network</td>
<td>Unknown</td>
</tr>
<tr>
<td>IX</td>
<td>Surface of collagen II-containing fibrils</td>
<td>Attaches functional groups to fibrils</td>
</tr>
<tr>
<td>X</td>
<td>Hexagonal networks, fibril-associated</td>
<td>Calcium binding</td>
</tr>
<tr>
<td>XI</td>
<td>Core of collagen II-containing fibrils</td>
<td>Tensile strength</td>
</tr>
<tr>
<td>XII</td>
<td>Fibril-associated</td>
<td>Stress-induced wound repair</td>
</tr>
<tr>
<td>XIII</td>
<td>Type II membrane protein</td>
<td>Vasculogenesis and inflammation</td>
</tr>
<tr>
<td>XIV</td>
<td>Fibril-associated</td>
<td>Regulation of fibril growth in developing tendons</td>
</tr>
<tr>
<td>XV</td>
<td>Multiplexin, supramolecular assembly</td>
<td>Stabilization of skeletal muscle cells</td>
</tr>
<tr>
<td>XVI</td>
<td>Fibril-associated</td>
<td>Stabilization of fibroblasts</td>
</tr>
<tr>
<td>XVII</td>
<td>Type II membrane protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>XVIII</td>
<td>Multiplexin, supramolecular assembly</td>
<td>Structural integrity of basement membrane</td>
</tr>
<tr>
<td>XIX</td>
<td>Fibril-associated</td>
<td>Skeletal muscle differentiation</td>
</tr>
<tr>
<td>XX</td>
<td>Fibril-associated</td>
<td>Unknown</td>
</tr>
<tr>
<td>XXI</td>
<td>Fibril-associated</td>
<td>Formation of vascular matrix</td>
</tr>
<tr>
<td>XXII</td>
<td>Fibril-associated</td>
<td>Tissue junction marker</td>
</tr>
<tr>
<td>XXIII</td>
<td>Type II membrane protein</td>
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<td>XXIV</td>
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<td>XXV</td>
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</tr>
<tr>
<td>XXVII</td>
<td>Fibril-forming</td>
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</tr>
<tr>
<td>XXVIII</td>
<td>Supramolecular assembly unknown</td>
<td>Unknown</td>
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Table 1.2. Table of Proteoglycans. From Couchman and Pataki (2012)

<table>
<thead>
<tr>
<th>Proteoglycan</th>
<th>Core Protein Size, kDa</th>
<th>Type of GAG Chains</th>
<th>Human Chromosome Localization</th>
<th>Tissue Location</th>
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<tbody>
<tr>
<td>Glypicans</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Glypican 1</td>
<td>56</td>
<td>HS</td>
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<td>59</td>
<td>HS</td>
<td>Chromosome: 7 location: 7q22.1</td>
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<tr>
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<td>Chromosome: X location: Xq26.1</td>
<td>GPI-anchored cell surface</td>
</tr>
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<td>Chromosome: X location: Xq26.1</td>
<td>GPI-anchored cell surface</td>
</tr>
<tr>
<td>Glypican 5</td>
<td>59</td>
<td>HS</td>
<td>Chromosome: 13 location: 13q32</td>
<td>GPI-anchored cell surface</td>
</tr>
<tr>
<td>Glypican 6</td>
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<td>HS</td>
<td>Chromosome: 13 location: 13q32</td>
<td>GPI-anchored cell surface</td>
</tr>
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<td>Syndecans</td>
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<td></td>
<td></td>
</tr>
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<td>Syndecan-1</td>
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</tr>
<tr>
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<td>Chromosome: 8 location: 8q22-23</td>
<td>Transmembrane, extracellular</td>
</tr>
<tr>
<td>Syndecan-3</td>
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<td>Chromosome: 1 location: 1pter-p22.3</td>
<td>Transmembrane, extracellular</td>
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<tr>
<td>Syndecan-4</td>
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<td>HS</td>
<td>Chromosome: 20 location: 20q12</td>
<td>Transmembrane, extracellular</td>
</tr>
<tr>
<td>Lecticans</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggrecan</td>
<td>208–220</td>
<td>CS/KS</td>
<td>Chromosome: 15 location: 15q26.1</td>
<td>Extracellular</td>
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<tr>
<td>Versican (0/1/2/3 isoforms)</td>
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<td>Chromosome: 5 location: 5q14.3</td>
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<tr>
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<td>CS</td>
<td>Chromosome: 1 location: 1q31</td>
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<tr>
<td>SLRPcs</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decorin</td>
<td>36</td>
<td>CS/DS</td>
<td>Chromosome: 12 location: 12q21.33</td>
<td>Extracellular</td>
</tr>
<tr>
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<td>DS/CS</td>
<td>Chromosome: X location: Xq28</td>
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</tr>
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<td>42</td>
<td>KS</td>
<td>Chromosome: 1 location: 1q32</td>
<td>Extracellular, intracellular</td>
</tr>
<tr>
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<td>KS</td>
<td>Chromosome: 12 location: 12q21.3-q22</td>
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</tr>
<tr>
<td>Keratocan</td>
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<td>KS</td>
<td>Chromosome: 12 location: 12q22</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Mimecan</td>
<td>25</td>
<td>KS</td>
<td>Chromosome: 9 location: 9q22</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Others</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Thrombomodulin</td>
<td>58</td>
<td>CS</td>
<td>Chromosome: 20 location: 20p11.2</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>CD44 (19 isoforms)</td>
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<td>CS/DS</td>
<td>Chromosome: 11 location: 11p13</td>
<td>Transmembrane, extracellular, intracellular</td>
</tr>
<tr>
<td>NG2/CSPG4</td>
<td>251</td>
<td>CS</td>
<td>Chromosome: 15 location: 15q24.2</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Invariant chain</td>
<td>31</td>
<td>CS</td>
<td>Chromosome: 5 location: 5q32</td>
<td>Cell surface, intracellular</td>
</tr>
<tr>
<td>Neuroglycan-C</td>
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<td>CS</td>
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<td>Transmembrane</td>
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<tr>
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<td>Chromosome: 21 location: 21q22.3</td>
<td>Extracellular</td>
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<tr>
<td>Perlecian</td>
<td>400–450</td>
<td>HS</td>
<td>Chromosome: 2 location: 1p36.1-p34</td>
<td>Extracellular</td>
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<tr>
<td>Agrin</td>
<td>212</td>
<td>HS</td>
<td>Chromosome: 1 location: 1p36.33</td>
<td>Transmembrane, extracellular</td>
</tr>
<tr>
<td>Betaglycan</td>
<td>110</td>
<td>HS/CS</td>
<td>Chromosome: 1 location: 1p33-p32</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>SY2</td>
<td>20</td>
<td>KS</td>
<td>Chromosome: 1 location: 1q21.2</td>
<td>Transmembrane</td>
</tr>
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<td>Serglycin</td>
<td>10–19</td>
<td>HS/CS</td>
<td>Chromosome: 10 location: 10q22.1</td>
<td>Intracellular</td>
</tr>
<tr>
<td>Endocan</td>
<td>50</td>
<td>DS</td>
<td>Chromosome: 5 location: 5q11.2</td>
<td>Circulating extracellular</td>
</tr>
<tr>
<td>Neuruplin-I</td>
<td>130</td>
<td>HS/CS</td>
<td>Chromosome: 10 location: 10p12</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Type IX collagen</td>
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<td>CS</td>
<td>Chromosome: 6 location: 6q12-q14</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Testican 1</td>
<td>48</td>
<td>HS/CS</td>
<td>Chromosome: 5 location: 5q31.2</td>
<td>Extracellular</td>
</tr>
<tr>
<td>Testican 2</td>
<td>45</td>
<td>HS/CS</td>
<td>Chromosome: 10 location: 10pter-q25.3</td>
<td>Extracellular</td>
</tr>
</tbody>
</table>

CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; GPI, glycosylphosphatidylinositol; HS, heparan sulfate; KS, keratan sulfate; SLRP, small leucine-rich family of proteoglycans.
Figure 1.1. Skeletal muscle structure. A) Gross muscle structure. B). Sarcomere, or contractile unit of the muscle fiber. C). Close-up view of sarcomere.
Myosin-Actin interaction. Myosin heads attached to the actin filament bind to adenosine triphosphate (ATP), which causes a change in the conformation of the myosin head, causing it release the actin filament and to become “cocked” (1). The ATP is then hydrolyzed, with the resulting adenosine diphosphate (ADP) and inorganic phosphate (Pi) remaining tightly bound to the myosin head (2). The inorganic phosphate is released when the myosin head binds to the actin, which in turn allows the myosin head to bind more tightly to the actin filament (3). The release of Pi (3-4) causes a conformational change in the myosin head, ratcheting back to the structure it held previous to binding with ATP (5). This movement is referred to as the “power stroke”, and results in the movement of the actin filament against the myosin filament. The myosin head remains tightly bound to the actin filament until it binds to ATP and the cycle starts over (6). Adapted from Aronson and Krum (2012).
Figure 1.3. Glycolysis and the Krebs cycle. From Nissim et al. (2012).
Figure 1.4. Skeletal muscle development. A. Embryonic development – myogenic precursor cells from the somite migrate to form limb and trunk muscles. B. Posthatch or postnatal growth – satellite cells are located between the basement membrane and the sarcolemma, where they are capable of proliferating, differentiating, and donating their nuclei to existing myofibers for muscle growth or repair. From Partridge (2009).
Figure 1.5. Myogenic regulatory factor profile. Expression of myogenic regulatory factors in satellite cells. Age is thought to possibly impact satellite cell function at 1., 2., and 3. From Shefer et al. (2006).
**Figure 1.7. Fibronectin structure.** Fibronectin is a homodimer that contains several binding domains. The RGD binding domain is required for integrin binding to fibronectin. From Hardin et al. (1999).
Figure 1.8. Glycosaminoglycan structure. Hyaluronic acid is a polymer of N-acetylglucosamine and glucuronic acid with a 1-3 and 1-4 linkage. Chondroitin sulfate is composed of a 1-4 and 1-3 linkage of N-acetylglucosamine and glucuronic acid. Dermatan sulfate is composed of a 1-4 and 1-3 linkage of N-acetylglucosamine and iduronic acid. Keratan sulfate is a 1-3 and 1-4 linkage of N-acetyl glucosamine and galactose. Heparan sulfate is a 1-4 linkage of N-acetylglucosamine and glucuronic acid. From Yamada and Toyoda (2013).
Figure 1.9. Structure of syndecan-4. Syndecan-4 contains a core protein with a cytoplasmic, transmembrane, and extracellular domain. Attached to the core protein are three heparan sulfate chains and two N-glycosylation chains. Adapted from Velleman et al. (2012).
Glypican-1

Figure 1.10. Structure of glypican-1. Glypican-1 is a cell-surface proteoglycan linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. The extracellular domain has a globular cysteine-rich region and has three heparin sulfate and 3 N-glycosylation chains attached. Adapted from Velleman et al. (2012).
CHAPTER 2

CHANGES IN PROLIFERATION, DIFFERENTIATION, FIBROBLAST GROWTH FACTOR 2 RESPONSIVENESS AND EXPRESSION OF SYNDECAN-4 AND GLYPICAN-1 WITH TURKEY SATELLITE CELL AGE


I. Introduction

Satellite cells, or adult myoblasts, are mesodermal-derived multipotential stem cells required for muscle growth, maintenance, and repair. Located between the sarcolemma and the basement membrane (Mauro 1961), these cells contribute to the maintenance of muscle mass, muscle growth and muscle repair by proliferating, aligning, and fusing with existing myofibers (Moss and LeBlond 1970). Satellite cells predominantly exist in a quiescent state but during the growth and regeneration of skeletal muscle, the satellite cells are activated to proliferate and differentiate (Schultz 1978; Schultz and McCormick 1994). The ability of satellite cells to proliferate and differentiate is not static and is influenced by age (Velleman et al. 2010). Satellite cells are a heterogeneous population of cells within a muscle. This heterogeneity can result in variable activation, proliferation, differentiation, and responsiveness to the stimulatory or
inhibitory effects of growth factors. The functional significance of satellite cell heterogeneity with age is not well understood and how populations of satellite cells may change in terms of their expression of key macromolecules involved in the regulation of proliferation and differentiation is also unclear.

Satellite cell proliferation and differentiation has been shown to be differentially affected by the heparan sulfate proteoglycans syndecan-4 and glypican-1 (Brandan et al. 1996; Song et al. 2010, 2012a; Velleman et al. 2006, 2007, 2013; Zhang et al. 2007, 2008). Syndecan-4 is a transmembrane heparan sulfate proteoglycan with a central core protein containing a cytoplasmic domain, transmembrane domain, and extracellular domain with three heparan sulfate chains at Ser\(^{38}\), Ser\(^{65}\), and Ser\(^{67}\) (Zhang et al. 2008) and two N-linked glycosylation chains at Asn\(^{124}\) and Asn\(^{139}\) (Song et al. 2011) in turkeys (meleagris gallopavo). Glypican-1 is an extracellular heparan sulfate proteoglycan linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor that contains a globular cysteine-rich domain. In turkeys, attached to the core protein are three heparan sulfate chains at positions Ser\(^{483}\), Ser\(^{485}\), and Ser\(^{487}\) (Zhang et al. 2007) and three N-linked glycosylation chains at Asn\(^{76}\), Asn\(^{113}\), and Asn\(^{382}\) (Song et al. 2010).

Syndecan-4 can initiate intracellular signaling in response to the binding of fibroblast growth factor 2 (FGF2) through its cytoplasmic domain (Volk et al. 1999; Horowitz et al. 2002; Zhang et al. 2003), though in turkey myogenic satellite cells it may also function during proliferation in a FGF2-independent manner (Velleman et al. 2007). The cytoplasmic domain has three regions, one variable and two highly conserved regions (C1 and C2). The variable regions of the syndecan-4 cytoplasmic domain interact with each other to form a homodimer structure (Lee et al. 1998; Shin et al.
2012a) which is stabilized by the binding of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to the variable region. Syndecan-4 then forms oligomers activating protein kinase C alpha (PKC$\alpha$) through the binding of the catalytic domain of PKC$\alpha$ to the variable region of the syndecan-4 cytoplasmic domain (Oh et al. 1997; Horowitz et al. 1999; Couchman et al. 2002; Keum et al. 2004).

In skeletal muscle, Shin et al. (2012a) showed that satellite cells form syndecan-4 oligomers and the cytoplasmic domain is involved in both regulating PKC$\alpha$ activity and FGF2 responsiveness (Song et al. 2012a). Protein kinase C $\alpha$ is important in activating the Ras homologue gene family, member A (RhoA) pathway, which has been shown to cause the formation of stress fibers and focal adhesions (Ren et al. 1999; Rottner et al. 1999; Dovas et al. 2006; Shin et al. 2013). Song et al. (2012b) showed that the cytoplasmic domain of syndecan-4 modulates the formation of focal adhesions and stress fibers through focal adhesion kinase in satellite cells, and syndecan-4 interactions with $\alpha$-actinin are important in the formation of focal adhesions in fibroblasts (Okina et al. 2012) and syndecan-4 oligomers in muscle (Shin et al. 2012a). In satellite cells, syndecan-4 plays a primary role in satellite cell migration through RhoA signal transduction (Shin et al. 2013). The migration of satellite cells is crucial in the formation of multinucleated myotube formation and ultimately fiber formation, and may be affected with satellite cell age.

In contrast to syndecan-4 modulating FGF2 responsiveness and satellite cell migration during proliferation, glypican-1 plays a primary role in differentiation by sequestering FGF2 from its tyrosine kinase receptor. Fibroblast growth factor 2 is a potent inhibitor of differentiation (Dollenmeier et al. 1981). Glypican-1 can sequester
FGF2 by being shed into the extracellular matrix via the cleavage of its GPI anchor (Brandan et al. 1996; Velleman et al. 2013). The shed form of glypican-1 binds to FGF2, preventing FGF2 from binding with its receptor (Velleman et al. 2013). Glypican-1 can also sequester FGF2 from its tyrosine kinase receptor by the localization of glypican-1 in lipid rafts in the cell membrane. The FGF2 tyrosine kinase receptor is located in non-raft portions of the cell membrane and the lipid rafts containing the glypican-1-bound FGF2 sequesters FGF2 from its receptor (Gutiérrez and Brandan 2010). Satellite cells express the myogenic transcriptional regulatory factors in a precisely regulated manner and are required for muscle development. Proliferating satellite cells are positive for Pax7 and MyoD, while cells that are differentiating are Pax7+/MyoD+/myogenin+ or Pax7-/MyoD+/myogenin+ (Hasty et al. 1993; Rudnicki et al. 1993; Seale et al. 2000; Yablonka-Reuveni and Rivera 1994). Zammit et al. (2004) showed that cells that are Pax7+/MyoD+ can give rise to daughter cells that are Pax7+/MyoD-, indicating that not only are satellite cells heterogeneous but they are dynamic in their transcriptional signature. Glypican-1 and syndecan-4 have been shown to modulate the expression of the myogenic regulatory factors MyoD and myogenin (Shin et al. 2012b). Gutiérrez and Brandan (2010) showed that glypican-1 is required for terminal myogenesis, and glypican-1 deficient myoblasts express significantly reduced levels of myogenin preventing further differentiation.

Satellite cells are a heterogeneous population of cells and vary not only in their myogenic transcriptional regulatory factor signature, but also by the type of muscle they originate from (Zammit et al. 2002), their genetics (Liu et al. 2006), and their age (Collins et al. 2007; Velleman et al. 2010). Furthermore, the percentage of syndecan-4 or
glypican-1 positive satellite cells during proliferation and differentiation has been shown to be affected by selection for increased growth and by sex (Song et al. 2013).

Understanding how age affects satellite cell sub-populations and the expression of genes involved in proliferation and differentiation during muscle growth is important in maintaining satellite cell proliferation and differentiation which has been shown to decline prior to senescence (Velleman et al. 2010). The present study represents an initial step in determining age-related changes in satellite cell populations positive for syndecan-4 and glypican-1, the effect on the expression of syndecan-4, glypican-1, MyoD, and myogenin, and responsiveness to FGF2 during muscle growth.

II. Materials and methods

1. Satellite cells

Pectoralis major muscle satellite cells were isolated, expanded, and stored in liquid nitrogen from 1 d, 7 wk, and 16 wk posthatch Randombred Control Line 2 turkeys (RBC2) based on the method of Velleman et al. (2010) (1 d, 7 wk, and 16 wk cells, respectively). The RBC2 line is maintained at the Poultry Research Center of The Ohio Agricultural Research Development Center/The Ohio State University Wooster, OH without conscious selection for any trait and will thus not exhibit growth selection effects on satellite cell proliferation and differentiation of satellite cells as shown by Velleman et al. (2000). In the present study only male satellite cells were used to avoid any sex effects (Velleman et al. 2000).

2. Cell culture

Satellite cells from 1 d, 7 wk, and 16 wk posthatch male RBC2 turkeys were plated in 6-well, 24-well or 48-well cell culture plates (Greiner Bio-one, Monroe, NC,
USA) coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) at 36,000, 15,000, and 9,000 cells per well, respectively. Cells were grown in a 95% air/5% CO₂ incubator (Thermo Fisher Scientific, Pittsburgh, PA) at 37°C. Cells were cultured in plating medium composed of Dulbecco’s Modified Eagle’s Medium (DMEM: Sigma-Aldrich) with 10% chicken serum (Gemini BioProducts, West Sacramento, CA, USA), 5% horse serum (Gemini BioProducts), 1% antibiotic/antimycotic (Gemini BioProducts), and 0.1% gentamicin (Gemini BioProducts). After 24 h of attachment, the plating medium was changed to a feeding medium composed of McCoy’s 5A medium (Sigma-Aldrich) containing 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic, and 0.1% gentamicin. Medium was changed every 24 h for 96 h or until the cell cultures reached 60 to 65% confluency, when differentiation was induced by changing to a low-serum media composed of DMEM containing 3% horse serum, 1% antibiotic/antimycotic, 0.1% gentamicin, 0.1% gelatin (Sigma-Aldrich), and 1 mg/ml bovine serum albumin (BSA: Sigma-Aldrich). Media was changed every 24 h until 72 h of differentiation.

