THE ROLE OF SYNDICAN-4 IN MUSCLE GROWTH AND DEVELOPMENT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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ABSTRACT

Muscle formation is a complex process that is regulated by the interactions between muscle cells and the extracellular matrix (ECM). The ECM is composed of fibrous proteins like the collagens and non-fibrous proteins including the proteoglycans. The proteoglycans can be divided into four groups: heparan sulfate, dermatan sulfate, chondroitin sulfate, and keratan sulfate proteoglycans, according to the type of side chains attached to the core protein. Heparan sulfate proteoglycans are required during muscle growth and development. Glypicans and syndecans are two main families of heparan sulfate proteoglycans. Six glypicans are expressed in mammals, but only glypican-1 is expressed in skeletal muscle. Four syndecans compose the syndecan family, and all of them are expressed in skeletal muscle. In the current study, focus was placed on glypican-1 and syndecan-4 for their essential role in muscle growth and development. Both glypican-1 and syndecan-4 are composed of a core protein and covalently attached glycosaminoglycan (GAG) and N-linked glycosylated (N-glycosylated) chains. The N-glycosylated chains play important roles in protein folding and cell membrane localization. Glypican-1 N-glycosylated chains were shown to have important roles in regulating turkey satellite cell proliferation, differentiation, and cellular responsiveness to fibroblast growth factor 2 (FGF2) by interacting with the GAG chains. In contrast, syndecan-4 N-glycosylated chains increased satellite cell proliferation by
interacting with GAG chains but had no effect on satellite cell differentiation. Syndecan-4 regulates satellite cell proliferation in an FGF2-independent manner. The serine residue in the C1 region and the tyrosine residue in the variable region of the cytoplasmic domain of syndecan-4 are two critical amino acids in modulating satellite cell proliferation and differentiation. The deletion of the cytoplasmic domain of syndecan-4 increased cellular responsiveness to FGF2 and further studies demonstrated that the serine residue in the C1 domain of the syndecan-4 cytoplasmic domain is critical in regulating cellular responsiveness to FGF2. The cytoplasmic domain of syndecan-4 is also critical in modulating the activity of protein kinase C alpha (PKCα) which is a critical kinase in RhoA pathway regulation of cell growth and development. Both syndecan-4 and PKCα are important focal adhesion components. Focal adhesions are the areas that cells stably attach to substrates and contain cell membrane receptors including integrins, cytoskeleton proteins and signaling proteins. The cytoplasmic domain of syndecan-4 and the N-glycosylated and GAG chains affect cell membrane localization of β1-integrin and β-actin but have no function on the formation of vinculin-containing focal adhesions. The discovery that syndecan-4 had no effect on satellite cell apoptosis further supported this conclusion as focal adhesions are critical in the survival of adherent cells. This study provides new information on the role of glypican-1 and syndecan-4 in muscle growth and development which will benefit the domestic animal industries in terms of selection strategy and also human health in terms of muscle maintenance and regeneration.
DEDICATION

This document is dedicated to my family.
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I finish with a final silence of gratitude for my life.
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**Abstracts**


**FIELDS OF STUDY**

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CHAPTER 1: LITERATURE REVIEW

1.1 Background

Tissue and organ formation begins in the fertilized oocyte to form a multifunctional organism. During animal embryogenesis, three germ layers are formed: ectoderm, endoderm, and mesoderm. The ectoderm differentiates to form skin and the nervous system, the endoderm forms the gastrointestinal tract, respiratory tract, endocrine glands and organs, and the mesoderm forms bone, connective tissue, and muscle.

Muscle is the contractile tissue of an animal which generates forces and movements. It composes about 50% of an animal’s body weight. As people age, they lose skeletal muscle mass. By the age of 60-70 years, about 25 to 30% of skeletal muscle mass will be lost because of a decrease in muscle fiber number and size (Brooks and Faulkner, 1994; Frontera et al., 2000). With decreased muscle mass, people have reduced muscle strength, increased health problems and decreased quality of life due to a sedentary lifestyle.

In animal science, muscle is converted into meat. The consumption of poultry meat has increased significantly in the past decades and is expected to continue to increase. The poultry industry has invested significant capital into developing higher yielding poultry lines with increased breast meat yield which is the most economically valuable muscle. However, the selection for growth and breast muscle yield has resulted
in meat quality problems such as pale, soft, exudative condition which results in a loss of water holding capacity. It is estimated that pale, soft, exudative meat costs about $200 million in economic loss to processors per year (Lubritz, 2007). To decrease muscle mass loss with aging, increase poultry meat yield, and maintain a high quality meat product requires an understanding of the mechanisms of how skeletal muscle growth and development are regulated.