3. Proliferation assay

Every 24 h beginning at 24 h through 96 h of proliferation, 24-well cell culture plates with four wells per treatment were harvested, rinsed with phosphate buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, and 7.81 mM Na₂HPO₄, pH 7.08), air dried for 15 min and stored at -70°C until analysis. Proliferation was quantified by measuring the DNA concentration per well as described by McFarland et al. (1995). Cell culture plates were thawed for 10 to 15 min prior to analyses. Cells were then trypsinized using 200 µl of 0.05% trypsin (Invitrogen, Carlsbad, CA, USA) in 1x TNE buffer (10mM Tris, 1 mM EDTA, and 2 M NaCl) with gentle tapping for 7 min. Plates
were then stored at -70°C overnight, removed and allowed to thaw for 10 to 15 min, after which 1.8 ml of Hoechst Dye buffer (0.2 µg/ml Hoechst 33258 (Sigma-Aldrich) in 1x TNE) was added to each well. Plates were then wrapped in foil and incubated at room temperature (RT) with shaking for 1 to 2 h, and then optical density (OD) was measured using a Fluorskan Ascent FL scanner (Thermo Fisher Scientific, Waltham, MA, USA) with an excitation of 365 nm and an emission of 460 nm. A standard curve was generated using a range of 0.1 to 1.2 µg double stranded calf thymus DNA (Sigma-Aldrich) to determine sample DNA concentration. Proliferation assays were independently repeated four times.

4. Differentiation assay

Every 24 h beginning at 96 h of proliferation (0 h of differentiation) through 72 h of differentiation, 48-well cell culture plates with five wells per treatment were harvested, rinsed with PBS, and stored at -70°C until analysis. Differentiation was quantified by measuring creatine kinase concentration using a modified method of Yun et al. (1997). In brief, at the time of the differentiation assay, cell culture plates were thawed for 10 to 15 min, and then 500 µl of creatine kinase buffer [20 mM glucose (Thermo Fisher Scientific), 10 mM Mg acetate (Thermo Fisher Scientific), 1.0 mM adenosine diphosphate (Sigma-Aldrich), 10 mM adenosine monophosphate (Sigma-Aldrich), 20 mM phosphocreatine (Calbiochem, San Diego, CA, USA), 0.5 U/ml hexokinase (Worthington Biochemical, Lakewood, NJ, USA), 1 U/ml of glucose-6-phosphate dehydrogenase (Worthington Biochemical), 0.4 mM thio-nicotinamide adenine dinucleotide (Oriental Yeast Co., Tokyo, Japan), and 1 mg/ml BSA (Sigma-Aldrich)] was added to each well. Optical density was then measured on a BioTek ELx800 (BioTek,
Winooski, VT, USA) plate reader at an OD of 405nm. A standard curve was generated using 0 to 60 mU creatine phosphokinase (Sigma-Aldrich) to measure sample concentration. Experiments were repeated independently three times.

5. Fibroblast growth factor 2 responsiveness during proliferation

Cells were plated in 24-well cell culture plates as described above. Twenty four h following the attachment, the media was changed to a serum-free defined media containing 0 or 20 ng/ml of FGF2 (PeproTech, Rocky Hill, NJ, USA) and changed every 24 h. Every 24 h through 96 h of proliferation, FGF2 responsiveness was quantified by measuring DNA concentration as previously described in the proliferation assay. Experiments were repeated independently two times.

6. Fibroblast growth factor 2 responsiveness during differentiation

Cells were plated in 48-well cell culture plates as described above. At 96 h of proliferation, media was changed to a low-serum fusion media described above and either 0 or 20 ng/ml FGF2. Media was changed every 24 h and sampling times were every 24 h beginning at 96 h of proliferation (0 h of differentiation) through 72 h of differentiation. Differentiation was quantified by measuring the creatine kinase concentration as described for the differentiation assay. The assay was repeated independently two times.

7. Flow cytometry

Cells cultured in 6-well plates were removed from the incubator at 72 h of proliferation and 48 h of differentiation, rinsed twice with PBS, and scraped using a cell scraper (Corning, Tewksbury, MA, USA). Cells were then centrifuged at 600 × g for 10 min and resuspended in PBS plus 1% BSA at a concentration of 500 cells per µl. The polyclonal antibodies goat anti-human syndecan-4 (sc-33913, Santa Cruz
Biotechnologies, Santa Cruz, CA, USA) and goat anti-human glypican-1 (sc-14645, Santa Cruz Biotechnologies) were conjugated to phycoerythrin (PE) using a PhycoLink R-Phycoerythrin conjugation kit (PJ31K, Prozyme, Hayward, CA, USA) following the manufacturer’s instructions. Three replicates of the resuspended cells were incubated in a 1:250 dilution of antibody at RT in the dark with gentle shaking for 1.5 h. The cells were then spun for 10 min at 628 x g and the supernatant was decanted without disturbing the cell pellet, rinsed 2Xin PBS with centrifugation for 10 min at 628 x g with the supernatant removed. The cells were then resuspended and measured for syndecan-4 and glypican-1 positive satellite cell populations using a Guava EasyCyte Flow Cytometer (EMD Millipore, Billerica, MA, USA). This assay was repeated independently five times.

8. Western blotting

Protein was isolated by incubating 24-well cell culture plates with 6 wells per sample on ice with RIPA lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 150 mM NaCl, 1 mM EDTA, 2 mM Na$_3$VO$_4$, and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)). The cell lysates were centrifuged for 15 min at 10,000 x g at 4°C, and the supernatant was transferred to new tubes. Protein concentration was measured using the Bio-Rad protein determination assay (Bio-Rad, Hercules, CA, USA) with a BSA standard curve. Thirty µg of protein was combined with 5x loading buffer (0.125 M Tris-HCl, pH 6.8, 4.1% SDS, 20% glycerol, 2% β-mercaptoethanol, and 0.001% bromophenol blue), boiled for 5 min, and then loaded on a 12% sodium dodecyl sulfate polyacrylamide gel with a 5% stacking gel and run according to the method of Laemmli (1970). Protein was then
transferred from the gel to an activated polyvinylidenedifluoride membrane (EMD Millipore) and the membrane was incubated in a 5% non-fat dry milk in Tris-buffered saline-Tween 20 (TBST: 20 mM Tris base, 200 mM NaCl, 0.1% Tween-20, pH 7.4) blocking solution for 1 h, then incubated with primary antibody diluted in blocking solution overnight at 4°C with shaking. The membrane was then washed 3X with TBST for 5 min at RT with shaking and then incubated for 1 h with secondary antibody diluted in blocking solution at RT with shaking. The membrane was then washed 3X with TBST for 5 min at RT with shaking, and then developed with Lumi-Phos WB (Thermo Fisher Scientific) for 5 min at RT. Images were acquired using the Bio-Rad ChemiDoc XRS, with band densities analyzed using Quantity One 4.6.6 (Bio-Rad) and normalized against β-actin. Primary antibodies and dilutions were: rabbit anti-chicken MyoD (gift from Bruce Paterson, National Institute of Health, Bethesda, MD) 1:15,000; rabbit anti-chicken myogenin (gift from Bruce Paterson, National Institute of Health) 1:15,000; and mouse monoclonal anti-β-actin clone AC-15 (Sigma A5441) 1:10,000. Secondary antibodies and dilutions were: donkey anti-rabbit IgG (Santa Cruz, Sc-2315) 1:15,000 for MyoD and myogenin; and goat anti-mouse IgG (Santa Cruz, sc-2008) 1:10,000 for β-actin. Protein was isolated from three independent experiments and western blots were repeated at least twice for each protein.

9. Measurement of number of nuclei per myotube

Cells cultured in 24-well cell culture plates with two replicate wells per treatment were removed from the cell culture incubator through both proliferation and differentiation. The cell culture wells were rinsed 2X with PBS, and then fixed with 250 µl of 10% formalin at RT for 30 min with the wells rinsed 2X with PBS. The cell
cultures were then incubated with 250 µl of 2 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS for 30 min at RT. Following this incubation, cells were rinsed twice with PBS and photographs were taken using ImagePro software (Media Cybernetics, Rockville, MD, USA) using an Olympus IX70 fluorescent microscope (Olympus America, Center Valley, PA, USA). Both fluorescent and bright field digital images were taken using a QImaging Retiga EXi Fast 1394 digital camera (QImaging, Surrey, BC, Canada), and the average number of nuclei per myotube was determined by counting the nuclei in 20 myotubes per treatment per sampling time. This experiment was repeated twice.

10. RNA isolation, reverse transcription, and real-time quantitative polymerase chain reaction

Total RNA was isolated using RNAzol RT (Molecular Research Center, Inc, Cincinnati, OH, USA) following the manufacturer’s instructions. The RNA concentration was measured using a nanodrop spectrophotometer (Thermo Fisher Scientific) and cDNA was synthesized using moloney murine leukemia virus reverse transcriptase (MMLV; Promega, Madison, WI, USA). 0.8 µg of total RNA, 1 µl of 50 µM Oligo dT (Operon, Huntsville, AL, USA), and nuclease-free water to 13.5 µl total volume was incubated at 70°C for 5 min, then cooled. Eleven and one-half µl of reaction mixture containing (5 µl of 5x MMLV buffer, 1 µl of 10 mM deoxynucleoside triphosphate mix, 0.25 µl of 40 U/µl RNAsin, 1 µl of 200 U/µl MMLV reverse transcriptase, and nuclease-free water up to 11.5 µl) was added and then incubated at 55°C for 60 min and then 90°C for 10 min. Real-time quantitative PCR (RT-qPCR) was performed using DyNAmo Hot Start SYBR Green (Finnzymes, Ipswich, MA, USA)
according to manufacturer’s instructions. In brief, 2 µl of cDNA was added to 10 µl of 2x master mix, 1 µl of a 1:1 mix of 10 µM forward and reverse primers, and 7 µl of nuclease free water. The reaction was run using a DNA Engine Opticon 2 real-time machine (MJ Research, Waltham, MA, USA). Primers for syndecan-4, glypican-1, MyoD, myogenin, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) are listed in Table 2.1. Cycle threshold was constant for GAPDH. Amplification specificity was ensured by resolving randomly selected samples from all RT-qPCR reactions on a 1% agarose gel and all primers have been previously confirmed for specificity by DNA sequence confirmation of the amplified product. Standard curves were produced using serial dilutions of purified PCR products for each gene. Amount of sample cDNA for each treatment was determined by comparing the results to the appropriate standard curve, and then normalizing to GAPDH expression. Sample concentrations fell within the standard curves. The RNA was isolated from three separate experiments and RT-qPCR was done using three replicates per sample for each gene.

11. Statistical analysis

Proliferation assays, differentiation assays, nuclei number, flow cytometry, RT-qPCR, and western blots were all analyzed within time using the general linear model in SAS (SAS Institute Inc., Cary, NC, USA), with differences between means evaluated using a student’s t-test. The model accounted for the effect of age. Fibroblast growth factor 2 responsiveness assays for proliferation and differentiation were analyzed within time using the mixed model in SAS with the model including the fixed effects of age, FGF2, and their interactions. Values were considered statistically significant at $P<0.05$. 

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III. Results

1. Age effects on satellite cell proliferation and differentiation

Proliferation was significantly decreased in the pectoralis major satellite cells from the 16 wk old birds compared to 1 d or 7 wk old cells at all times during proliferation (Figure 2.1A). There was no significant difference in proliferation between the pectoralis major satellite cells from 1 d and 7 wk old muscle. These data indicate that pectoralis major muscle satellite cell proliferation significantly declined by 16 wk of age. The differentiation into multinucleated myotubes was also affected by age (Figure 2.1B). At 48 and 72 h of differentiation, the 7 wk cells had significantly lower levels of creatine kinase than the 1 d cells, though there was no significant difference at 0 or 24 h of differentiation. The 16 wk cells had significantly lower creatine kinase levels than the 1 d and the 7 wk cells at all times during differentiation. Differentiation was also significantly decreased in the 16 wk cells compared to the 1 d and 7 wk cells. In summary, both proliferation and differentiation was decreased by 16 wk posthatch, while differentiation was decreased in the 7 wk cells at 48 and 72 h of differentiation.

Proliferating cells of all age groups exhibited attachment and an elongated phenotype, as is expected during proliferation. Brightfield images of the 1 d, 7 wk and 16 wk old satellite cells at 72 h of proliferation are shown in Figure 2.2A through C. Myotube diameter decreased with age with the average myotube width being 20.8 ± 1.7 µm in 1 d cells, 18.3 ± 1.1 µm in 7 wk cells, and 14.3 ± 0.5 µm in 16 wk cells at 72 h of differentiation (Figure 2.2D through F). The number of nuclei per myotube was counted during differentiation (Table 2.2). The 16 wk cells had reduced nuclei number in the myotubes beginning at 48 h of differentiation compared to the 1 d and 7 wk old
myotubes. The 7 wk old number of nuclei per myotube was significantly reduced from that of the 1 d cells at only 72 h of differentiation. Decreased myotube width and decreased number of nuclei per myotube suggest that 16 wk cells have reduced differentiation compared to the 1 d or 7 wk cells.

2. Percentage of syndecan-4 and glypican-1 positive cells, and expression of syndecan-4 and glypican-1

The percentage of cells expressing syndecan-4 and glypican-1 during proliferation and differentiation was age-dependent (Figure 2.3). For syndecan-4, at 72 h of proliferation there was no significant difference between the 1 d, 7 wk, and 16 wk cells (Figure 2.3A). At 48 h of differentiation, the percentage of 16 wk syndecan-4 positive cells was significantly decreased compared to that of 1 d cells while there was no difference between the 7 wk and 1 d cells. The percentage of glypican-1 positive cells was also differentially expressed with age (Figure 2.3B). At 72 h of proliferation, there was no significant difference between 1 d, 7 wk, and 16 wk pectoralis major satellite cells. At 48 h of differentiation, there was a significant decrease in the number of cells positive for glypican-1 compared to the 1 d and 7 wk cells, while there was no difference between the 1 d and 7 wk cells. Thus, the percentage of satellite cells expressing syndecan-4 or glypican-1 decreased by 16 wk.

Expression of mRNA for syndecan-4 and glypican-1 in the 1 d, 7 wk, and 16 wk old cells was measured every 24 h beginning at 72 h of proliferation through 72 h of differentiation (Figure 2.4). The mRNA expression of syndecan-4 at 7 wk of age was decreased at all sampling times compared to the 1d and 16 wk old satellite cells. Interestingly, the 16 wk old satellite cells had higher syndecan-4 expression at 96 h of
proliferation and 72 h of proliferation than the 1 d old satellite cells (Figure 2.4A). In contrast to syndecan-4, glypican-1 mRNA expression was decreased in the 16 wk cells compared to the 1d and 7wk old satellite cells at 72 h of proliferation, and 24 h and 48 h of differentiation. At 72 h of differentiation, expression of glypican-1 in 1 d and 7 wk cells was significantly different; though there was no difference between either the 1 d or 7 wk cells and the 16 wk cells. Expression of glypican-1 was higher in 7 wk cells compared to the 1 d satellite cells at 24 and 48 h of differentiation, but at 72 h of differentiation the 1 d and 16 wk old satellite cells expressed more glypican-1 than 7 wk old satellite cells. Syndecan-4 expression was decreased in 7 wk versus 1 d cells and increased in 16 wk cells throughout proliferation and differentiation, while glypican-1 expression was increased in the 7 wk cells and decreased in the 16 wk cells compared to the 1 d cells at 24 and 48 h of differentiation.

3. Age effects on the expression of myogenic transcriptional regulatory factors

MyoD and myogenin

The mRNA expression of MyoD and myogenin was measured every 24h from 72 h of proliferation through 72 h of differentiation (Figure 2.5A and B). MyoD was expressed at low levels during proliferation and increased during differentiation, indicating that there are satellite cells that are proliferating as well as differentiating. Throughout differentiation, the 16 wk old satellite cells expressed the lowest levels of MyoD. Only at 48 h of differentiation did the 7 wk old satellite cells have higher MyoD expression than the 1 d satellite cells. At 72 and 96 h of proliferation and 24 h of differentiation, myogenin mRNA expression was significantly lower in 7 wk cells compared to the 1 d cells, and in the 16 wk cells compared to the 1 d or 7 wk cells
Figure 5B. Myogenin expression was decreased in the 16 wk cells at all times, and was similar between 1 d and 7 wk cells at 48 h of differentiation whereas the 1 d cells had lower expression than the 7 wk cells at 72 h of differentiation. These data suggest that the mRNA expression of MyoD and myogenin was differentially expressed with age and in general decreased in the 16 wk cells compared to the 1 d and 7 wk cells.

Western blot analysis of total protein lysates (Figure 2.5C) from 1 d, 7 wk, and 16 wk cells at 72 h of proliferation through 72 h of differentiation was done. The relative density of the protein bands after normalization to β-actin is shown in Figures 5D and E. MyoD and myogenin protein levels began to decrease at 48 h of differentiation with the 16 wk old cells showing the lowest relative levels. MyoD levels were higher in the 16 wk cells compared to the 1 d and 7 wk cells at 72 and 96 h of proliferation and 24 h of differentiation. Myogenin expression was similar between 1 d and 7 wk cells at 72 h of proliferation, while it was higher in the 1 d than 7 wk samples at 96 h of proliferation and 24 h of differentiation. The 16 wk old satellite cells had higher protein levels of myogenin than the other satellite cells at 72 and 96 h of proliferation but began to decline in myogenin levels at 24 h of differentiation. In summary, MyoD and myogenin protein expression in 16 wk cells was highest earlier than the 1 d or 7 wk cells and decreased by 48 and 72 h of differentiation.

4. Age effects on satellite cell responsiveness to fibroblast growth factor 2 during proliferation and differentiation.

The addition of exogenous FGF2 had no significant effect on proliferation at 24 or 48 h, though there was an effect of age on proliferation (Figure 2.6A). However, at 72 and 96 h of proliferation, there was a significant effect of FGF2 and an interaction
between the effect of age and FGF2. The 1 d and 7 wk satellite cells were more responsive to exogenous FGF2 at 72 h and 96 h of proliferation compared to the 16 wk cells. At 0 h of differentiation, prior to the addition of FGF2, there was an effect of age on differentiation (Figure 2.5B). At 24, 48, and 72 h of differentiation there was a significant effect of age and FGF2, as well as an interaction between age and FGF2. At 72 h of differentiation the 16 wk old cells were the most responsive to FGF2 with a 63% decrease in differentiation compared to 0.1 and 6.8% in the 1 d and 7 wk old satellite cells, respectively. In contrast, at 24 and 48 h of differentiation the 1 d and 7 wk satellite cells were more responsive to FGF2 inhibitory effects on differentiation. These data demonstrated that both proliferation and differentiation were differentially affected by FGF2 in 1 d, 7 wk and 16 wk old satellite cells.

**IV. Discussion**

Until recently myogenic satellite cells were considered to be a homogenous population of cells functioning in postnatal or posthatch muscle growth and muscle regeneration. Satellite cells are now known to be a heterogeneous population of multipotential stem cells. The satellite cells can vary in their expression of genes, proliferation and differentiation, the degree of maintaining a stem cell phenotype, and ability to transdifferentiate into cell lineages other than muscle. The satellite cell niche population also changes with animal age. Satellite cells are primarily active during the growth phase of animals, declining to less than 5% of the total myofiber nuclei and become largely quiescent (Hawke and Garry 2001). During the growth phase of animals, it is not well understood how satellite cell changes may influence the postnatal or posthatch growth of muscle.
The cell membrane-associated heparan sulfate proteoglycans, syndecan-4 and glypican-1 are differentially expressed during the proliferation and differentiation of satellite cells (Velleman et al. 2006, 2013; Zhang et al. 2007, 2008; Song et al. 2010, 2011, 2013; Shin et al. 2013). Song et al. (2013) showed by separating the syndecan-4 and glypican-1 satellite cell populations using flow cytometry that satellite cell subpopulations expressing syndecan-4 or glypican-1 were affected by stage of proliferation and differentiation, selection for increased body weight, and sex. Because syndecan-4 and glypican-1 play important roles in myogenic satellite cell proliferation, differentiation, and FGF2 signal transduction, the objective of the present study was to determine the effects of age during the growth phase of the pectoralis major muscle on the syndecan-4 and glypican-1 satellite cell subpopulations, proliferation and differentiation, FGF2 responsiveness, and the expression of the myogenic regulatory factors MyoD and myogenin.

Proliferation and differentiation were significantly decreased in the 16 wk old satellite cells at all sampling times. There was no significant difference between the 1 d and 7 wk old satellite cells during proliferation. Velleman et al. (2010) showed in a muscle development study from 1 d through 54 wk of age using isolated turkey primary pectoralis major satellite cells that proliferation was significantly decreased beginning at 8 wk of age. In the Velleman et al. (2010) study, primary satellite cells were isolated from 4 wk and 8 wk old birds. Thus, the results from the present study suggest that proliferation changes may begin between 7 and 8 wk of age. In the present study, differentiation was reduced in the 7 wk old satellite cells compared to the 1 d satellite cells. However, decreased levels of differentiation may occur as early as 4 wk of
posthatch age in the turkey (Velleman et al. 2010). These data demonstrate that there are significant changes in the ability of satellite cells to contribute to posthatch muscle growth well before the satellite cells become senescent. In turkeys, 16 wk of age is the approximate market age and the birds reach sexual maturity between 40 to 45 wk of age. Changes in satellite cell activity preceding senescence are not unique to the turkey and have been reported in other species (Barani et al. 2003; Li et al. 2011).

The percentage of satellite cells positive for syndecan-4 and glypican-1 decreased with age. These results represent the first demonstration of age-dependent changes in syndecan-4 and glypican-1 satellite cell populations. Changes in the size of syndecan-4 and glypican-1 satellite cell subpopulations will affect both the proliferation and differentiation of the satellite cells. Syndecan-4 is critical in the activation and proliferation of satellite cells (Cornelison et al. 2004), and their migration by mediating Rho A signal transduction (Shin et al. 2013). The migration of satellite cells is critical in the formation of multinucleated myotubes leading to subsequent muscle fiber formation. Glypican-1, on the other hand, affects the differentiation process through the sequestration of FGF2 from its tyrosine kinase receptor (Gutiérrez and Brandan 2010; Velleman et al. 2013). Fibroblast growth factor 2 is a strong inhibitor of differentiation and preventing FGF2 from binding to its receptor will permit differentiation to take place. Furthermore, myoblasts deficient in glypican-1 have decreased expression of the muscle transcriptional regulatory factor myogenin (Gutiérrez and Brandan 2010). Myogenin expression is necessary for further muscle cell differentiation (Brunetti and Goldfine 1990).
In addition to differences in the syndecan-4 and glypican-1 positive satellite cell populations with age, there were also age-associated changes in the mRNA and protein expression of the myogenic regulatory factors MyoD and myogenin. By 16 wk of age during differentiation, the expression of both MyoD and myogenin was decreased compared to the younger satellite cells at both the protein and mRNA levels. Protein levels of MyoD and myogenin showed that the 16 wk cells expressed more MyoD and myogenin earlier than 1 d or 7 wk cells, but levels then decreased. The 1 d and 7 wk cells had more sustained expression of MyoD and myogenin. The mRNA and protein expression were not always directly correlated. It is possible that the protein levels detected during proliferation were caused by earlier translation of MyoD and myogenin mRNA. Regardless of the difference between the protein and mRNA expression, these data further support that older satellite cells in the growing animal have a reduced capacity to proliferate and differentiate.

It is possible that a reduction in the expression of the heparan sulfate proteoglycans syndecan-4 and glypican-1 may be partially involved in the changes in MyoD and myogenin expression. Shin et al. (2012b) used small interfering RNA to knockdown the gene expression of syndecan-4 and glypican-1 to assay the effect on turkey pectoralis major satellite cell myogenic transcriptional regulatory expression. The knockdown of syndecan-4 gene expression increased MyoD expression during proliferation only, whereas glypican-1 decreased both MyoD and myogenin during both proliferation and differentiation. The change in the expression of these factors in combination with the altered expression of syndecan-4 and glypican-1 maybe correlated
with the decrease in nuclei number per myotube and reduced myotube diameter found in the present study with increased satellite cell age.

Both syndecan-4 and glypican-1 can bind to FGF2 at their heparan sulfate chains and function as regulators of FGF2 signal transduction (Volk et al. 1999; Gutiérrez and Brandan 2010; Velleman et al. 2013). Yablonka-Reuveni et al. (1999) found that FGF2 plays a critical role in the recruitment of satellite cells into proliferation. However, the proliferative capacity of older satellite cells can be restored by the addition of FGF2 (Shefer et al. 2006). In the present study, the response to FGF2 during both proliferation and differentiation was differentially affected by age. During proliferation, the 16 wk old satellite cells were the least responsive to FGF2. However, during differentiation the 1 d posthatch satellite cells responded the earliest to the inhibitory effects of FGF2 on differentiation followed by the 7 wk and then the 16 wk old satellite cells. Since glypican-1 is a regulator of FGF2 binding to its receptor during differentiation and the glypican-1 satellite cell population is decreased in 16 wk old cells during differentiation, it is possible that the decreased levels of glypican-1 permits more FGF2 to bind to its receptor further inhibiting differentiation in the older satellite cells.

In summary, the present study demonstrated that satellite cell age during the phase of muscle growth has a significant effect on satellite cell proliferation, differentiation, myogenic transcriptional regulatory factor expression, FGF2 responsiveness, and expression of syndecan-4 and glypican-1. As satellite cell age increases, proliferation and differentiation decline, as does the syndecan-4 and glypican-1 positive satellite cell populations. Responsiveness to FGF2 is also age-dependent, with younger cells being more responsive to FGF2 during proliferation and responding earlier
to FGF2 during differentiation. Because syndecan-4 and glypican-1 are key molecules in the regulation of the factors shown to be affected by age-related changes in satellite cells, further studies are needed to determine if the maintenance of syndecan-4 and glypican-1 satellite cell populations will sustain muscle cell proliferation and differentiation. Ultimately, these findings could lead to the development of treatments for muscle mass maintenance and repair with aging in humans.
V. References


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<td>GAPDH</td>
<td>5’-GAG GGT AGT GAA GGC TGC TG-3’ (forward) 5’-CCA CAA CAC GGT TGC TGT AT-3’ (reverse)</td>
<td>504-523</td>
<td>200 bp</td>
</tr>
</tbody>
</table>

¹ Primers were designed from the following GenBank accession numbers: Glypican-1, AY551002.2; Syndecan-4, AY852251.1; MyoD, AY641567.1; Myogenin, AY560111.3; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), U94327.1
Table 2.2. Average number of nuclei per myotube

<table>
<thead>
<tr>
<th>Age</th>
<th>1 d</th>
<th>7 wk</th>
<th>16 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h D</td>
<td>1.75 ±0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.45 ±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25 ±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 h D</td>
<td>1.80 ±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.80 ±0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.60 ±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>48 h D</td>
<td>7.75 ±1.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.80 ±1.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.75 ±0.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>72 h D</td>
<td>19.30 ±3.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.55 ±1.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.90 ±0.25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-c</sup> Data was analyzed within time, and averages without common letters represent values that are statistically different at P<0.05. Average values plus or minus the standard error of the mean are shown. D = differentiation.
Figure 2.1. Effect of age on satellite cell proliferation and differentiation.
Proliferation (A) and differentiation (B) of satellite cells from 1 d, 7 wk, and 16 wk pectoralis major muscle from posthatch randombred control line 2 male turkeys.
Average values were graphed with bars representing the standard error of the mean.
Means without common letters represent values that are statistically different at P<0.05.
D = differentiation, P = proliferation.
Figure 2.2. Photographic plate of the effect of age on satellite cell proliferation and differentiation. Effect of age on proliferation and differentiation of satellite cells from 1 d, 7 wk, and 16 wk pectoralis major muscle from posthatch randombred control line 2 (RBC2) male turkeys. Images A through C were taken at 72 h of proliferation, and images D through F were taken at 72 h of differentiation. Images A and D are of cells isolated from 1 d posthatch RBC2 turkeys; images B and E are 7 wk RBC2 posthatch satellite cells; and images C and F are 16 wk RBC2 posthatch satellite cells. The arrows highlight myotubes formed during differentiation. The scale bar represents 100 µm. D = differentiation, P = proliferation.
Figure 2.3. Effect of age on satellite cell populations expressing syndecan-4 and glypican-1. Effect of age on (A) syndecan-4 and (B) glypican-1 pectoralis major satellite cell populations as measured by flow cytometry during proliferation (P) and differentiation (D). Satellite cells were isolated from 1 d, 7 wk, and 16 wk posthatch randombred control line 2 male turkeys. Average values were graphed with bars representing the standard error of the mean. Means without common letters represent values that are statistically different at P<0.05.
Figure 2.4. **Effect of age on syndecan-4 and glypican-1 mRNA expression.** The mRNA expression of (A) syndecan-4 and (B) glypican-1 during proliferation (P) and differentiation (D). Pectoralis major satellite cells were isolated from 1 d, 7 wk, and 16 wk posthatch randombred control line 2 male turkeys. Average values were graphed with bars representing the standard error of the mean. Means without common letters represent values that are statistically different at P<0.05.
Figure 2.5. Effect of age on MyoD and myogenin mRNA and protein expression. mRNA and protein expression of the myogenic regulatory transcription factors MyoD and myogenin during proliferation (P) and differentiation (D) of 1 d, 7 wk, and 16 wk posthatch pectoralis major satellite cells isolated from randombred control line 2 (RBC2) turkeys. (A) and (B) show mRNA expression of MyoD and myogenin, respectively. Average values were graphed with bars representing the standard error of the mean. Bars without common letters are significantly different at P<0.05. C) Western blot analysis of MyoD, myogenin, and β-actin during proliferation and differentiation of 1 d, 7 wk, and 16 wk posthatch pectoralis major satellite cells isolated RBC2. (D) and (E) represent relative densitometric values for each protein band obtained in the Western analysis for MyoD (D) and myogenin (E). Relative density values were normalized to β-actin.
Figure 2.6. Effect of fibroblast growth factor 2 and age on satellite cell proliferation and differentiation. Fibroblast growth factor 2 (FGF2) responsiveness during (A) proliferation and (B) differentiation of pectoralis major satellite cells isolated from 1 d, 7 wk, and 16 wk posthatch randombred control line 2 male turkeys. Average values were graphed with bars representing the standard error of the mean. Data was analyzed within time to evaluate age, FGF2 treatment, and the interaction between age and FGF2 treatment. A = age, F = FGF2 treatment; AF = age x FGF2 treatment. Values were significantly different at P<0.05. D = differentiation, P = proliferation.
CHAPTER 3

THE EFFECT OF SYNGECA-4 AND GLYPCAN-1 ON AGE-RELATED CHANGES IN MYOGENIC SATELLITE CELL PROLIFERATION, DIFFERENTIATION, AND FIBROBLAST GROWTH FACTOR 2 RESPONSIVENESS

I. Introduction

Adult myoblasts, also called satellite cells, are a type of multipotential stem cell that are required for muscle growth, maintenance, and repair. Satellite cells are a mesodermal-derived stem cell located between the muscle basement membrane and the sarcolemma (Mauro, 1961). Though satellite cells are typically quiescent, during muscle growth and repair they are activated, causing them to proliferate and differentiate (Schultz, 1978; Schultz and McCormick, 1994). Satellite cells contribute to muscle growth, maintenance, and repair by donating their nuclei to existing myofibers, increasing protein synthesis levels and resulting in muscle growth through muscle fiber hypertrophy (Moss and LeBlond, 1970). While these satellite cells are involved in muscle hypertrophy, it is important to note that satellite cells are a heterogeneous population of cells. Satellite cells have been shown to vary in their expression of myogenic regulatory factors (Kuang et al., 2007; Li et al., 2011), proliferative capacity (Maier et al., 2012), expression of cell surface molecules (Song et al., 2013; Chapman et al., 2013; Harthan et al., 2013) and cytoskeletal molecules (Baroffio et al., 1995). It is
not well understood how satellite cell heterogeneity affects their function and ability to proliferate and differentiate.

Satellite cells have also been shown to change with age (Collins et al., 2007; Velleman et al. 2010; Li et al., 2011). In turkeys, satellite cell proliferation was shown to be significantly decreased by 8 wk of age and differentiation was significantly decreased by 4 wk of age (Velleman et al., 2010). Expression of turkey pectoralis major muscle heparan sulfate proteoglycans syndecan-4 and glypican-1 have been shown to decline in satellite cells isolated from 16 wk old poults compared to those isolated from 1 d old poults (Harthan et al., 2013), and mRNA expression of syndecan-4 and glypican-1 also decreased in turkey pectoralis major muscle from Randombred control line 2 (RBC2) (Sporer et al., 2011). These observations indicate that changes in satellite cell populations occur relatively early during growth, as a 16 wk old turkey is still actively growing. The functional significance of the decline in syndecan-4 and glypican-1 expression is not well understood, though it may be correlated with the age-related decline in satellite cell proliferation and differentiation. Syndecan-4 and glypican-1 have been shown to differentially regulate satellite cell proliferation and differentiation (Brandan et al., 1996; Song et al., 2010, 2012a; Velleman et al., 2006, 2007, 2013; Zhang et al., 2007, 2008).

Syndecan-4 is required for the activation and proliferation of satellite cells (Cornelison et al., 2004). A transmembrane heparan sulfate proteoglycan, syndecan-4 contains a central core protein with an extracellular domain, transmembrane domain and cytoplasmic domain that contains one variable region and two conserved regions (C1 and C2). In turkeys, attached to the extracellular domain are three heparan sulfate chains at
Ser$^{38}$, Ser$^{65}$, and Ser$^{67}$ (Zhang et al., 2008) and two N-linked glycosylation chains attached at Asn$^{124}$ and Asn$^{139}$ (Song et al., 2011). Upon binding to fibroblast growth factor 2 (FGF2), syndecan-4 initiates intracellular signaling through its cytoplasmic domain (Volk et al., 1999; Horowitz et al., 2002; Zhang et al., 2003). This causes the variable regions of the cytoplasmic domain to interact with each other to form homodimers (Lee et al., 1998; Shin et al., 2012a). The homodimer structures are stabilized by phosphatidylinositol 4,5-bisphosphate (PIP$_2$) binding to the variable regions. The syndecan-4 homodimers then form oligomers, which results in the activation of protein kinase C alpha (PKC$\alpha$) by the binding of the variable region of the syndecan-4 cytoplasmic domain to the catalytic domain of PKC$\alpha$ (Oh et al., 1997, Horowitz et al., 1999; Couchman et al., 2002; Keum et al., 2004; Shin et al., 2012a; Song et al., 2012a). Shin et al. (2012a) showed that syndecan-4 oligomers form in turkey satellite cells, while Song et al. (2012a) demonstrated that the cytoplasmic domain functions in regulating PKC$\alpha$ activity and FGF2 responsiveness.

Protein kinase C $\alpha$ is involved in the activation of the Ras homolog gene family member A (RhoA) pathway, which induces the formation of stress fibers and focal adhesions (Ren et al., 1999; Rottner et al., 1999; Dovas et al., 2006; Shin et al., 2013). In turkey satellite cells, focal adhesion and stress fiber formation are modulated by the cytoplasmic domain of syndecan-4 by focal adhesion kinase (Song et al., 2012b). The interaction of syndecan-4 with $\alpha$-actinin is important in the formation of focal adhesions in fibroblasts (Okina et al., 2012), as well as syndecan-4 oligomers in muscle (Shin et al., 2012a). Focal adhesion and stress fiber formation are crucial in migration, and satellite cell migration is critical in the formation of multinucleated myotubes and myofibers.
Syndecan-4 has been shown to affect migration through RhoA signal transduction (Shin et al., 2013). Satellite cell migration is important not only in myotube and myofiber formation, but also in muscle regeneration (Schultz et al., 1988). Age-related declines in the ability of satellite cells to migrate could result in decreased muscle repair and regeneration and potentially result in sarcopenia. Also critical in muscle repair and regeneration is the ability of satellite cells to differentiate to form myotubes.

Glypican-1 plays a primary role in satellite cell differentiation by sequestering FGF2, a potent inhibitor of differentiation (Dollenmeier et al., 1981) from its tyrosine kinase receptor. Glypican-1 is an extracellular heparan sulfate proteoglycan that is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. The central core protein of glypican-1 contains a globular cysteine-rich domain and, in turkeys, has three heparan sulfate chains attached at Ser\(^{483}\), Ser\(^{485}\), and Ser\(^{487}\) (Zhang et al., 2007) and three N-linked glycosylation chains attached at Asn\(^{76}\), Asn\(^{113}\), and Asn\(^{382}\) (Song et al., 2010). Two mechanisms of glypican-1-mediated FGF2 sequestration are known. Glypican-1 can be shed from the cell membrane via the cleavage of the GPI anchor (Brandan et al., 1996; Velleman et al., 2013). This shed form of glypican-1 binds to FGF2 and prevents it from binding to its tyrosine kinase receptor (Velleman et al., 2013). Glypican-1 is also located in lipid raft portions of the cell membrane, preventing FGF2 from binding to its tyrosine kinase receptors located in non-raft portions of the cell membrane (Gutiérrez and Brandan, 2010). Cells that are deficient in glypican-1 do not undergo terminal myogenesis (Gutiérrez and Brandan, 2010).

Glypican-1 and syndecan-4 have also been shown to affect the expression of myogenic regulatory factors MyoD and myogenin (Shin et al., 2012b). Proliferative
satellite cells are positive for both Pax7 and MyoD, while differentiating satellite cells are positive for Pax7, MyoD, and myogenin or MyoD and myogenin (Hasty et al., 1993; Rudnicki et al, 1993; Seale et al., 2000; Yablonka-Reuveni and Rivera, 1994). Myogenin expression is required for muscle cell differentiation (Brunetti and Goldfine, 1990). Glypican-1 deficient myoblasts express significantly reduced levels of myogenin, preventing differentiation (Gutiérrez and Brandan, 2010). Shin et al. (2012b) reported that MyoD and myogenin expression decreased when glypican-1 expression was knocked down, while MyoD expression increased during proliferation when syndecan-4 expression was knocked down. Thus, changes in the percentage of satellite cells expressing syndecan-4 and glypican-1 may affect satellite cell proliferation and differentiation with age.

The role of syndecan-4 and glypican-1 in the age-related changes in the ability of satellite cells to proliferate and differentiate is supported by the critical roles these molecules have been shown to play in proliferation, differentiation, and responsiveness to FGF2. Syndecan-4 is important in satellite cell activation (Cornelison et al., 2004), proliferation (Velleman et al., 2007), and migration (Shin et al., 2013), while glypican-1 is important for differentiation (Brandan et al., 1996; Gutiérrez and Brandan, 2010; Velleman et al., 2013). Fibroblast growth factor 2 is important in the activation of satellite cells (Bischoff, 1986; Yablonka-Reuveni et al., 1999) and also stimulates proliferation and inhibits differentiation (Dollenmeier et al., 1981). Declining expression of these proteoglycans has been hypothesized to, in part, be associated with changes in proliferation and differentiation. The objective of the present study was to determine the effect of syndecan-4 and glypican-1 expression on satellite cell proliferation,
differentiation and FGF2 responsiveness during growth of the turkey pectoralis major muscle.