1.2 Muscle

There are three types of muscle: smooth muscle, cardiac muscle, and skeletal muscle. Among them, cardiac muscle and skeletal muscle are also called striated muscle because of the transverse banding pattern under the microscope (Figure 1.1).

1.2.1 Smooth muscle

Smooth muscle composes walls of hollow organs and tubes, such as the stomach, bladder, uterus, and blood vessels. The contraction of smooth muscle is controlled by the autonomic nervous system, hormones, and autocrine/paracrine agents. There is one nucleus in the center of the spindle-shaped cells. The contraction of smooth muscle is slow and fatigue resistant.

1.2.2 Cardiac muscle

Cardiac muscle is only located in the heart. It is controlled by the autonomic nervous system, hormones, and autocrine/paracrine agents which is the same as smooth
muscle. The cardiac muscle cell has a rectangular shape with only one centrally located nucleus. The contraction of cardiac muscle is strong, involuntary, and rhythmical.

1.2.3 Skeletal muscle

Skeletal muscle is the largest tissue in the body and it composes almost half of the human body mass (Huard et al., 2002). Skeletal muscle attaches to bones through tendons, and is responsible for supporting and moving the skeleton. The contraction of skeletal muscle is controlled by the central nervous system, and thus is under voluntary control. Skeletal muscle cells are multinucleated cylindrically shaped long fibers, ranging from 1 to 40 mm. The nuclei are usually located parallel to the long axis of the fiber beneath the sarcolemma.

The muscle fibers are surrounded by connective tissues (Figure 1.2). Outside of the individual muscle is a connective tissue layer called epimysium, which protects muscles from friction against other muscles and bones. Each muscle contains muscle fiber bundles (fasciculus), and each of the muscle fiber bundles are surrounded by perimysium. The perimysium is responsible for shaping and organizing the muscle fibers, as well as transmitting forces within the muscle. Each of the individual muscle fibers is surrounded by a connective tissue sheath called endomysium. The endomysium covers muscle cell membrane and contains nerves, capillaries, and lymphatics.

Muscle fibers are composed of myofibrils which contain sarcomeres, the basic unit of the contractile structure. Sarcomeres are composed of thick filaments and thin filaments. They compose about 65% of the protein in the myofibril and they are
responsible for muscle contraction. The thick filaments are composed of myosin, and the thin filaments contain actin, as well as troponin and tropomyosin.

Upon receiving action potential signals from the nervous system, calcium is released from the sarcoplasmatic reticulum. The released calcium binds to troponin and changes the conformation of binding between tropomyosin and actin which allows the binding of myosin and actin. The ATP-driven interactions between myosin and actin lead to the contraction of the sarcomeres, and the whole muscle.

1.3 Skeletal muscle growth and development

Muscle cells are derived from mesenchymal stem cells. Some of these stem cells proliferate, align with each other, and then fuse to form multinucleated myotubes. After the formation of primary myofibrils, secondary myofibrils form and then align with each other to form functional mature muscle fibers. This process happens during embryonic period and is called hyperplasia. At the time of hatch, muscle fiber number has been determined (Smith, 1963). Posthatch muscle growth (hypertrophy) is characterized by the enlargement of existing muscle fiber size. Satellite cells are responsible for hypertrophy and muscle regeneration.

1.3.1 Myogenesis

In the early stage of embryonic development, ectoderm, mesoderm, and endoderm layers form through a process called gastrulation. Mesoderm will form bone, connective tissue, and muscle. On both sides of the neural tube, the mesoderm is divided into four different layers: axial mesoderm, intermediate mesoderm, paraxial mesoderm, and the
lateral plate mesoderm. Almost all of the embryonic skeletal muscles are derived from the paraxial mesoderm. Paraxial mesoderm separates into somites which are clusters of cells. Cells from the dorsal part of the somites form the dermomyotome. Muscle progenitor cells delinate from the four edges of the dermomyotome and migrate to the areas that muscle will form and then become myoblasts through the action of the myogenic regulatory factors. These myoblasts increase the expression of Myf5, Mrf4, and MyoD, and differentiate into myocytes through the action of myogenin, Mrf4, and MyoD. The myocytes eventually fuse and mature into multinucleated muscle fibers.

Wnt, sonic hedgehog (Shh), and transcription factor Pax3 are involved in the early myogenesis in which mesodermal cells migrate into the areas of muscle formation and are determined into mitotically dividing myoblasts. Wnt and Shh function to commit mesoderm cells to the myogenic lineage, and Pax3 promotes determined cells to migrate and proliferate (Goulding et al., 1994). Fibroblast growth factor (FGF), Shh, and Pax7 are involved in late myogenesis, in which myoblasts are terminally differentiated and aligned into myotubes. Fibroblast growth factor regulates myoblast proliferation and differentiation (Clegg et al., 1987; Sheehan and Allen, 1999). Shh regulates myoblast proliferation and induces myosin heavy chain expression, and Pax7 is specific for satellite cells.