II. Materials and methods

1. Satellite cells

   Satellite cells were isolated from the pectoralis major muscle of 1 d, 7 wk and 16 wk posthatch RBC2 turkeys (1 d, 7 wk, and 16 wk cells, respectively) based on the method of Velleman et al. (2010). Isolated satellite cells were expanded and stored in liquid nitrogen. The RBC2 line is a non-growth selected line representative of a 1966 turkey (Nestor et al., 1969) and is maintained at The Ohio State University’s Ohio Agricultural Research and Development Center Poultry Research Center in Wooster, OH with no conscious selection for any trait. Therefore, satellite cells isolated from this line do not exhibit any growth selection effects on satellite cell proliferation and differentiation (Velleman et al., 2000). To avoid the effect of sex, only male satellite cells were used in the current study (Velleman et al., 2000; Song et al., 2013).

2. Cell culture and transfection

   Satellite cells isolated from 1 d, 7 wk, and 16 wk posthatch male RBC2 turkeys were plated in 0.1% gelatin-coated (Sigma-Aldrich, St. Louis, MO, USA) 24 well or 48 well cell culture plates (Greiner Bio-one, Monroe, NC, USA). Cells were plated at 15,000 and 9,000 cells per well, respectively, and were grown at 37°C in a 95% air/5% CO₂ incubator (Thermo Fisher Scientific, Pittsburg, PA, USA). Cells were plated in a plating medium composed of Dulbecco’s Modified Eagle’s Medium (DMEM: Sigma-Aldrich) containing 10% chicken serum (Gemini Bio-Products, West Sacramento, CA, USA) and 5% horse serum (Gemini BioProducts). The cells were allowed a 24 h
attachment period, after which they were transiently transfected with empty pCMS-EGFP vector (CON), syndecan-4, glypican-1 or both syndecan-4 and glypican-1. For the transfection, 0.6 or 0.3 µg of CON, syndecan-4, glypican-1 or syndecan-4 with glypican-1 were used with 1 or 0.5 µl of Optifect (Invitrogen, Carlsbad, CA, USA) for the 24 and 48 well plates, respectively, according to the manufacturer’s instructions. After 5 h of transfection, the plating medium was removed and replaced with a feeding medium composed of McCoy’s 5A medium (Sigma-Aldrich) with 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic (Gemini BioProducts) and 0.1% gentamicin (Gemini BioProducts). Medium was changed every 24 h until the cells reached 60 to 65% confluency at approximately 72 h post transfection (PT). Differentiation was then induced by changing the medium to a low-serum medium composed of DMEM containing 3% horse serum, 1% antibiotic/antimycotic, 0.1% gentamicin, 0.1% gelatin (Sigma-Aldrich), and 1 mg/ml bovine serum albumin (BSA: Sigma-Aldrich). Thereafter, medium was changed every 24 h until 72 h of differentiation. Transfection efficiency was visually estimated by green fluorescent protein expression from the PCMS-EGFP vector, and was at or above 50%.

3. Proliferation Assay

Every 24 h beginning at 0 h through 72 h PT, cell culture plates with four wells per treatment were harvested, rinsed with phosphate buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, and 7.81 mM Na₂HPO₄, pH 7.08), air dried for 15 min and stored at -70°C until analysis. The DNA concentration per well was measured by the method of McFarland et al. (1995) as a means of quantifying proliferation. In brief, cell culture plates were removed from the freezer and allowed to thaw for 10 to 15 min before
the addition of 200 µl of 0.05% trypsin (Invitrogen) in 1x TNE buffer (10mM Tris, 1 mM EDTA, and 2 M NaCl, pH 7.4) per well. Plates were incubated at room temperature (RT) for 7 min with gentle tapping, then stored at -70°C overnight. Cell culture plates were then removed from the freezer and thawed for 10 to 15 min before the addition of 1.8 ml of Hoechst Dye buffer (0.2 µg/ml Hoechst 33258 (Sigma-Aldrich) in 1x TNE) per well. Plates were then wrapped in foil and incubated with shaking at RT for 1 to 2 h. After incubation, optical density (OD) was measured using a Fluorskan Ascent FL scanner (Thermo Fisher Scientific) with an excitation of 365 nm and an emission of 460 nm. Sample DNA concentration was measured using a standard curve generated using a range of 0.1 to 1.2 µg double stranded calf thymus DNA (Sigma-Aldrich). Proliferation assays were repeated eight times.

4. Differentiation assay

Cell culture plates with five wells per treatment were harvested every 24 h beginning at 72 h PT through 72 h of differentiation. Plates were rinsed with PBS and stored at -70°C until analysis. Differentiation was quantified by measuring creatine kinase concentration using a modified method of Yun et al. (1997). In brief, cell culture plates were thawed for 10 to 15 min, then 500 µl of creatine kinase buffer [20 mM glucose (Thermo Fisher Scientific), 10 mM Mg acetate (Thermo Fisher Scientific), 1.0 mM adenosine diphosphate (Sigma-Aldrich), 10 mM adenosine monophosphate (Sigma-Aldrich), 20 mM phosphocreatine (Calbiochem, San Diego, CA, USA), 0.5 U/ml hexokinase (Worthington Biochemical, Lakewood, NJ, USA), 1 U/ml of glucose-6-phosphate dehydrogenase (Worthington Biochemical), 0.4 mM thio-nicotinamide adenine dinucleotide (Oriental Yeast Co., Tokyo, Japan), and 1 mg/ml BSA (Sigma-
were added to each well. A BioTek ELx800 (BioTek, Winooski, VT, USA) plate reader was then used to measure OD at 405 nm. Sample concentration was measured using a standard curve generated with 0 to 60 mU creatine phosphokinase (Sigma-Aldrich). Experiments were repeated five times.

5. Fibroblast growth factor 2 responsiveness during proliferation

Cells were plated as previously described in 24 well cell culture plates. After the 24 h attachment period, in lieu of feeding medium, medium was changed to a serum-free defined medium (McFarland et al., 2006) containing either 0 or 20 ng/ml of FGF2 (PeproTech, Rocky Hill, NJ, USA). Medium was changed every 24 h until 72 h PT, with sampling times at 24 and 72 h PT. Fibroblast growth factor 2 responsiveness was quantified by measuring DNA concentration as previously described. Experiments were independently repeated six times.

6. Fibroblast growth factor 2 responsiveness during differentiation

Cells were plated as previously described in 48 well cell culture plates. At 72 h PT, medium was changed to a low-serum fusion medium described above with either 0 or 20 ng/ml of FGF2 added. Medium was changed every 24 h with sampling times at 0 h and 48 h of differentiation. Creatine kinase concentration was measured as previously described above to quantify differentiation. The assay was independently repeated four times.

7. Flow cytometry

Cells were cultured in 24 well plates and removed from the 95% air/5% CO₂ incubator at either 72 h PT or 48 h of differentiation. Wells were rinsed twice with PBS and scraped using a cell scraper (Corning, Tewksbury, MA, USA). The cells were then
centrifuged at 628 × g for 10 min and resuspended in PBS plus 1% BSA at a concentration of approximately 500 cells per µl. A PhycoLink R-Phycoerythrin conjugation kit (PJ31K, Prozyme, Hayward, CA, USA) was used to conjugate polyclonal goat anti-human syndecan-4 (sc-33913, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and goat anti-human glypican-1 (sc-14645, Santa Cruz Biotechnologies) to phycoerythrin following the manufacturer’s instructions. Three replicate samples of the resuspended cells were incubated in a 1:250 dilution of antibody for 1.5 h at RT in the dark with gentle shaking. Following the incubation, cells were spun for 10 min at 628 × g, and then the supernatant was decanted without disturbing the cell pellet. Cells were then rinsed 2X with PBS with centrifugation at 628 × g for 10 min between supernatant removal. Cells were then resuspended and the percentage of cells expressing syndecan-4 or glypican-1 was measured using a Guava EasyCyte Flow Cytometer (EMD Millipore, Billerica, MA, USA). The assay was repeated three times.

8. RNA isolation and real-time quantitative PCR

Total RNA was isolated from cells grown in 24-well cell culture plates at 72 h post-transfection and 48 h of differentiation using RNAzol RT (Molecular Research Center, Inc, Cincinnati, OH, USA) following the manufacturer’s instructions. The concentration of the isolated RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Moloney murine leukemia virus reverse transcriptase (MMLV: Promega, Madison, WI, USA) was used to synthesize cDNA. In brief, 0.8 µg of total RNA, 1 µl of 50 µM Oligo dT (Operon, Huntsville, AL, USA), and nuclease-free water to 13.5 µl total volume were incubated at 70°C for 5 min, then cooled. A reaction mixture volume of 11.5 µl containing 5 µl of 5x MMLV buffer, 1 µl of 10 mM
deoxynucleoside triphosphate mix, 0.25 µl of 40 U/µl RNAsin, 1 µl of 200 U/µl MMLV reverse transcriptase, and nuclease-free water up to 11.5 µl was added to each tube. The sample was then incubated at 55°C for 60 min and then 90°C for 10 min. Real-time quantitative PCR (RT-qPCR) was performed according to manufacturer’s instruction using DyNAmo Hot Start SYBR Green (Finnzymes, Ipswich, MA, USA). Each reaction contained 2 µl of cDNA, 10 µl of 2x master mix, 1 µl of a 1:1 mix of 10 µM forward and reverse primers, and 7 µl of nuclease free water. Reactions were run using a RNA Engine Opticon 2 real-time machine (MJ Research, Waltham, MA, USA). Primers are listed in Table 1 for syndecan-4, glypican-1, MyoD, myogenin, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Amplification specificity was confirmed by resolving randomly selected samples from all RT-qPCR reactions on 1% agarose gel. Primer specificities have been previously confirmed by DNA sequencing. Standard curves were produced using serial dilutions of purified PCR products for each gene. Sample concentrations were determined by comparing results to the appropriate standard curve. Data were then normalized to average GAPDH expression, the cycle threshold of which remained constant throughout. The RNA was isolated from at least three separate experiments and RT-qPCR was performed using three replicates per sample for each gene.

9. Statistical analysis

Overexpression of syndecan-4 and glypican-1, and RT-qPCR were analyzed within time and within age using the general linear model in SAS (SAS Institute Inc., Cary, NC, USA), with a student’s t-test used to evaluate differences between means. The model accounted for the effect of transfection. Proliferation and differentiation assays
were analyzed within time using the mixed model in SAS. The model included the fixed effects of age, transfection, and their interactions. Fibroblast growth factor 2 responsiveness assays for proliferation and differentiation, as well as RT-qPCR during FGF2 responsiveness, were also analyzed within time using the mixed model in SAS with the model including the fixed effects of age, FGF2, transfection, and their interactions. Values were considered statistically significant at $P<0.05$.

III. Results

1. Effect of overexpression of syndecan-4, glypican-1 and syndecan-4 with glypican-1 on satellite cell proliferation and differentiation

Syndecan-4 and glypican-1 mRNA overexpression was confirmed by RT-qPCR (Figure 1). Cells that were transfected with syndecan-4 expressed syndecan-4 mRNA at significantly higher levels than those transfected with the CON vector (Figure 1A, 1B), while those that were transfected with glypican-1 had significantly increased glypican-1 mRNA expression (Figure 1C, 1D). Overexpression of syndecan-4 with glypican-1 at the protein level was confirmed by flow cytometry (Figure 2). In transfected populations, syndecan-4 with glypican-1 expression was increased at least 30% at the protein level during both proliferation (Figure 2A, 2B) and differentiation (Figure 2C, 2D).

There was no effect of transfection with either syndecan-4 (Figure 3A), glypican-1 (Figure 3B), or both syndecan-4 with glypican-1 (Figure 3C) on satellite cell proliferation at 0 h PT or 48 h PT. There was an effect of transfection with syndecan-4 (Figure 3A) at 24 h PT and at 72 h PT, and an interaction between age and transfection at 72 h PT. There was no significant effect of transfection with syndecan-4 in the 16 wk
cells at any time during proliferation. Transfection with syndecan-4 significantly decreased proliferation in 1 d cells at 24 h PT and 72 h PT and in 7 wk cells at 24, 48, and 72 h PT. There was no significant effect of transfection with syndecan-4 on 1 d cells at 0 or 48 h PT, or on 7 wk cells at 0 h PT. In the cells transfected with glypican-1 (Figure 3B), there was an effect of transfection at both 24 h PT and 72 h PT, as well as an interaction between age and transfection. Transfection with glypican-1 had no significant effect at 0 h PT or 48 h PT in any of the age groups, while there was a significant effect of glypican-1 transfection at 24 h PT in the 7 wk cells, but not in the 1 d or 16 wk cells. At 72 h PT, transfection with glypican-1 resulted in a decrease in proliferation in the 1 d and 7 wk cells, but had no significant effect on proliferation in the 16 wk cells. There was an effect of transfection on the satellite cells transfected with both syndecan-4 with glypican-1 (Figure 3C) at 24 h PT, and an interaction between transfection and age at 72 h PT. Transfection with both syndecan-4 with glypican-1 significantly decreased proliferation in the 1 d and 7 wk cells at 72 h PT, but increased proliferation in the 16 wk cells. There was an effect of age on all transfections at all sampling times. These data suggest that transfection with syndecan-4 with glypican-1 has no effect on satellite cell proliferation in the 16 wk cells at 72 h PT, while decreasing proliferation in the 1 d and 7 wk cells at 72 h PT.

During differentiation, there was an effect of age at all times in the cells transfected with syndecan-4 compared to CON. At 24 h of differentiation, there was also an effect of transfection and an interaction of transfection by age (Figure 4A). Transfection with syndecan-4 had no effect on the 1 d or 7 wk cells, but resulted in an increase in differentiation in the 16 wk cells at 72 h of differentiation. In the cells
transfected with glypican-1, there was also an age effect at all times, as well as an interaction of age by transfection at 24 h of differentiation (Figure 4B). Transfection with glypican-1 did not have a significant effect on differentiation in any of the cell ages at any time during differentiation. The cells that were transfected with syndecan-4 with glypican-1 had an effect of age at all times and an interaction of age by transfection at 24 h of differentiation (Figure 4C). At 72 h of differentiation, differentiation was decreased in the 1 d and 7 wk cells transfected with syndecan-4 with glypican-1 compared to those transfected with CON. The 16 wk cells transfected with both syndecan-4 with glypican-1 had increased differentiation compared to those transfected with CON.

2. Effect of overexpression of syndecan-4, glypican-1, and syndecan-4 with glypican-1 on fibroblast growth factor 2 responsiveness

In satellite cells overexpressing syndecan-4 compared to the CON empty vector and treated with either 0 ng/ml FGF2 or 20 ng/ml FGF2 (Figure 5A), there was an effect of transfection, FGF2, age, and an interaction of age by transfection and age by FGF2 at 24 h PT. At 72 h PT, there was only an effect of FGF2 and age and an interaction of age and FGF2. These data indicate that overexpression of syndecan-4 does not significantly affect the ability of 7 wk and 16 wk satellite cells to respond to FGF2 at 72 h PT, though it does decrease the response to FGF2 in 1 d cells at 24 and 72 h PT. In the satellite cells transfected with glypican-1 compared to CON (Figure 5B), there was an effect of transfection, FGF2, and age and an interaction of age by transfection, age by FGF2, and transfection by FGF2 at 24 h PT. At 72 h PT, there was an effect of age and FGF2 and interactions of age by transfection and age by FGF2. These data suggest that overexpression of glypican-1 increased FGF2 responsiveness in 1 d and 7 wk cells at 72
h PT but decreased FGF2 responsiveness in 16 wk cells. At 24 h PT, transfection with glypican-1 also decreased FGF2 responsiveness in 1 d cells. In the cells overexpressing syndecan-4 with glypican-1 (Figure 5C), there was an effect of age, FGF2, transfection, and interactions of age by transfection, age by FGF2, and a three way interaction of age by transfection by FGF2 at 24 h PT. There was an effect of age and FGF2 and an interaction of age by FGF2 at 72 h PT. Taken together, these data suggest that overexpression of syndecan-4 with glypican-1 had no effect on FGF2 responsiveness at 72 h PT, but decreased the cells response to FGF2 at 24 h PT in the 1d cells.

At 0 h of differentiation, overexpression of syndecan-4 compared to CON had an effect of age and FGF2. At 48 h of differentiation, there was an effect of age and FGF2 as well as interactions of age by transfection, age by FGF2, and a three way interaction of age by transfection by FGF2 (Figure 6A). Transfection with syndecan-4 caused an increase in creatine kinase levels in 1 d, 7 wk, and 16 wk satellite cells at 48 h of differentiation with the addition of 20 ng/ml FGF2. At 0 h of differentiation, overexpression of syndecan-4 had no effect on differentiation in 1 d, 7 wk, or 16 wk cells, whereas at 48 h of differentiation, differentiation was decreased in 16 wk cells not treated with FGF2 and increased in 1 d, 7 wk, and 16 wk cells exposed to FGF2.

In the cells transfected with glypican-1 compared to those transfected with CON, there was an effect of age, FGF2, transfection, an interaction of age by transfection, and a three way interaction of age by FGF2 by transfection at 0 h of differentiation (Figure 6B). At 48 h of differentiation, there was an effect of age, FGF2, and a three way interaction between age, FGF2, and transfection. The 1 d, 7 wk, and 16 wk satellite cells transfected with glypican-1 and treated with 20 ng/ml FGF2 had increased differentiation when
compared with those transfected with CON at 48 h of differentiation. Overexpression of glypican-1 increased differentiation in 1 d and 16 wk cells treated with FGF2 at 0 h of differentiation, and in 1 d, 7 wk, and 16 wk cells treated with FGF2 at 48 h of differentiation.

Overexpression of syndecan-4 with glypican-1 resulted in an effect of age, FGF2, transfection, and a three way interaction of age, FGF2, and transfection at 0 h of differentiation (Figure 6C). At 48 h of differentiation, there was an effect of age, FGF2, transfection, as well as an interaction of age by FGF2 and a three way interaction of age by FGF2 by transfection. Transfection with syndecan-4 with glypican-1 had no effect on creatine kinase levels in 1 d or 16 wk cells treated with 20 ng/ml FGF2, but did increase creatine kinase in 7 wk cells. In those cells not treated with FGF2, transfection with syndecan-4 with glypican-1 resulted in a decrease in creatine kinase compared to those transfected with CON. Overexpression of syndecan-4 with glypican-1 increased differentiation in 7 wk cells at 0 h of differentiation, as well as in 1 d and 16 wk cells treated with FGF2. At 48 h of differentiation, overexpression of syndecan-4 with glypican-1 caused a decrease in differentiation in 1 d, 7 wk and 16 wk cells without FGF2 while increasing differentiation in 7 wk cells treated with FGF2.

3. Effect of overexpression of syndecan-4, glypican-1 and syndecan-4 with glypican-1 on MyoD, myogenin and MRF4 mRNA expression

In 7 wk cells, overexpressing syndecan-4 increased the mRNA expression of MyoD at 72 h PT, whereas in 1 d cells transfected with syndecan-4, glypican-1, and syndecan-4 with glypican-1 decreased MyoD expression, and there was no effect on MyoD expression in 16 wk cells (Figure 7A). At 48 h of differentiation, there was no
effect on MyoD expression in any of the cell ages (Figure 7B). Myogenin mRNA expression was decreased at 72 h PT in 1 d cells overexpressing syndecan-4, glypican-1, and syndecan-4 with glypican-1 and decreased in 7 wk cells transfected with glypican-1 and syndecan-4 with glypican-1. There was no effect on 16 wk cells (Figure 7C). At 48 h of differentiation, 1 d cells transfected with glypican-1 showed decreased myogenin mRNA expression, while transfection of 7 wk cells with syndecan-4 increased myogenin expression and there was no effect on the 16 wk cells compared to the cells transfected with the CON empty vector (Figure 7D). At 72 h PT, the 1 d cells had decreased MRF4 mRNA expression when overexpressing syndecan-4, glypican-1, and syndecan-4 with glypican-1, while 7 wk cells had increased MRF4 expression when overexpressing syndecan-4. In the 16 wk cells there was no effect of transfection (Figure 7E). In 7 wk cells, there was significantly decreased MRF4 expression in the cells overexpressing syndecan-4 and glypican-1 at 48 h of differentiation (Figure 7F). There was no effect of overexpression on either the 1 d or 16 wk cells at 48 h of differentiation.

Overexpression of syndecan-4 (Figure 8A, B) and glypican-1 (Figure 8C, D) was confirmed in the FGF2 responsiveness assays using RTqPCR. Treatment with exogenous FGF2 increased the expression of MyoD mRNA in 1 d, 7 wk, and 16 wk cells at 72 h PT (Figure 9A). There was no significant difference between 1 d, 7 wk, and 16 wk cells overexpressing syndecan-4, glypican-1, or syndecan-4 with glypican-1 without exogenous FGF2. In the 1 d cells exposed to exogenous FGF2, overexpression of both syndecan-4 and syndecan-4 with glypican-1 increased MyoD expression, while in 7 wk cells an increase in MyoD was observed in those cells overexpressing glypican-1 and syndecan-4 with glypican-1, and in 16 wk cells an increase in MyoD was observed only
in those cells overexpressing syndecan-4 with glypican-1. At 48 h of differentiation (Figure 9B), there was no significant effect of overexpression of syndecan-4, glypican-1, or syndecan-4 with glypican-1, though there was an effect of both age and FGF2 on MyoD expression.

Exogenous FGF2 was also found to increase myogenin mRNA concentration in 1 d, 7 wk, and 16 wk cells at 72 h PT (Figure 10A). There was no significant effect of overexpression of syndecan-4, glypican-1, or syndecan-4 with glypican-1 on myogenin expression in the 1 d, 7 wk and 16 wk cells not exposed to exogenous FGF2 at 72 h PT. In the 1 d cells, myogenin expression increased when cells overexpressed syndecan-4 and syndecan-4 with glypican-1, while in 7 wk cells overexpression of syndecan-4, glypican-1 and syndecan-4 with glypican-1 increased myogenin expression. In 16 wk cells myogenin expression was only increased in cells overexpressing syndecan-4 with glypican-1. At 48 h of differentiation (Figure 10B), exogenous FGF2 resulted in a decrease in myogenin expression in 1 d, 7 wk, and 16 wk cells. In 1 d cells, overexpression of syndecan-4 and glypican-1 resulted in decreased myogenin expression when exposed to either 0 or 20 ng/ml FGF2. The 7 wk cells that expressed decreased myogenin compared to the CON were those that overexpressed glypican-1 with 0 ng/ml FGF2 and those that overexpressed syndecan-4, glypican-1, and syndecan-4 with glypican-1 with 20 ng/ml FGF2. Decreased myogenin expression was also found in the 16 wk cells overexpressing glypican-1 without exogenous FGF2 and those overexpressing syndecan-4 and glypican-1 with exogenous FGF2.

Expression of MRF4 mRNA was increased in 1 d, 7 wk, and 16 wk cells exposed to exogenous FGF2 at 72 h PT (Figure 11A). In 1 d cells not exposed to FGF2, MRF4
expression was higher in the cells overexpressing syndecan-4 and glypican-1 than in those transfected with the CON empty vector. In the 1 d cells exposed to exogenous FGF2, overexpression of syndecan-4 and syndecan-4 with glypican-1 resulted in elevated MRF4 expression. In 7 wk cells exposed to FGF2, overexpression of glypican-1 and syndecan-4 with glypican-1 resulted in increased MRF4 and in 16 wk cells, overexpression of syndecan-4 with glypican-1 resulted in increased MRF4 and overexpression of glypican-1 resulted in decreased MRF4 expression. During differentiation, exposure to FGF2 had no effect on MRF4 mRNA expression (Figure 11B). In 1 d cells, overexpression of syndecan-4, glypican-1, and syndecan-4 with glypican-1 resulted in increased MRF4 expression, while in 7 wk cells there was no effect of transfection in those without FGF2, whereas overexpression of syndecan-4, glypican-1, and syndecan-4 with glypican-1 decreased MRF4 expression in those cells exposed to FGF2. The expression of MRF4 was increased in 16 wk cells overexpressing syndecan-4 and glypican-1 and not exposed to FGF2, while MRF4 expression was decreased in 16 wk cells overexpressing glypican-1 while exposed to exogenous FGF2.

IV. Discussion

The proportion of satellite cells that express syndecan-4 and glypican-1 has been shown to decrease from 1 d to 16 wk of age (Harthan et al., 2013). Turkeys during this time are still actively growing and do not reach sexual maturity until after 40 wk of age. Syndecan-4 and glypican-1 play critical roles in satellite cell proliferation, differentiation, and FGF2 responsiveness. Syndecan-4 is important in satellite cell activation and proliferation (Cornelison et al., 2004), as well as in satellite cell migration by the modulation of the RhoA signal transduction pathway (Shin et al., 2013). Glypican-1 is
primarily responsible for promoting differentiation by sequestering FGF2 from its tyrosine kinase receptors (Brandan et al., 1996; Gutiérrez and Brandan, 2010; Velleman et al., 2013). Satellite cells are also a dynamic heterogeneous population of cells that have altered expression of myogenic regulatory factors and cell surface proteins such as syndecan-4 and glypican-1 depending on their developmental stage (Hasty et al. 1993; Rudnicki et al. 1993; Yablonka-Reuveni and Rivera 1994; Seale et al. 2000; Velleman et al., 2010; Harthan et al., 2013). Satellite cells that express one myogenic regulatory factor profile are also capable of giving rise to cells that express a different profile (Zammit et al., 2004). Expression of syndecan-4 and glypican-1 has also been shown to affect the expression of the myogenic regulatory factors MyoD, myogenin, and MRF4 (Shin et al., 2012b). Change in satellite cell populations is important because it affects the ability of satellite cells to become activated, proliferate and differentiate and contribute to muscle growth. The objective of the present study was to determine whether increasing syndecan-4 and glypican-1 expression in 16 wk cells results in increased proliferation, differentiation, and FGF2 responsiveness.

Song et al. (2013) previously reported that syndecan-4 and glypican-1 expression differs by developmental stage of the satellite cell, as well as between lines and between sexes. In the current study, the 1 d cells and 7 wk cells that overexpressed syndecan-4, glypican-1, and syndecan-4 with glypican-1 had decreased proliferation at 72 h PT, whereas the 16 wk cells overexpressing syndecan-4, glypican-1, and syndecan-4 with glypican-1 had no difference. These data indicate that 1 d and 7 wk cells were affected similarly by the overexpression of syndecan-4, glypican-1, and syndecan-4 with glypican-1 during proliferation, while the 16 wk cells were unaffected. Satellite cells are
a heterogeneous population of cells, and while 16 wk cells have smaller subpopulations that express syndecan-4 and glypican-1 than the 1 d or 7 wk cells, it does not appear that increasing the expression of syndecan-4 and glypican-1 has a significant effect on satellite cell proliferation in 16 wk cells.

During differentiation, there was no significant difference between the 1 d, 7 wk, and 16 wk cells that were overexpressing syndecan-4, glypican-1, and syndecan-4 with glypican-1 compared to those transfected with the CON empty vector. Previous studies have demonstrated that transfecting 7 wk cells with glypican-1 results in a growth curve that has higher proliferation early on that levels off at 72 h PT (Velleman et al., 2007; Zhang et al., 2007; Song et al., 2010). Overexpression of glypican-1 also results in an increase in differentiation early during differentiation that levels off to become similar to (Zhang et al., 2007; Song et al., 2010) or lower than (Velleman et al., 2007) cells transfected with CON. While the current study does not show a significant effect on proliferation and differentiation, the data follows the same trend as previous studies. Thus, the current study demonstrates that overexpressing syndecan-4 and glypican-1 does not significantly impact the ability of 16 wk cells to proliferate and differentiate.

Both syndecan-4 and glypican-1 can regulate FGF2 signal transduction by binding FGF2 to their heparan sulfate chains (Volk et al., 1999; Gutiérrez and Brandan, 2010; Velleman et al., 2013). Fibroblast growth factor 2 has been shown to increase the number of satellite cells capable of proliferating (Yablonka-Reuveni et al., 1999) and is a stimulator of proliferation (Dollenmeier et al., 1981). The proliferative capacity of older satellite cells can be increased by the addition of exogenous FGF2 (Shefer et al., 2006). Because of the role of syndecan-4 and glypican-1 in the regulation of FGF2 signal
transduction, it was thought that increasing the expression of syndecan-4 and glypican-1 in 16 wk cells would increase the satellite cell response to FGF2. The results from the present study showed no increased responsiveness to FGF2 during proliferation or differentiation with the transfection of syndecan-4 with glypican-1 in the 1 d, 7 wk, or 16 wk cells. Overexpression of syndecan-4 decreased FGF2 responsiveness in 1 d cells, while overexpression of glypican-1 increased FGF2 responsiveness in 1 d and 7 wk cells at 72 h of proliferation. Thus, syndecan-4 and glypican-1 expression does not appear to be a limiting factor in responsiveness to FGF2 in the 1 d, 7 wk, and 16 wk cells.

Understanding how syndecan-4 and glypican-1 affect the expression of myogenic regulatory factors MyoD, myogenin, and MRF4 is also important in understanding effects of syndecan-4 and glypican-1 on satellite cell function. The present study indicates that expression of MyoD, myogenin, and MRF4 are decreased at 72 h proliferation in all overexpressing 1 d cells compared to the control 1 d cells and myogenin in glypican-1 overexpressing cells at 48 h of differentiation. In 7 wk cells, MyoD and MRF4 expression is increased in cells overexpressing syndecan-4, while myogenin is decreased in cells overexpressing glypican-1 and syndecan-4 with glypican-1 at 72 h of proliferation and increased in cells overexpressing syndecan-4 at 48 h of differentiation. The 16 wk cells were unaffected by overexpression at all times as compared to the cells transfected with the control vector. Knockdown studies on the effect of glypican-1 and syndecan-4 on the expression of MyoD, myogenin and MRF4 showed that knockdown of syndecan-4 resulted in increased MyoD, myogenin and MRF4 expression, while knockdown of glypican-1 caused a decrease in MyoD, myogenin and MRF4 expression (Shin et al., 2012b). Based on these studies, it was expected that the
overexpression of syndecan-4 would result in decreased MyoD, myogenin and MRF4 expression, while the overexpression of glypican-1 would result in increased MyoD, myogenin and MRF4 expression. This suggests that overexpression of syndecan-4 and glypican-1 has little to no effect on the mRNA expression in 7 wk and 16 wk cells, or in the 1 d cells at 48 h of differentiation. In the current study, there was no significant effect of overexpression of syndecan-4, glypican-1, or syndecan-4 with glypican-1 on proliferation or differentiation. Coupled with the requirement of MyoD for proliferation and myogenin for differentiation (Hasty et al., 1993; Rudnicki et al., 1993; Seale et al., 2000; Yablonka-Reuveni and Rivera, 1994), and the presence of MRF4 during proliferation (Shin et al., 2012b), it stands to reason that myogenic regulatory factor expression would remain unchanged.

The increase in MyoD and MRF4 mRNA expression observed in the 1 d, 7 wk, and 16 wk cells that overexpress syndecan-4 with glypican-1 and are also treated with exogenous FGF2 should lead to an increase in proliferation in these cells at 72 h PT. However, no significant increase in proliferation was observed. This could possibly be due to post-transcriptional regulation of MyoD and MRF4, resulting in increased MyoD and MRF4 mRNA expression with no increase at the protein level. The same trend is true in myogenin, where increased myogenin mRNA should result in increased differentiation. No significant increase in differentiation was observed, which points to a possible post-transcriptional regulation of the myogenic regulatory factors MyoD, myogenin and MRF4.

Identifying the molecules responsible for the age-related changes in proliferation and differentiation during muscle growth could be critical in developing strategies to
maintain satellite cell populations with age. Though typically quiescent, satellite cells are activated during muscle growth, causing them to proliferate and differentiate (Schultz, 1978; Schultz and McCormick, 1994) and contribute to muscle growth by donating their nuclei to existing myofibers (Moss and LeBlond, 1970). Satellite cells have been shown to change with age (Collins et al., 2007; Velleman et al. 2010; Li et al., 2011). Satellite cells decline to less than 5% of the total myofiber nuclei and become largely quiescent (Hawke and Garry 2001). Satellite cell proliferation was shown to be significantly decreased by 8 wk of age and differentiation was significantly decreased by 4 wk of age in turkeys (Velleman et al., 2010), while expression of syndecan-4 and glypican-1 has been shown to decrease between satellite cells isolated from 1 d old pouls and those from 16 wk old pouls (Harthan et al., 2013). The present study sought to identify the effects of altered syndecan-4 and glypican-1 expression on satellite cell proliferation and differentiation. The present study determined that syndecan-4 and glypican-1 do not significantly increase satellite cell proliferation and differentiation in the 16 wk cells. Further work is needed to identify other age-related changes in satellite cell function, as well as other molecules that may be involved in the age-related changes in satellite cell proliferation, differentiation, FGF2 responsiveness, and expression of myogenic regulatory factors.
**V. References**


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1 Primers were designed from the following GenBank accession numbers: Glypican-1, AY551002.2; Syndecan-4, AY852251.1; MyoD, AY641567.1; Myogenin, AY560111.3; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), U94327.1
Figure 3.1. Effect of overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on syndecan-4 and glypican-1 mRNA expression. Effect of transfection with syndecan-4, glypican-1, or syndecan-4 and glypican-1 on (A, C) syndecan-4 and (B, D) glypican-1 mRNA expression in satellite cells isolated from the pectoralis major muscle of 1 d-old, 7 wk-old, and 16 wk-old randombred control line 2 turkeys. Average values were graphed with bars representing the standard error of the mean. Values were considered significantly different at P<0.05. PT = post transfection, D = differentiation, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1.
Figure 3.2. Effect of overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on the percentage of cells expressing syndecan-4 and glypican-1. Effect of transfection with syndecan-4, glypican-1, or syndecan-4 and glypican-1 on (A, C) syndecan-4 and (B, D) glypican-1 positive pectoralis major satellite cell populations. Protein expression was measured using flow cytometry during proliferation at 72 h post transfection (A, B) and at 48 h of differentiation (C, D). Satellite cells were isolated from 1 d-old, 7 wk-old, and 16 wk-old posthatch randombred control line 2 male turkeys. Average values were graphed with bars representing the standard error of the mean. Values were considered significantly different at P<0.05. PT = post transfection, D = differentiation, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1.
Figure 3.3. Effect of overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on proliferation. Proliferation of satellite cells from pectoralis major muscle from 1 d-old, 7 wk-old, and 16 wk-old posthatch randombred control line 2 turkeys transfected with pCMS-EGFP and either syndecan-4 (A), glypican-1 (B), or syndecan-4 and glypican-1 (C). Average values were graphed with bars representing the standard error of the mean. Data were analyzed within time to evaluate age, transfection, and the interaction between age and transfection. A = age, T = transfection; AT = age x transfection. Values were considered significantly different at P<0.05. PT = post transfection, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1.
Figure 3.4. Effect of overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on differentiation. Differentiation of satellite cells from pectoralis major muscle from 1 d-old, 7 wk-old and 16 wk-old posthatch randombred control line 2 turkeys transfected with pCMS-EGFP and either syndecan-4 (A), glypican-1 (B), or syndecan-4 and glypican-1 (C). Average values were graphed with bars representing the standard error of the mean. Data were analyzed within time to evaluate age, transfection, and the interaction between age and transfection. A = age, T = transfection; AT = age × transfection. Values were considered significantly different at P<0.05. PT = post transfection, D = differentiation, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1.
Figure 3.5. Effect of fibroblast growth factor 2 and overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on proliferation. Fibroblast Growth Factor 2 (FGF2) responsiveness during proliferation of satellite cells from pectoralis major muscle from 1 d-old, 7 wk-old and 16 wk-old posthatch randombred control line 2 turkeys transfected with pCMS-EGFP and either syndecan-4 (A), glypican-1 (B), or syndecan-4 and glypican-1 (C). Average values were graphed with bars representing the standard error of the mean. Data were analyzed within time to evaluate age, FGF2, transfection, and the interaction between age and transfection, age and FGF2, transfection and FGF2 and age, transfection and FGF2. A = age, T = transfection; AT = age × transfection; AF = age × FGF2; TF = transfection × FGF2; AFT = age × transfection × FGF2. Values were considered significantly different at P<0.05. PT = post transfection, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1.
Figure 3.6. Effect of fibroblast growth factor 2 and overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on differentiation. Fibroblast Growth Factor 2 (FGF2) responsiveness during differentiation of satellite cells from pectoralis major muscle from 1 d-old, 7 wk-old and 16 wk-old posthatch randombred control line 2 turkeys transfected with pCMS-EGFP and either syndecan-4 (A), glypican-1 (B), or syndecan-4 and glypican-1 (C). Average values were graphed with bars representing the standard error of the mean. Data were analyzed within time to evaluate age, FGF2, transfection, and the interaction between age and transfection, age and FGF2, transfection and FGF2 and age, transfection and FGF2. A = age, T = transfection; AT = age × transfection; AF = age × FGF2; TF = transfection × FGF2; AFT = age × transfection × FGF2. Values were considered significantly different at P<0.05. PT = post transfection, D = differentiation, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1.
Figure 3.7. Effect of overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on MyoD, myogenin and MRF4 mRNA expression. Effect of transfection with syndecan-4, glypican-1, or syndecan-4 and glypican-1 on (A, B) MyoD, (C, D) myogenin, and (E, F) MRF4 mRNA expression in satellite cells isolated from the pectoralis major muscle of 1 d-old, 7 wk-old and 16 wk-old randombred control line 2 turkeys at 72 h PT (A, C, E) and 48 h of differentiation (B, D, F). Average values were graphed with bars representing the standard error of the mean. Data were analyzed within age to evaluate the effect of transfection. Values without common letters were considered statistically different at P<0.05. PT = post transfection, D = differentiation, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1.
Figure 3.8. Effect of fibroblast growth factor 2 and overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on syndecan-4 and glypican-1 mRNA expression. Effect of fibroblast growth factor 2 (FGF2) and transfection with syndecan-4, glypican-1, or syndecan-4 and glypican-1 on (A, B) syndecan-4 and (C, D) glypican-1 mRNA expression in satellite cells isolated from the pectoralis major muscle of 1 d-old, 7 wk-old and 16 wk-old randombred control line 2 turkeys. Average values were graphed with bars representing the standard error of the mean. Data were analyzed within age to determine the effect of transfection. Values were considered statistically different at P<0.05. PT = post transfection, D = differentiation, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1, 20 FGF2 = grown in media with 20 ng/ml FGF2, 0 FGF2 = grown in media with 0 ng/ml FGF2.
Figure 3.9. Effect of fibroblast growth factor 2 and overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on MyoD mRNA expression. Effect of fibroblast growth factor 2 (FGF2) and transfection with syndecan-4, glypican-1, or syndecan-4 and glypican-1 on MyoD mRNA expression in satellite cells isolated from the pectoralis major of 1 d-old, 7 wk-old and 16 wk-old randombred control line 2 turkeys at 72 h PT (A) and 48 h of differentiation (B). Average values were graphed with bars representing the standard error of the mean. Values without common letters were considered statistically different at P<0.05. PT = post transfection, D = differentiation, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1, 20 FGF2 = grown in media with 20 ng/ml FGF2, 0 FGF2 = grown in media with 0 ng/ml FGF2.
Figure 3.10. Effect of fibroblast growth factor 2 and overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on myogenin mRNA expression. Effect of fibroblast growth factor 2 (FGF2) and transfection with syndecan-4, glypican-1, or syndecan-4 and glypican-1 on myogenin mRNA expression in satellite cells isolated from the pectoralis major of 1 d-old, 7 wk-old and 16 wk-old randombred control line 2 turkeys at 72 h PT (A) and 48 h of differentiation (B). Average values were graphed with bars representing the standard error of the mean. Values without common letters were considered statistically different at P<0.05. PT = post transfection, D = differentiation, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1, 20 FGF2 = grown in media with 20 ng/ml FGF2, 0 FGF2 = grown in media with 0 ng/ml FGF2.
Effect of fibroblast growth factor 2 and overexpression of syndecan-4, glypican-1, and syndecan-4 and glypican-1 on MRF4 mRNA expression. Effect of fibroblast growth factor 2 (FGF2) and transfection with syndecan-4, glypican-1 or syndecan-4 and glypican-1 on MRF4 mRNA expression in satellite cells isolated from the pectoralis major of 1 d-old, 7 wk-old and 16 wk-old randombred control line 2 turkeys at 72 h PT (A) and 48 h of differentiation (B). Average values were graphed with bars representing the standard error of the mean. Values without common letters were considered statistically different at P<0.05. PT = post transfection, D = differentiation, CON = pCMS-EGFP, S4 = syndecan-4, G1 = glypican-1, S4G1 = syndecan-4 and glypican-1, 20 FGF2 = grown in media with 20 ng/ml FGF2, 0 FGF2 = grown in media with 0 ng/ml FGF2.
CHAPTER 4