1.3.2 Muscle regeneration

Muscle regeneration is a complex process, and it is still not well understood. Adult muscle wound healing has three overlapping phases: inflammation, tissue formation, and tissue remodeling (Huard et al., 2002). Also with the healing process,
scar tissue forms. At the site of injury, many growth factors, for example, FGF, transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), and hepatocyte growth factor (HGF) are up-regulated (Husmann et al., 1996; Grounds, 1999), and Pax7 expressing satellite cells are activated. The activation of satellite cells is similar to embryonic myogenesis, which is controlled by Pax3, Pax7, Myf5, and MyoD. Quiescent satellite cells do not express any myogenic regulatory factors. The up-regulation of MyoD and Myf5 leads to the proliferation of satellite cells (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994; Cornelison and Wold, 1997; Cooper et al., 1999). At this stage, these satellite cells are called myoblasts. The up-regulation of myogenin and Mrf4 (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994; Cornelison and Wold, 1997) and down-regulation of Pax7 (Olguin and Olwin, 2004) lead to the differentiation of these myoblasts. These differentiated myoblasts either fuse with each other to form new myofibers or fuse to existing damaged myofibers for muscle regeneration (Hawke and Garry, 2001; Chargé and Rudnicki, 2004).

1.3.3 Satellite cell

Satellite cells were first described by Mauro (1961) and were named due to their peripheral location in skeletal muscle fibers: between the basement membrane and the sarcolemma. Satellite cells are undifferentiated mononuclear myogenic precursor cells. The progenitor cells from the dermomyotome give rise to satellite cells (Armand et al., 1983; Gros et al., 2005), but whether this is the only source of satellite cells is still unclear.
Satellite cells have the ability to self-renew, maintain, repair and increase muscle fiber size by proliferation, differentiation and fusion with existing muscle fibers (Moss and LeBlond, 1971; Kuang et al, 2007). Satellite cells are normally quiescent in adults (Schultz et al., 1978; Cornelison and Wold, 1997). However, when skeletal muscle tissue is injured or heavily used, the satellite cells become active and re-enter the cell cycle (Changé and Rudnicki, 2004; Dhawan and Rando, 2005). Satellite cells proliferate, fuse with damaged myofibers or form new myofibers through a process similar to fetal muscle formation (Parker et al., 2003).

Quiescent satellite cells express the transcription factor Pax7 (Seale et al., 2000), cell adhesion protein M-cadherin (Irintchev et al., 1994), myogenic regulatory factor Myf5 (Tajbakhsh et al., 1997; Beauchamp et al., 2000; Shefer et al., 2006) and saliomucin CD34 (Beauchamp et al., 2000). Cell membrane proteins syndecan-3 and syndecan-4 (Cornelison et al., 2001), lysenin (Nagata et al., 2006) and caveolin 1 (Volonte et al., 2005) are also quiescent satellite cell markers. When activated, satellite cells begin to express muscle-specific proteins like desmin and myogenic transcription factors, such as MyoD, myogenin (Füchtbauer and Westphal, 1992; Grounds et al., 1992; Yablonka-Reuveni and Rivera, 1994), and MRF4 (Smith et al., 1993, 1994). Myf5, Pax7, M-cadherin, and CD34 are also expressed in activated satellite cells. Figure 1.3 illustrates the expression of satellite cell membrane markers at different stages of myogenesis.

The activities of satellite cells can be regulated by their extracellular matrix. The extracellular matrix is secreted by cells and feeds back information to the cell by sending
signals into the cell. Bischoff (1990) demonstrated that the behavior of satellite cells can be regulated by their extracellular matrix. The number of satellite cells decreases in aged animals, however, the capacity to self-renew, proliferate and differentiate remains the same (Shefer et al., 2006). Satellite cells from young animals showed decreased activation when they are grafted to an aged animal whereas satellite cells from aged animal had enhanced activation when they are grafted to a young animal (Zacks and Sheff, 1982; Carlson and Faulkner, 1989).