THE EFFECT OF NUTRITIONAL STATUS AND MYOGENIC SATELLITE CELL AGE ON PROLIFERATION, DIFFERENTIATION, AND EXPRESSION OF MYOGENIC TRANSCRIPTIONAL REGULATORY FACTORS AND HEPARAN SULFATE PROTEOGLYCANS SYNDENCAN-4 AND GLYPICAN-1

I. INTRODUCTION

Posthatch muscle growth is mediated by an adult myoblast population termed satellite cells. Satellite cells are a population of mesodermal-derived multipotential stem cells located between the sarcolemma and the basement membrane (Mauro, 1961). Satellite cells proliferate, align and fuse with existing muscle fibers during the growth and regeneration of muscle (Moss and LeBlond, 1970). Although satellite cells exist predominantly in a quiescent state, they are activated to proliferate and differentiate during muscle growth and regeneration (Schultz, 1978; Schultz and McCormick, 1994). Satellite cell proliferation and differentiation are affected by age (Velleman et al., 2010a; Harthan et al., 2013). Nutritional status has been shown to affect pectoralis major (p. major) satellite cell proliferation and differentiation in broilers (Halevy et al., 2000; Powell et al., 2013). How nutrient status affects p. major satellite cells at different ages during the growth of the bird is not well understood.
In broilers, p. major satellite cells have a period of maximal activity that lasts only for the first week posthatch, after which satellite cell activity rapidly declines with increasing age (Halevy et al., 2000). Standard industry procedures for handling and transportation during this immediate posthatch period can result in poult's that are feed deprived for up to 72 h posthatch. There is evidence that feed restrictions during the time of maximal satellite cell activity has detrimental effects on satellite cell function and muscle growth. Broiler chicks that were feed deprived for 48 h posthatch had decreased satellite cell proliferation, body weight and breast muscle weight, as well as altered breast muscle morphology (Halevy et al., 2000). Similarly, Velleman et al. (2010b) found that broilers that were 20% feed restricted for 2 wk posthatch had altered muscle organization, increased fat deposition and necrosis, increased MyoD expression and decreased myogenin expression. Mozdziak et al. (2002) and Halevy et al. (2003) have shown that immediate posthatch feed deprivation for either 2 or 3 d decreased satellite cell mitotic activity. Heparan sulfate proteoglycan expression also decreased in broilers feed deprived for the first 3 d posthatch (Velleman and Mozdziak, 2005). Because satellite cells are multipotential mesodermal stems cells, it is possible that they may transdifferentiate into other mesodermal cell types such as adipocytes. Asakura et al. (2001) demonstrated that satellite cells in vitro can transdifferentiate into adipogenic and osteogenic cell lineages by altering nutrient conditions during the culturing of the satellite cells.

Satellite cell proliferation and differentiation is regulated by myogenic transcriptional regulatory factors, which include but are not limited to: myogenic determination factor 1 (MyoD), myogenin, and myogenic regulatory 4 (MRF4).
Proliferating satellite cells express both Pax7 and MyoD, while differentiating satellite cells express Pax7, MyoD and myogenin or MyoD and myogenin (Hasty et al., 1993; Rudnicki et al, 1993; Seale et al., 2000; Yablonka-Reuveni and Rivera, 1994). Expression of myogenin is necessary for muscle cell differentiation (Brunetti and Goldfine, 1990). The formation of muscle fibers from multinucleated myotubes has been shown to be regulated by MRF4 (Hinterberger et al., 1991), but recent evidence suggests that MRF4 may play a role in satellite cell proliferation (Shin et al., 2012).

Myogenic transcriptional regulatory factor expression is affected by the heparan sulfate proteoglycans syndecan-4 and glypican-1. Syndecan-4 is primarily involved in satellite cell migration (Shin et al., 2013), which is required for satellite cell alignment prior to multinucleated myotube formation. Glypican-1 is primarily involved in facilitating differentiation (Brandan et al., 1996; Gutiérrez and Brandan, 2010; Velleman et al., 2013). Myoblasts deficient in glypican-1 express significantly reduced levels of myogenin, which is required for differentiation (Gutiérrez and Brandan, 2010). Shin et al. (2012) reported that the expression of MyoD and myogenin decreased when glypican-1 was knocked down. In contrast, MyoD expression increased during proliferation when syndecan-4 was knocked down. Furthermore, the expression of both MyoD and myogenin is affected by immediate posthatch nutrition. Velleman et al. (2010b) found during an immediate posthatch 20% feed restriction lasting 2 wk, broiler p. major muscle expression of MyoD was increased and myogenin was decreased. This upregulation of MyoD was hypothesized by Velleman et al. (2010b) to reflect a possible increase in satellite cell proliferation to compensate for reduced differentiation due to the lower expression of myogenin.
In vivo nutrient restrictions can be simulated in vitro by restricting the essential amino acid methionine (Met) as shown by (Powell et al., 2013). In this defined medium, cysteine (Cys) concentration was also altered proportionally to Met. Replicating feed restrictions in vitro allows one to determine the effect of restricting nutrition directly on the satellite cell without any interference from other cell types. This will reduce the transsulfuration of Met to Cys, as Cys is the only amino acid that can be synthesized from Met (Scott et al., 1982). The inclusion of Cys, which is important in disulfide bond formation for protein synthesis, reduces Met requirements in diets (Baker, 2006). Powell et al. (2013) showed that isolated broiler satellite cells grown in Met and Cys restricted medium resulted in decreased proliferation and differentiation in a dose-dependent manner. The objective of the current study was to provide new information on the effect of nutritional status and p. major satellite cell age on the proliferation and differentiation of satellite cells.

II. MATERIALS AND METHODS

1. Satellite Cells

Satellite cells were isolated from the p. major muscle of 1 d, 7 wk and 16 wk posthatch Randombred Control Line 2 turkeys (RBC2; 1 d, 7 wk and 16 wk cells, respectively) and then expanded and stored in liquid nitrogen based on the method of Velleman et al. (2010a). The RBC2 line is maintained at the Poultry Research Center at the Ohio Agricultural Research and Development Center/The Ohio State University in Wooster, OH without conscious selection for any traits. Therefore, there are no growth selection effects on satellite cell proliferation and differentiation, as shown by Velleman
et al. (2000). Only male satellite cells were used to avoid any sex effects (Velleman et al., 2000; Song et al., 2013).

2. Cell Culture

Satellite cells from 1 d, 7 wk, and 16 wk posthatch RBC2 turkeys were plated in 24-well or 48-well cell culture plates (Greiner Bio-one, Monroe, NC, USA) coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) at 15,000 and 9,000 cells per well, respectively. Cells were cultured in plating medium composed of Dulbecco’s Modified Eagle’s Medium (DMEM: Sigma-Aldrich) with 10% chicken serum (Gemini BioProducts, West Sacramento, CA, USA), 5% horse serum (Gemini BioProducts), 1% antibiotic/antimycotic (Gemini BioProducts) and 0.1% gentamicin (Gemini BioProducts) and grown at 37°C in a 95% air/5% CO₂ incubator (Thermo Fisher Scientific, Pittsburg, PA). After 24 h of attachment, the medium was changed to the defined feeding medium used in Powell et al. (2013) containing either 30, 7.5, 3, or 0 mg/L Met with 3.2 mg/L Cys per 1 mg/L Met. Standard DMEM contains 30 mg/L Met and 96 mg/L Cys, and this treatment served as the control. Medium was changed every 24 h until 96 h of proliferation when the cells reached 60 to 65% confluency. Differentiation was then induced by changing the medium to a low-serum medium composed of Met and Cys free DMEM (Corning-Cellgro Corp., Manassas, VA) containing 3% horse serum, 1% antibiotic/antimycotic, 0.1% gentamicin, 0.1% gelatin (Sigma-Aldrich), 1 mg/ml bovine serum albumin (BSA: Sigma-Aldrich) and 30, 7.5, 3, or 0 mg/L Met with 3.2 mg/L Cys per 1 mg/L Met. Medium was then changed every 24 h until 72 h of differentiation.
3. Proliferation Assay

Every 24 h beginning at 24 h of proliferation through 96 h of proliferation, 24-well cell culture plates were harvested, rinsed with phosphate buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, 7.81 mM Na₂HPO₄, pH 7.08), air dried for 15 min and stored at -70°C until analysis. Proliferation was determined by measuring the concentration of DNA per well by the method of McFarland et al. (1995). In brief, plates were removed from the freezer and allowed to thaw for 10 to 15 min before 200 μl of 0.05% trypsin (Invitrogen, Carlsbad, CA, USA) in 1x TNE buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4) was added to each well. Plates were then incubated at room temperature (RT) for 7 min with gentle tapping and stored at -70°C overnight. Plates were removed from the freezer and allowed to thaw for 10 to 15 min before the addition of 1.8 mL of Hoechst Dye buffer (0.2 μg/mL Hoechst 33258 [Sigma-Aldrich] in 1x TNE) to each well. The cell culture plates were then wrapped in foil and incubated at RT with shaking for 1 to 2 h. Optical density was measured after the incubation using a Fluoroskan Ascent FL scanner (Thermo Fisher Scientific) with an excitation of 365 nm and an emission of 460 nm. A standard curve with a range of 0.1 to 1.2 μg double stranded calf thymus DNA (Sigma-Aldrich) was used to determine sample DNA concentration. Proliferation assays were independently repeated 6 times.

4. Differentiation assay

Cells were plated and grown in 48-well cell culture plates as described in the Cell Culture section with the exception that the defined feeding medium was substituted for feeding medium composed of McCoy’s 5A containing 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic and 0.1% gentamicin. Plates with five wells per
treatment were harvested every 24 h beginning at 96 h of differentiation through 72 h of differentiation. Plates were then rinsed with PBS and stored at -70°C until analysis.

Differentiation was quantified by measuring creatine kinase concentration using a modified method of Yun et al. (1997). In brief, plates were thawed for 10 to 15 min and 500 μL of creatine kinase buffer [20 mM glucose (Thermo Fisher Scientific), 10 mM Mg acetate (Thermo Fisher Scientific), 1.0 mM adenosine diphosphate (Sigma-Aldrich), 10 mM adenosine monophosphate (Sigma-Aldrich), 20 mM phosphocreatine (Calbiochem, San Diego, CA, USA), 0.5 U/mL hexokinase (Worthington Biochemical, Lakewood, NJ, USA), 1 U/mL of glucose-6-phosphate dehydrogenase (Worthington Biochemical), 0.4 mM thio-nicotinamide adenine dinucleotide (Oriental Yeast Co., Tokyo, Japan), and 1 mg/mL BSA (Sigma-Aldrich)] were added to each well. Optical density at 405 nm was measured using a BioTek ELx800 (BioTek, Winooski, VT, USA) plate reader. A standard curve with 0 to 100 mU creatine phosphokinase (Sigma-Aldrich) was used to measure sample concentrations. The differentiation assay was repeated independently 4 times.

5. Microscopy

Photographs were taken at 96 h of proliferation and 48 h of differentiation using an Olympus IX70 microscope (Olympus America, Center Valley, PA, USA) equipped with a QImaging Retiga EXi Fast 1394 digital camera (QImaging, Surrey, BC, Canada). Images were captured using CellSens software (Olympus America). Satellite cell length and myotube width were also measured using CellSens, and 10 measurements were taken per treatment per sample time.
6. RNA Isolation and Real-time Quantitative Polymerase Chain Reaction

Total RNA was isolated from 24-well cell culture plates at 96 h of proliferation and 48 h of differentiation using RNAzol RT (Molecular Research Center, Inc, Cincinnati, OH, USA) following the manufacturer’s instructions. A nanodrop spectrophotometer (Thermo Fisher Scientific) was used to measure the concentration of the RNA. Moloney murine leukemia virus reverse transcriptase (MMLV: Promega, Madison, WI, USA) was used to synthesize the cDNA following the manufacturer’s instructions. In brief, 0.8 μg of total RNA, 1 μL of 50 μM Oligo dT (Operon, Huntsville, AL, USA) and nuclease-free water to 13.5 μL total volume was incubated at 70°C for 5 min and then cooled on ice. A reaction mixture containing 5 μL of 5x MMLV buffer, 1 μL of 10 mM deoxynucleoside triphosphate mix, 0.25 μL of 40 U/μL RNAsin, 1 μL of 200 U/μL MMLV reverse transcriptase and nuclease-free water up to 11.5 μL was added to each tube. The sample tubes were then incubated at 55°C for 60 min and 90°C for 10 min. Real-time quantitative PCR (RT-qPCR) was performed using DyNAmo Hot Start SYBR green (Finnzymes, Ipswich, MA, USA) according to the manufacturer’s instructions. Each reaction contained 2 μL of cDNA, 10 μL of 2x master mix, 1 μL of a 1:1 mix of 10 μM forward and reverse primers and 7 μL of nuclease free water. Reactions were run using a DNA Engine Opticon 2 real-time machine (MJ Research, Waltham, MA, USA). Primers for glyceraldehydes-4-phosphate-dehydrogenase (GAPDH), syndecan-4, glypican-1, MyoD, myogenin and MRF4 are listed in Table 4.1. Amplification specificity was confirmed by resolving randomly selected samples from all RT-qPCR reactions on a 1% agarose gel. Primer specificities have been previously confirmed by DNA sequencing. Serial dilutions of purified PCR products were used to
produce standard curves for each gene, which were used to determine sample concentrations. Data was then normalized to the average GAPDH expression, the cycle threshold of which remained constant throughout. RNA was isolated from 10 separate experiments and RT-qPCR was performed using three replicates per sample per gene.

7. Statistical analysis

Proliferation assays, differentiation assays, and RT-qPCR were analyzed within time using the general linear model in SAS (SAS Institute Inc., Cary, NC, USA) with differences between means evaluated using a student’s t-test. The model accounted for the effect of Met and Cys concentrations. Satellite cell length and myotube width were analyzed both within age as well as within Met and Cys concentrations. Differences between the means were evaluated using a student’s t-test. The models accounted for the effect of Met and Cys concentrations and the effect of age, respectively. Values were considered statistically significant at $P<0.05$.

III. RESULTS

1. Effect of Nutrition and Age on Pectoralis Major Satellite Cell Proliferation

In the 1 d cells (Figure 4.1A), treatment with 0 mg/L Met/0 mg/L Cys resulted in significantly decreased proliferation at both 72 and 96 h of proliferation compared to all other treatments. At 96 h of proliferation, treatment with 7.5 mg/L Met/24 mg/L Cys had significantly less proliferation than the control 30 mg/L Met/96 mg/L Cys, and proliferation in those treated with 3 mg/L Met/9.6 mg/L Cys was significantly lower than in both 30 mg/L Met/96 mg/L Cys and 7.5 mg/L Met/24 mg/L Cys. Similar to the 1 d cells, the 7 wk cells (Figure 4.1B) treated with 0 mg/L Met/0 mg/L Cys had significantly less proliferation at 72 and 96 h of proliferation than the other treatments. Treatment of 7
wk cells with 30 mg/L Met/96 mg/L Cys and 7.5 mg/L Met/24 mg/L Cys resulted in proliferation levels that were statistically similar at 96 h of proliferation. Proliferation was also similar in the 7 wk cells treated with 7.5 mg/L Met/24 mg/L Cys and 3 mg/L Met/9.6 mg/L Cys at 96 h of proliferation. Treatment with 0 mg/L Met/0 mg/L Cys also resulted in proliferation that was significantly less than in cells treated with 30 mg/L Met/96 mg/L Cys at 48 h of proliferation. Proliferation was not significantly impacted by Met and Cys concentration in the 16 wk cells (Figure 4.1C) at 72 h of proliferation or earlier. At 96 h of proliferation, however, proliferation was significantly decreased in the 16 wk cells treated with 3 mg/L Met/9.6 mg/L Cys and further decreased in those treated with 0 mg/L Met/0 mg/L Cys. In summary, all three ages are affected by decreased levels of Met and Cys, with proliferation at 96 h being most significantly affected in 1 d cells compared to the 16 wk and 7 wk cells.

Microscopic analysis at 96 h of proliferation showed differences in the morphology of satellite cells based on treatment and age during proliferation (Figure 4.2). Proliferating satellite cells are elongated with an elliptical morphology and then align prior to fusion. In the 1 d and 7 wk cells, cell length was significantly decreased in the 0 mg/L Met/0 mg/L Cys treatment compared to the other treatments (Table 4.2). In the 16 wk cells, however, there was no statistical difference in cell length between any of the treatments and the control 30 mg/L Met/96 mg/L Cys treatment. With regard to age, in the control 30 mg/L Met/96 mg/L Cys treatment, there was no significant difference in satellite cell length between the 1 d and 7 wk cells, while the 16 wk cells were significantly smaller than both the 1 d and the 7 wk cells. In the 7.5 mg/L Met/24 mg/L Cys treatment, the 1 d cells were not significantly different from either the 7 wk or the 16
wk cells, though the 16 wk cells were significantly smaller than the 7 wk cells. There was no significant difference in satellite cell length between the ages at the 3 mg/L Met/9.6 mg/L Cys or the 0 mg/L Met/0 mg/L Cys treatments.

2. Effect of Nutrition and Age on Pectoralis Major Satellite Cell Differentiation

During differentiation, the 1 d cells (Figure 4.3A) that were treated with 0 mg/L Met/0 mg/L Cys medium had significantly decreased levels of differentiation at 48 and 72 h. Treatment of the 1 d cells with 7.5 mg/L Met/24 mg/L Cys and 3 mg/L Met/9.6 mg/L Cys medium resulted in significantly increased differentiation compared to the control 30 mg/L Met/96mg/L Cys at 48 and 72 h of differentiation. The differentiation of the 7 wk cells (Fig. 3B) was also reduced by the 0 mg/L Met/0 mg/L Cys treatment resulting in significantly decreased levels of creatine kinase at 24, 48 and 72 h. Treatment of the 7 wk cells with 7.5 mg/L Met/24 mg/L Cys and 3 mg/L Met/9.6 mg/L Cys medium resulted in increased differentiation compared to the control 30 mg/L Met/96mg/L Cys at 48 h of differentiation. In contrast, the 3 mg/L Met/9.6 mg/L Cys treatment had decreased differentiation in the 7 wk cells at 72 h of differentiation compared to the control 30 mg/L Met/96mg/L Cys and 7.5 mg/L Met/24 mg/L Cys medium. The 16 wk cells (Fig. 3C) had significantly decreased differentiation when treated with the medium without Met or Cys compared to the control at 24, 48 and 72 h of differentiation. Differentiation was significantly lower in the 3 mg/L Met/9.6 mg/L Cys treated cells at 48 h of differentiation than in the cells treated with the control 30 mg/L Met/96mg/L Cys medium. Concentrations of Met below the control 30 mg/L except for the 0 mg/L concentration significantly increased differentiation in the 1 d
satellite cells but did not increase differentiation in the 7 wk and 16 wk satellite cells. Cultures with 0 mg/L Met and Cys had limited differentiation at all ages.

Satellite cells treated with 0 mg/L Met/0 mg/L Cys had less myotube formation (Figure 4.4, images d, h, and l). Myotubes are indicated by the arrows in Figure 4.4, images a and g. The myotubes that did form had significantly reduced diameters than in the 30 mg/L Met/96 mg/L Cys, 7.5 mg/L Met/24 mg/L Cys, and 3 mg/L Met/9.6 mg/L Cys treatments (Table 4.3). Myotube width was statistically similar in the 1 d cells treated with 30 mg/L Met/96 mg/L Cys, 7.5 mg/L Met/24 mg/L Cys, and 3 mg/L Met/9.6 mg/L Cys, but was reduced in the 0 mg/L Met 0 mg/L Cys treatment. In the 7 wk satellite cells, myotube width was largest in the 30 mg/L Met/96 mg/L Cys, then 3 mg/L Met/9.6 mg/L Cys, then 7.5 mg/L Met/24 mg/L Cys and smallest in the 0 mg/L Met/0 mg/L Cys. Myotube width significantly decreased in a dose-dependent manner in the 16 wk cells. With regard to age, myotube width was significantly smaller in the 16 wk cells compared to the 1 d or 7 wk cells in the control 30 mg/L Met/96 mg/L Cys treatment, while both the 7 wk and the 16 wk cells had myotubes that were significantly smaller than the 1 d cells when treated with 7.5 mg/L Met/24 mg/L Cys. At both 3 mg/L Met/9.6 mg/L Cys and 0 mg/L Met/0 mg/L Cys, the myotubes formed in the 7 wk cells were significantly smaller than those in the 1 d cells, while the 16 wk cells were significantly smaller than both the 1 d and 7 wk cells.
3. Effect of Nutrition and Age on Pectoralis Major Satellite Cell Expression of Syndecan-4, Glypican-1 and Myogenic Transcriptional Regulatory Factors MyoD, Myogenin and MRF4

Syndecan-4 mRNA expression was unaffected by Met and Cys treatment in the 7 wk cells at 96 h of proliferation (Figure 4.5A). Syndecan-4 expression was decreased in the 1 d cells treated with 7.5 mg/L Met/24 mg/L Cys and 0 mg/L Met/0 mg/L Cys and increased in the 16 wk cells treated with 7.5 mg/L Met/24 mg/L Cys and 3 mg/L Met/9.6 mg/L Cys compared to those treated with the control 30 mg/L Met/96 mg/L Cys at 96 h of proliferation. At 48 h of differentiation (Figure 4.5B), syndecan-4 expression was decreased in the 1 d cells at 0 mg/L Met/0 mg/L Cys, the 7 wk cells at 3 mg/L Met/9.6 mg/L Cys, and the 16 wk cells at 3 mg/L Met/9.6 mg/L Cys and 0 mg/L Met/0 mg/L Cys compared to the 30 mg/L Met/96 mg/L Cys. Glypican-1 mRNA expression was also affected by Met and Cys concentration (Figure 4.5C), with the 1 d, 7wk, and 16 wk cells having decreased glypican-1 expression when treated with 0 mg/L Met/0 mg/L Cys and 7 wk cells having increased expression when treated with 7.5 mg/L Met/24 mg/L Cys media at 96 h of proliferation. The same pattern of glypican-1 expression was seen in the 1 d and 7 wk cells at 48 h of differentiation (Figure 4.5D), though the 16 wk cells expressed significantly less glypican-1 when treated with 7.5 mg/L Met/24 mg/L Cys and 3 mg/L Met/9.6 mg/L Cys and even further decreased expression when treated with 0 mg/L Met/0 mg/L Cys. Treatment with varying levels of Met and Cys differentially affected the expression of syndecan-4 and glypican-1 mRNA in satellite cells of different ages.
Expression of the myogenic transcriptional regulatory factors MyoD, myogenin and MRF4 were also differentially affected by Met and Cys concentration with treatment and age. At 96 h of proliferation (Figure 4.6A), MyoD expression was significantly decreased in the 1 d cells when treated with 0 mg/L Met/0 mg/L Cys, increased in 7 wk cells treated with 7.5 mg/L Met/24 mg/L Cys and not significantly affected in 16 wk cells as compared to those cells treated with the control 30 mg/L Met/96 mg/L Cys. MyoD expression was only affected by the 0 mg/L Met/0 mg/L Cys treatment in the 7 wk cells at 48 h of differentiation (Figure 4.6B). In the 1 d cells at 96 h of proliferation, myogenin expression was impacted by Met and Cys concentration in the same manner as MyoD expression was affected (Figure 4.6C). Myogenin expression decreased significantly with in a dose-dependent manner with Met and Cys concentration in both the 7 wk and 16 wk satellite cells at 96 h of proliferation. At 48 h of differentiation, myogenin expression increased only in the 1 d cells treated with 3 mg/L Met/9.6 mg/L Cys and decreased in the 16 wk cells treated with 3 mg/L Met/9.6 mg/L Cys and was even further decreased in those treated with 0 mg/L Met/0 mg/L Cys (Figure 4.6D). Methionine and Cys concentration had no effect on myogenin expression in the 7 wk cells at 48 h of differentiation. Expression of MRF4 was increased in 1 d and 7 wk cells treated with 0 mg/L Met/0 mg/L Cys at both 96 h of proliferation (Figure 4.6E) and 48 h of differentiation (Figure 4.6F). The expression of MRF4 in 16 wk cells was only affected by Met and Cys concentration at 48 h of differentiation, whereas treatment with 7.5 mg/L Met/24 mg/L Cys and 3 mg/L Met/9.6 mg/L Cys resulted in decreased expression compared to the 30 mg/L Met/96 mg/L Cys control treatment. In summary, treatment with varying levels of Met and Cys results in differential expression of the myogenic
transcriptional regulatory factors MyoD, myogenin and MRF4 in an age-dependent manner.

IV. DISCUSSION

There have been several studies investigating the effects of feed restrictions on muscle growth in poultry. These previous studies have shown that feed restrictions or feed deprivations that take place in the immediate posthatch period generally result in decreased muscle growth and altered breast muscle morphology (Halevy et al., 2000; Velleman et al., 2010b). Velleman et al. (2010b) observed increased fat depots in the p. major muscle in feed restricted broilers, suggesting that the satellite cells may be induced by the feed restriction to transdifferentiate to adipogenic cellular pathway rather than forming muscle. Satellite cells have been shown to be capable of transdifferentiating to an adipogenic lineage when cultured under certain nutrient conditions (Asakura et al., 2001).

Previous studies have also shown that satellite cells are affected by nutrition during the immediate posthatch period, indicating that the critical period for maintenance of satellite cell populations for muscle growth is during the first week posthatch (Halevy et al, 2000, 2001, 2003; Mozdziak et al., 2002; Moore et al., 2005a; Velleman et al., 2010b). Halevy et al. (2003) reported that turkey poults that were feed deprived for 48 h posthatch had decreased body weight and breast muscle weight than their full-fed counterparts. This study also showed that satellite cells isolated from the feed deprived poults had decreased proliferation that peaked 2 d later in the starved poults than in the full fed poults. The starved poults also had a decreased number of satellite cells per g of breast muscle compared to those that were not feed deprived. Satellite cell mitotic
activity in turkey poults feed deprived for 72 h posthatch was highest at 6 d of age, while mitotic activity was highest at d 3 in the full fed poults and declined through 10 d of age (Moore et al., 2005a). A 72 h posthatch feed deprivation was also shown to decrease body weight and myofiber cross-sectional area in turkey poults compared to those that were not feed deprived (Moore et al., 2005a). Previous studies have also shown that satellite cells isolated from turkey poults of various ages differ in proliferation and differentiation (Velleman et al., 2010a; Harthan et al., 2013). Proliferation is decreased in turkey satellite cells between 7 wk and 8 wk posthatch (Velleman et al., 2010a; Harthan et al., 2013), while differentiation decreases by 4 wk posthatch (Velleman et al., 2010a). What is not well understood is how nutrition directly affects satellite cells at different developmental ages.

In the present study, satellite cells isolated from 1 d, 7 wk and 16 wk old turkeys to measure the effect of nutrient restriction on p. major satellite cells. During proliferation, decreasing concentrations of Met and Cys resulted in decreased proliferation, which was most pronounced in the 1 d cells and became less evident as satellite cell age increased. Decreased proliferation results in fewer satellite cells that are available for differentiation, thereby limiting the amount of satellite cells available to differentiate into multinucleated myotubes and contribute to muscle growth. These observations offer a possible explanation for the cause of the decreased body weight reported in previous studies resulting from feed deprivation during the immediate posthatch period (Halevy et al., 2003; Moore et al., 2005a, 2005b; Velleman et al., 2010b).
Nutrient restriction was also determined to have an effect on satellite cell differentiation. The results from the present study showed that lower concentration of Met and Cys but not 0 mg/L increased differentiation in the 1 d cells, while having little or no effect on differentiation in the 7 wk and 16 wk cells. These data support that satellite cells at different developmental ages have different nutrient requirements for differentiation and proliferation. The decreased proliferation and increased differentiation observed in the 1 d satellite cells treated with reduced concentrations of Met and Cys may result in a significantly reduced pool of satellite cells as the satellite cell pool fails to renew itself and the available satellite cells differentiate to form myotubes. This could be caused by decreased levels of Met and Cys resulting in fewer cells proliferating and more of these cells differentiating, culminating in a significant decrease in the number of satellite cells available for muscle growth and an overall depletion of the satellite cell pool. The lack of a significant effect of Met and Cys restriction on proliferation and differentiation in older satellite cells, particularly 16 wk cells, lends support to the recommendation of Moore et al. (2005a,b) to formulate more specific diets to maximize satellite cell growth in age-dependent manner. The maximization of satellite cell growth could result in increased muscle growth, decreased myopathies, and increased feed efficiency, which would be highly beneficial for poultry producers.

Methionine and Cys restriction also differentially affected the expression of the heparan sulfate proteoglycans syndecan-4 and glypican-1. Syndecan-4 is primarily involved in mediating satellite cell migration through the RhoA signal pathway (Shin et al., 2013). Migration is critical in satellite cell function: without migration, satellite cells
would be unable to align and differentiate into myotubes. Glypican-1 is key macromolecule in promoting differentiation by regulating fibroblast growth factor 2 (FGF2) by sequestering the binding of FGF2 to its tyrosine kinase receptor (Brandan et al., 1996; Gutiérrez and Brandan, 2010; Velleman et al., 2013). The function of both of these heparan sulfate proteoglycans is thus critical in satellite cell proliferation and differentiation. The decrease in the expression of syndecan-4 is suggestive of decreased cell migration necessary for alignment preceding myotube formation, whereas the decrease in glypican-1 expression may lead to reduced differentiation.

Syndecan-4 and glypican-1 have also been shown to affect the expression of myogenic transcriptional regulatory factors MyoD, myogenin and MRF4 (Shin et al., 2012). The knockdown of syndecan-4 results in increased MyoD, myogenin and MRF4 expression during proliferation, while glypican-1 knockdown decreased MyoD, myogenin, and MRF4 expression during both proliferation and differentiation. The expression of syndecan-4, glypican-1 and the myogenic transcriptional regulatory factors is important as MyoD is required for proliferation, myogenin is required for differentiation (Hasty et al., 1993; Rudnicki et al., 1993; Seale et al., 2000; Yablonka-Reuveni and Rivera, 1994) and MRF4 has been shown to be expressed during proliferation (Shin et al., 2012). The current study shows that nutrition affects the expression of each myogenic transcriptional regulatory factor differently, and also affects each age differently. These data indicate that nutrition has a direct effect on the regulation of satellite cell proliferation and differentiation through the regulation of expression of the myogenic transcriptional regulatory factors MyoD, myogenin and MRF4.
In conclusion, nutrient restriction appears to have a greater impact on 1 d cells than it has on 7 wk or 16 wk cells. These data indicate that older satellite cells are less responsive to nutrition during the proliferation and differentiation. Understanding the response of satellite cells to nutritional status could result in the development of diets that maximize satellite cell potential leading to enhanced muscle mass accretion. Moore et al. (2005a,b) suggested that diets should be developed for different developmental stages to maximize satellite cell growth. The results from the present study support this suggestion. Although the current study shows that Met and Cys requirements of satellite cells change with age, the determination of optimal Met and Cys levels in feed to maximize satellite cell activity still needs to be determined. Optimizing satellite cell activity during the immediate posthatch period will result in increased muscle mass accretion and could potentially increase producer profits by either increasing breast muscle yield or decreasing time to market.
V. REFERENCES


Table 4.1. Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence&lt;sup&gt;1&lt;/sup&gt;</th>
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<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glypican-1</td>
<td>5'-CTT GTC GCT GTG GCA GAT CGG-3' (forward)</td>
<td>880-900</td>
<td>176 bp</td>
</tr>
<tr>
<td></td>
<td>5'-CTG CTG GAG CTT TTT GTG CTG A-3' (reverse)</td>
<td>1034-1055</td>
<td></td>
</tr>
<tr>
<td>Syndecan-4</td>
<td>5'-CTA CCC TGG CTC TGG AGA CCT-3' (forward)</td>
<td>102-122</td>
<td>234 bp</td>
</tr>
<tr>
<td></td>
<td>5'-TCA TTG TCC AGC ATG GTG TTT-3' (reverse)</td>
<td>315-335</td>
<td></td>
</tr>
<tr>
<td>Myogenin</td>
<td>5'-CCT TTC CCA CTC CTC TCC AAA-3' (forward)</td>
<td>813-833</td>
<td>175 bp</td>
</tr>
<tr>
<td></td>
<td>5'-GAC CTT GGT CGA AGA GCA ACT-3' (reverse)</td>
<td>967-987</td>
<td></td>
</tr>
<tr>
<td>MyoD</td>
<td>5'-GAC GGC ATG ATG GAG TAC AG-3' (forward)</td>
<td>553-572</td>
<td>201 bp</td>
</tr>
<tr>
<td></td>
<td>5'-AGC TTC AGC TGG AGG CAG TA-3' (reverse)</td>
<td>734-753</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GAG GGT AGT GAA GGC TGC TG-3' (forward)</td>
<td>504-523</td>
<td>200 bp</td>
</tr>
<tr>
<td></td>
<td>5'-CCA CAA CAC GGT TGC TGT AT-3' (reverse)</td>
<td>684-703</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Primers were designed from the following GenBank accession numbers: Glypican-1, AY551002.2; Syndecan-4, AY852251.1; MyoD, AY641567.1; Myogenin, AY560111.3; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), U94327.1
Table 4.2. Average satellite cell length in micrometers

<table>
<thead>
<tr>
<th>Age</th>
<th>1 day</th>
<th>7 week</th>
<th>16 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mg/L Met 96 mg/L Cys</td>
<td>90.26 ±4.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.96 ±5.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.48 ±6.25&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.5 mg/L Met 24 mg/L Cys</td>
<td>79.37 ±3.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88.23 ±3.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77.57 ±6.23&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 mg/L Met 9.6 mg/L Cys</td>
<td>78.22 ±6.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89.02 ±4.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.11 ±3.97&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0 mg/L Met 0 mg/L Cys</td>
<td>58.57 ±3.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.84 ±8.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.69 ±5.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-d</sup> satellite cell lengths within the same age without a common letter are significantly different (P< 0.05)
Table 4.3. Average myotube width in micrometers

<table>
<thead>
<tr>
<th>Age</th>
<th>1 day</th>
<th>7 week</th>
<th>16 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mg/L Met 96 mg/L Cys</td>
<td>39.05 ±4.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.09 ±2.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.16 ±1.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.5 mg/L Met 24 mg/L Cys</td>
<td>34.95 ±3.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.26 ±1.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.65 ±0.79&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 mg/L Met 9.6 mg/L Cys</td>
<td>39.39 ±1.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.57 ±2.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.36 ±0.77&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0 mg/L Met 0 mg/L Cys</td>
<td>21.09 ±1.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.20 ±1.24&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.08 ±0.51&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-d</sup> myotube widths within the same age without a common letter are significantly different (P< 0.05)
Figure 4.1. Effect of nutrition on proliferation. Proliferation of satellite cells from 1 d (A), 7 wk (B), and 16 wk (C) pectoralis major from posthatch randombred control line 2 turkeys treated with either 30, 7.5, 3, or 0 mg/L methionine (MET) with 3.2 mg/L cysteine (CYS) per 1 mg/L MET during proliferation. Average values were graphed with bars representing the standard error of the mean. Data was analyzed within time to evaluate the effect of MET and CYS treatment on proliferation. Values without common letters were considered significantly different at P < 0.05. P = proliferation.
Figure 4.2. Photographic plate of nutrient restriction during proliferation. Photographs of 1 d, 7 wk, and 16 wk satellite cells at 96 h of proliferation. Cells were treated with either 30 mg/L Met/96 mg/L Cys, 7.5 mg/L Met/24 mg/L Cys, 3 mg/L Met/9.6 mg/L Cys, or 0 mg/L Met/0 mg/L Cys. Scale bar represents 100 μm. The arrow in (a) indicate satellite cells that are aligning and in (c) indicate satellite cells that exhibit a rounded morphology. P = proliferation.
Figure 4.3. Effect of nutrition on differentiation. Differentiation of satellite cells from 1 d (A), 7 wk (B), and 16 wk (C) pectoralis major from posthatch randombred control line 2 turkeys treated with either 30, 7.5, 3, or 0 mg/L methionine (MET) with 3.2 mg/L cysteine (CYS) per 1 mg/L Met during differentiation. Average values were graphed with bars representing the standard error of the mean. Data was analyzed within time to evaluate the effect of treatment with MET and CYS on differentiation. Values were considered significant at $P < 0.05$. D = differentiation.
Figure 4.4. Photographic plate of the effect of nutrition during differentiation.
Photographs of 1 d, 7 wk, and 16 wk satellite cells at 48 h of differentiation. Cells were treated with either 30 mg/L Met/96 mg/L Cys, 7.5 mg/L Met/24 mg/L Cys, 3 mg/L Met/9.6 mg/L Cys, or 0 mg/L Met/0 mg/L Cys. Scale bar represents 100 μm. The arrows in a) and g) indicate myotubes that have formed. D = differentiation.
Figure 4.5. Syndecan-4 and glypican-1 mRNA expression. The mRNA expression of syndecan-4 (A,B) and glypican-1 (C,D) at 96 h of proliferation and 48 h of differentiation. Pectoralis major satellite cells were isolated from 1 d, 7 wk, and 16 wk old posthatch randombred control line 2 male turkeys and treated with different concentrations of methionine (MET) and cysteine (CYS). Average values were graphed with bars representing the standard error of the mean. Means without common letters represent values that are statistically different at P< 0.05. P = proliferation, D = differentiation.
Figure 4.6. MyoD, Myogenin, and MRF4 mRNA expression. The mRNA expression of MyoD (A,B) and myogenin (C,D) and MRF4 (E,F) at 96 h of proliferation and 48 h of differentiation. Pectoral major satellite cells were isolated from 1 d, 7 wk, and 16 wk old posthatch randombred control line 2 male turkeys and treated with different concentrations of methionine (MET) and cysteine (CYS). Average values were graphed with bars representing the standard error of the mean. Means without common letters represent values that are statistically different at P< 0.05. P = proliferation, D = differentiation.
CHAPTER 5

CONCLUSION

1. Introduction

Satellite cells were considered to be a homogenous population of cells until fairly recently. However, satellite cells are now known to be a heterogeneous population of multipotential stem cells that vary in their gene expression, proliferation, differentiation, maintenance of stem cell phenotype, and ability to transdifferentiate into other cell lineages. The satellite cell niche population, the satellite cells located in the niche between the sarcolemma and the basement membrane, also changes with animal age (Collins et al., 2007; Velleman et al. 2010; Li et al., 2011, Harthan et al., 2013). Satellite cells are primarily active during the growth phase of animals, declining to less than 5% of the total myofiber nuclei and become largely quiescent (Hawke and Garry, 2001). Turkeys are actively growing until they reach sexual maturity after 40 wk of age, well past market age. Changes that occur to satellite cell heterogeneity could be important in both the agricultural community and in the biomedical community, with potential implications in producer profitability and disease treatment and prevention.

Satellite cells are a dynamic heterogeneous population of cells that have altered expression of myogenic transcriptional regulatory factors and cell surface proteins such
as syndecan-4 and glypican-1 depending on their developmental stage (Hasty et al. 1993; Rudnicki et al. 1993; Yablonka-Reuveni and Rivera 1994; Seale et al. 2000; Velleman et al., 2010; Harthan et al., 2013). Syndecan-4 and glypican-1 play critical roles in satellite cell proliferation, differentiation, and FGF2 responsiveness. Syndecan-4 is important in satellite cell activation and proliferation (Cornelison et al., 2004), as well as in satellite cell migration by the modulation of the RhoA signal transduction pathway (Shin et al., 2013). Glypican-1 is primarily responsible for promoting differentiation by sequestering FGF2 from its tyrosine kinase receptors (Brandan et al., 1996; Gutiérrez and Brandan, 2010; Velleman et al., 2013). Expression of syndecan-4 and glypican-1 has also been shown to affect the expression of the myogenic transcriptional regulatory factors MyoD, myogenin and MRF4 (Shin et al., 2012b). Satellite cells that express one myogenic regulatory factor profile are also capable of giving rise to cells that express a different profile (Zammit et al., 2004). Change in satellite cell populations are important because it affects the function of satellite cells to become activated, proliferate and differentiate and contribute to muscle growth.

Studies have also shown that satellite cells are affected by nutrient status, indicating that the critical period for maintenance of satellite cell populations for muscle growth is during the first week posthatch (Halevy et al., 2000, 2001, 2003; Mozdziak et al., 2002; Moore et al., 2005a; Velleman et al., 2010b). Halevy et al. (2003) reported that turkey poults that were feed deprived for 48 h posthatch had decreased body weight and breast weight than their full-fed counterparts. This study also showed that satellite cells isolated from the feed deprived poults had a decreased number of satellite cells per g of breast muscle and proliferation that peaked 2 d later in the starved poults than in the full
fed poults. Satellite cell mitotic activity in turkey poults feed deprived for 72 h posthatch was highest at 6 d of age, while mitotic activity was highest at d 3 in full fed poults and declined through 10 d of age (Moore et al., 2005a). The objective of the current study was to determine how satellite cell changes and nutrient availability during growth may influence the postnatal or posthatch growth of muscle. The hypothesis was that satellite cell age affects satellite cell proliferation, differentiation, FGF2 responsiveness, and the expression of the cell surface proteoglycans syndecan-4 and glypican-1 as well as myogenic regulatory factor profile. It was also hypothesized that nutrient status affects satellite cell function through modulating syndecan-4 and glypican-1 expression and myogenic regulatory factor profile.

2. Effect of Age

Velleman et al. (2010) showed in a muscle development study from 1 d through 54 wk of age using isolated turkey primary pectoralis major satellite cells that proliferation was significantly decreased beginning at 8 wk of age. In the Velleman et al. (2010) study, primary satellite cells were isolated from 4 wk and 8 wk old birds. In the current study, proliferation and differentiation were significantly decreased in the 16 wk old satellite cells at all sampling times while there was no significant difference between the 1 d and 7 wk old satellite cells during proliferation. Thus, the results from the present study suggest that proliferation changes may begin between 7 and 8 wk of age. In the present study, differentiation was reduced in the 7wk old satellite cells compared to the 1 d satellite cells. However, decreased levels of differentiation may occur as early as 4 wk of posthatch age in the turkey (Velleman et al. 2010). These data demonstrate that there are significant changes in the ability of satellite cells to contribute to posthatch muscle
growth well before the satellite cells become senescent. In turkeys, 16 wk of age is the approximate market age, while the birds reach sexual maturity between 40 to 45 wk of age and are actively growing until this point. Changes in satellite cell activity prior to senescence are not unique to the turkey and have been reported in other species (Barani et al. 2003; Li et al. 2011).

Syndecan-4 is critical in the activation and proliferation of satellite cells (Cornelison et al. 2004), and their migration by mediating Rho A signal transduction (Shin et al. 2013). The migration of satellite cells is critical in the formation of multinucleated myotubes leading to subsequent muscle fiber formation. Glypican-1, on the other hand, affects the differentiation process through the sequestration of FGF2 from its tyrosine kinase receptor (Gutiérrez and Brandan 2010; Velleman et al. 2013). Fibroblast growth factor 2 is a strong inhibitor of differentiation and preventing FGF2 from binding to its receptor will permit differentiation to take place. Changes in the size of syndecan-4 and glypican-1 satellite cell subpopulations will affect both the proliferation and differentiation of the satellite cells. The percentage of satellite cells positive for syndecan-4 and glypican-1 decreased with age. These results represent the first demonstration of age-dependent changes in syndecan-4 and glypican-1 satellite cell populations. Furthermore, myoblasts deficient in glypican-1 have decreased expression of the muscle transcriptional regulatory factor myogenin (Gutiérrez and Brandan 2010). Myogenin expression is necessary for further muscle cell differentiation (Brunetti and Goldfine, 1990).

In addition to differences in the syndecan-4 and glypican-1 positive satellite cell populations with age, there were also age-associated changes in the mRNA and protein
expression of the myogenic regulatory factors MyoD and myogenin. By 16 wk of age during differentiation, the expression of both MyoD and myogenin was decreased compared to the younger satellite cells at both the protein and mRNA levels. Protein levels of MyoD and myogenin showed that the 16 wk cells expressed more MyoD and myogenin earlier than 1 d or 7 wk cells, but levels then decreased. The 1 d and 7 wk cells had more sustained expression of MyoD and myogenin. The mRNA and protein expression were not always directly correlated. It is possible that the protein levels detected during proliferation were caused by earlier translation of MyoD and myogenin mRNA. Regardless of the difference between the protein and mRNA expression, these data further support that older satellite cells in the growing animal have a reduced capacity to proliferate and differentiate.

The reduction in the expression of the heparan sulfate proteoglycans syndecan-4 and glypican-1 may be partially involved in the changes in MyoD and myogenin expression. The knockdown of syndecan-4 gene expression using small interfering RNA increased MyoD expression during proliferation only, whereas glypican-1 decreased both MyoD and myogenin during both proliferation and differentiation (Shin et al. 2012b). The change in the expression of these myogenic transcriptional regulatory factors, along with the altered expression of syndecan-4 and glypican-1, may be correlated with the decreased number of nuclei per myotube and reduced myotube diameter with increased satellite cell age as observed in the current study.

As satellite cell age increases, proliferation and differentiation decline, as does the syndecan-4 and glypican-1 positive satellite cell populations. Responsiveness to FGF2 is also age-dependent, with younger cells being more responsive to FGF2 during
proliferation and responding earlier to FGF2 during differentiation. Because syndecan-4 and glypican-1 are key molecules in the regulation of the factors shown to be affected by age-related changes in satellite cells, Specific Aim 3 explored the role of syndecan-4 and glypican-1 positive satellite cell populations on sustaining muscle cell proliferation and differentiation.

3. Effect of Syndecan-4 and Glypican-1 on Satellite Cell Function

The cell membrane-associated heparan sulfate proteoglycans, syndecan-4 and glypican-1 are differentially expressed during the proliferation and differentiation of satellite cells (Velleman et al. 2006, 2013; Zhang et al. 2007, 2008; Song et al. 2010, 2011, 2013; Shin et al. 2013). Both syndecan-4 and glypican-1 bind to FGF2 at their heparan sulfate chains and function as regulators of FGF2 signal transduction (Volk et al. 1999; Gutiérrez and Brandan 2010; Velleman et al. 2013). Yablonka-Reuveni et al. (1999) found that FGF2 plays a critical role in the recruitment of satellite cells into proliferation. However, the proliferative capacity of older satellite cells can be restored by the addition of FGF2 (Shefer et al. 2006).

In Specific Aim 1 of the current study, the response to FGF2 during both proliferation and differentiation was differentially affected by age. During proliferation, the 16 wk old satellite cells were the least responsive to FGF2. However, during differentiation the 1 d posthatch satellite cells responded the earliest to the inhibitory effects of FGF2 on differentiation followed by the 7 wk and then the 16 wk old satellite cells. Because glypican-1 is a regulator of FGF2 binding to its receptor during differentiation and the glypican-1 satellite cell population is decreased in 16 wk old cells
during differentiation, it is possible that the decreased levels of glypican-1 permits more FGF2 to bind to its receptor further inhibiting differentiation in the older satellite cells.

Song et al. (2013) previously reported that syndecan-4 and glypican-1 expression differs by developmental stage of the satellite cell, as well as between the RBC2 and the growth-selected F-line and between sex. The 1 d cells and 7 wk cells that overexpressed syndecan-4, glypican-1, and syndecan-4 with glypican-1 had decreased proliferation at 72 h PT, whereas the 16 wk cells overexpressing syndecan-4, glypican-1, and syndecan-4 with glypican-1 had no difference. These data indicate that 1 d and 7 wk cells were affected similarly by the overexpression of syndecan-4, glypican-1, and syndecan-4 with glypican-1 during proliferation, while the 16 wk cells were unaffected. While 16 wk cells have smaller subpopulations that express syndecan-4 and glypican-1 than the 1 d or 7 wk cells, it does not appear that increasing the expression of syndecan-4 and glypican-1 has a significant effect on satellite cell proliferation in 16 wk cells.

There was also no significant difference between the 1 d, 7 wk and 16 wk cells that were overexpressing syndecan-4, glypican-1, and syndecan-4 with glypican-1 compared to those transfected with the CON empty vector during differentiation. Previous studies have demonstrated that transfecting 7 wk cells with glypican-1 results in a growth curve that has higher proliferation early on that levels off at 72 h PT (Velleman et al., 2007; Zhang et al., 2007; Song et al., 2010). The overexpression of glypican-1 also results in an increase in differentiation early during differentiation that levels off to become similar to (Zhang et al., 2007; Song et al., 2010) or lower than (Velleman et al., 2007) cells transfected with CON. While the current study does not show a significant effect on proliferation or differentiation, the data does follow a similar trend as previous
studies, demonstrating that overexpressing syndecan-4 and glypican-1 does not significantly impact the function of 16 wk cells.

Syndecan-4 and glypican-1 can both regulate FGF2 signal transduction by binding FGF2 to their heparan sulfate chains (Volk et al., 1999; Gutiérrez and Brandan, 2010; Velleman et al., 2013). The proliferative capacity of older satellite cells can be increased by the addition of exogenous FGF2 (Shefer et al., 2006). Fibroblast growth factor 2 has been shown to increase the number of satellite cells capable of proliferating (Yablonka-Reuveni et al., 1999) and is a stimulator of proliferation (Dollenmeier et al., 1981). Because of the role of syndecan-4 and glypican-1 in the regulation of FGF2 signal transduction, it was thought that increasing the expression of syndecan-4 and glypican-1 in 16 wk cells would increase the satellite cell response to FGF2. The results from Specific Aim 3 of the present study show no increased responsiveness to FGF2 during proliferation or differentiation with the transfection of syndecan-4 with glypican-1 in the 1 d, 7 wk, or 16 wk cells. Overexpression of syndecan-4 decreased FGF2 responsiveness in 1 d cells, while overexpression of glypican-1 increased FGF2 responsiveness in 1 d and 7 wk cells at 72 h of proliferation. Therefore, syndecan-4 and glypican-1 expression does not appear to be a limiting factor in responsiveness to FGF2 in the 1 d, 7 wk, and 16 wk cells.

Understanding how syndecan-4 and glypican-1 affects the expression of the myogenic regulatory factors MyoD, myogenin and MRF4 is also important in understanding the effect of syndecan-4 and glypican-1 on satellite cell function. Expression of MyoD, myogenin, and MRF4, is decreased at 72 h proliferation in all overexpressing 1 d cells compared to the control 1 d cells and myogenin in glypican-1
overexpressing cells at 48 h of differentiation in the current study. In 7 wk cells, MyoD and MRF4 expression is increased in cells overexpressing syndecan-4, while myogenin is decreased in cells overexpressing glypican-1 and syndecan-4 with glypican-1 at 72 h of proliferation and increased in cells overexpressing syndecan-4 at 48 h of differentiation. The 16 wk cells were unaffected by overexpression at all times as compared to the cells transfected with the control vector. Knockdown studies on the effect of glypican-1 and syndecan-4 on the expression of MyoD, myogenin and MRF4 showed that knockdown of syndecan-4 resulted in increased MyoD, myogenin and MRF4 expression, while knockdown of glypican-1 caused a decrease in MyoD, myogenin and MRF4 expression (Shin et al., 2012b). Because of these studies, it was expected that the overexpression of syndecan-4 would result in decreased MyoD, myogenin and MRF4 expression, while the overexpression of glypican-1 would result in increased MyoD, myogenin and MRF4 expression. However, this was not the case, suggesting that the overexpression of syndecan-4 and glypican-1 has little to no effect on the mRNA expression of myogenic regulatory factors in 7 wk and 16 wk cells, or in the 1 d cells at 48 h of differentiation. There was also no significant effect of overexpression of syndecan-4, glypican-1, and syndecan-4 with glypican-1 on proliferation or differentiation. Thus, because of the requirement of MyoD for proliferation and myogenin for differentiation (Hasty et al., 1993; Rudnicki et al., 1993; Seale et al., 2000; Yablonka-Reuveni and Rivera, 1994), and the presence of MRF4 during proliferation (Shin et al., 2012b), it stands to reason that myogenic regulatory factor expression would remain largely unchanged.

The increase in MyoD and MRF4 mRNA expression observed in the 1 d, 7 wk, and 16 wk cells that overexpress syndecan-4 with glypican-1 and are also treated with
exogenous FGF2 should lead to an increase in proliferation in these cells at 72 h PT. However, no significant increase in proliferation is observed. The same is true in myogenin mRNA expression, where increased myogenin mRNA should result in increased differentiation. No significant increase in differentiation is observed, which points to a possible post-transcriptional regulation of the myogenic regulatory factors MyoD, myogenin and MRF4, resulting in increased MyoD and MRF4 mRNA expression with no increase at the protein level.

The current study demonstrated that syndecan-4 and glypican-1 do not significantly increase satellite cell proliferation and differentiation in the 16 wk cells. Further work is needed to identify other age-related changes in satellite cell function, as well as other molecules that may be involved in the age-related changes in satellite cell proliferation, differentiation, FGF2 responsiveness and the expression of myogenic regulatory factors.

4. Effect of Nutrient Status

There have been several studies investigating the impact of feed restrictions on muscle growth in poultry, indicating that feed restrictions that take place in the immediate posthatch period generally result in decreased muscle growth and altered breast muscle morphology (Halevy et al., 2000; Velleman et al., 2010b). Increased fat depots were observed in feed restricted broilers in Velleman et al. (2010b). This led to the hypothesis that the satellite cells that are responsible for posthatch muscle growth may be induced by nutrient restriction to transdifferentiate to adopt an adipogenic fate. Satellite cells have been shown to be capable of adopting such a fate when cultured under certain conditions (Asakura et al., 2001).
Halevy et al. (2003) reported that turkey poults that were feed deprived for 48 h posthatch had decreased body weight and breast weight than their full-fed counterparts. Starved poultcs also had a decreased number of satellite cells per g of breast muscle and proliferation that peaked 2 d later compared to those that were not feed deprived. Satellite cell mitotic activity in turkey poultcs feed deprived for 72 h posthatch was highest at 6 d of age, while mitotic activity was highest at d 3 in full fed poultcs and declined through 10 d of age (Moore et al., 2005a). A 72 h posthatch feed deprivation was also shown to decrease body weight and myofiber cross-sectional area in turkey poultcs compared to those that were not feed deprived (Moore et al., 2005a).

In Specific Aim 4 of the present study, satellite cells isolated from three different ages of turkey poultcs were used to quantify the effect of nutrient restriction on satellite cells at different ages. During proliferation, decreasing concentrations of Met and Cys resulted in decreased proliferation that was most pronounced in 1 d cells and became less pronounced as satellite cell age increased. Decreased proliferation resulted in fewer satellite cells that were available for differentiation, which limited the number of satellite cells available to fuse and contribute to muscle growth.

Nutrient restriction was also determined to have an effect on satellite cell differentiation. The results from Specific Aim 4 of the present study showed that lower concentrations of Met and Cys but not 0 mg/L increased differentiation in the 1 d cells, while having little or no effect on differentiation in the 7 wk and 16 wk cells. These data support that satellite cells at different developmental ages have different nutrient requirements for differentiation and proliferation. The decreased proliferation and increased differentiation seen in the 1 d satellite cells treated with reduced Met and Cys
medium may result in a significantly reduced pool of satellite cells as the satellite cell pool fails to replenish itself. Differentiation that is less affected might mean that the available satellite cells differentiate to form myotubes, effectively removing themselves from the satellite cell pool. The lack of significant effect of Met and Cys restriction on proliferation and differentiation in older satellite cells, particularly 16 wk cells, lends support to the recommendation of Moore et al. (2005a,b) to formulate more specific diets to maximize satellite cell growth. The maximization of satellite cell growth could result in increased producer profits by improving muscle growth, decreasing myopathies, and increasing feed efficiency.

Methionine and Cys restriction also differentially affected the expression of the heparan sulfate proteoglycans syndecan-4 and glypican-1. Syndecan-4 is primarily involved in mediating satellite cell migration through the RhoA signal pathway (Shin et al., 2013). Migration is critical in satellite cell function: without migration, satellite cells would be unable to align and differentiate into myotubes. Glypican-1 is key in promoting differentiation by preventing fibroblast growth factor, an inhibitor of differentiation (Dollenmeier, 1981), from binding to its receptor (Brandan et al., 1996; Gutiérrez and Brandan, 2010; Velleman et al., 2013). The function of both of these heparan sulfate proteoglycans is critical in satellite cell proliferation and differentiation, and the expression of syndecan-4 and glypican-1 mRNA can indicate how capable satellite cells at migrating and differentiating. Declining expression of syndecan-4 indicates decreased migration, while decreased glypican-1 expression points towards lower rates of differentiation.
Syndecan-4 and glypican-1 have also been shown to affect the expression of myogenic regulatory factors. The knockdown of syndecan-4 results in increased MyoD, myogenin and MRF4 expression during proliferation, while glypican-1 knockdown decreased MyoD, myogenin, and MRF4 during both proliferation and differentiation (Shin et al., 2012). The expression of syndecan-4, glypican-1 and the myogenic regulatory factors is important because MyoD is required for proliferation, myogenin is required for differentiation (Hasty et al., 1993; Rudnicki et al., 1993; Seale et al., 2000; Yablonka-Reuveni and Rivera, 1994) while MRF4 has been shown to be expressed during proliferation (Shin et al., 2012).

The results from Specific Aim 4 of the current study indicate that older cells are better equipped to deal with nutrient restriction while still maintaining proliferation and differentiation. Understanding the response of satellite cells to nutrient restriction could result in the development of diets that restrict nutrients at specific times to reduce feed costs while still maximizing satellite cell potential. Moore et al. (2005a,b) postulates that diets could be developed for different developmental stages to maximize satellite cell growth. Optimizing satellite cell growth early on could result in increased muscle growth, increased breast muscle yield and could potentially increase producer profits by either increasing yield or decreasing time to market.

5. Conclusion

Understanding how age affects satellite cell function is important in several ways. Changes that occur to the heterogeneity and functionality of satellite cells during the early stages of growth and while the animal is still growing have potential to lead to advances in treatment of muscular dystrophies and myopathies in both biomedical and...
agricultural applications. The formulation of diets that would take maximal advantage of these age related changes in satellite cell function and result in optimized growth has great agricultural potential. Optimizing muscle growth through diet manipulation could result in increased producer profit by reducing nutrient waste, increasing growth rate, increasing feed conversion and decreasing time to market.
6. References


BIBLIOGRAPHY


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