Growth factors in the extracellular matrix play a key role in regulating cell function. Growth factors can be produced and secreted by muscle cells and secreted to the extracellular matrix where they are bound to proteoglycans. Growth factors that are involved in regulating satellite cell growth and differentiation are TGF-β, IGF, HGF, and FGF (Hawke and Garry, 2001). Transforming growth factor beta inhibits satellite cell differentiation through the myogenic lineage (Allen and Boxhorn, 1987). Insulin like growth factor 1 increases proliferation and differentiation by increasing the expression of cell-cycle progression factors during proliferation (Engert et al., 1996) and induces myogenic regulatory factor expression during differentiation (Musaro and Rosenthal, 1999). Hepatocyte growth factor can stimulate quiescent satellite cells to enter the cell cycle, increase cell proliferation, and inhibit satellite cell differentiation (Allen et al., 1995; Gal-Levi et al., 1998; Miller et al., 2000). Fibroblast growth factor is a strong stimulator of satellite cell proliferation and inhibitor of satellite cell differentiation (Allen and Boxhorn, 1989; Yablonka-Reuveni et al., 1999).
Self-renewal is a unique and crucial property of satellite cells which gives satellite cells the ability to maintain the integrity of skeletal muscle throughout the lifespan of an animal. Collins et al. (2005) showed that satellite cells in grafted single isolated myofibers can proliferate and differentiate to generate large amount of muscle tissue, and expand the satellite cell population that is able to mediate further regeneration in the engrafted muscle after injury. This is the direct evidence for satellite cell self-renewal. There are two different mechanisms for satellite cell self-renewal: symmetric and asymmetric division of satellite cells (Collins et al., 2005; Dhawan and Rando, 2005). Symmetric division results in the activation and proliferation of both daughter cells (Figure 1.4a). The cells maintain Pax7 expression, and down-regulating MyoD will self-renew the cell and return to the quiescent state. Asymmetric division generates two daughters, one of them differentiates and the other one returns to the quiescent state (Figure 1.4b). The study from Shinin et al. (2006) supports satellite cell asymmetric division. They found that template DNA strands are selectively inherited in only one daughter cell after satellite cell proliferation and after injury using a mice single muscle fiber assay.

1.4 Extracellular matrix

Muscle growth is regulated by the accurate expression of muscle specific genes and the interaction of muscle cells with their extrinsic extracellular matrix environment. The extracellular matrix is not only a substrate for the cells to adhere to, but also an organized dynamic structure that regulates intracellular communication and cell behavior.
Extracellular matrix is composed of fibrous proteins and polysaccharides that are secreted by cells (Figure 1.5).

1.4.1 Fibrous protein

a. Collagen

Collagen is the most abundant protein in animal bodies and it is the major protein in the extracellular matrix. Collagens can interact with other collagens, glycoproteins, proteoglycans, cell surface receptors, and growth factors. Collagens fibers can also provide structural support to cells, contributes to the tensile strength, integrity and function of tissues. At least 29 different types of collagens have been identified (Söderhäll et al., 2007). All collagens consist of 3 coiled polypeptide α chain subunits, which form a right-handed triple helix. Among them, type I, II and III are the most abundant collagens.

In the skeletal muscle extracellular matrix, type I and III are the primary expressed collagens, and both of them have a central triple helical domain that consists of a repeating amino acid sequence of Gly-X-Y, X and Y can be any amino acids. Usually X and Y are proline and hydroxyproline which compose about 1/6 of the total sequences. The formation of most collagens is similar as type I collagen (Figure 1.6). Type I collagen is composed of two types of peptide chains: α1 and α2 chains. These two chains are called preprocollagen and they are formed during translation on ribosomes along the rough endoplasmic reticulum. These polypeptide chains are released in the rough endoplasmic reticulum lumen in which signal peptides on both N and C ends are cleaved. The polypeptides without signal peptides are called pro-α chain. Inside of the rough
endoplasmic reticulum lumen, lysine and proline are hydroxylated, and some specific hydroxylysines are glycosylated. Two $\alpha_2$ chains and one $\alpha_1$ chain form a triple helical structure which is called procollagen. After being transported to the Golgi apparatus, procollagen is packaged and secreted out of the cell by exocytosis. Tropocollagen is formed after the registration peptides are cleaved by procollagen peptidase. Tropocollagens assemble via hydrophobic and electrostatic interactions into head-to-tail arrays to form collagen fibrils. The adjacent collagen fibrils are displaced about a quarter of their length and this assembly is called a quarter-staggered array which causes a striated effect under the electron microscope. Collagen fibrils form collagen fibers.

b. Fibronectin

Fibronectin is a rod-like glycoprotein. Fibronectin is composed of two similar polypeptide chains that are attached by disulfide bonds (Mao and Schwarzbauer, 2005). Each fibronectin polypeptide chain contains three types of modules: type I, II, and III (Yamada, 1991). Typically, 12 type I modules, 2 type II modules and 15 to 17 type III modules compose each of the fibronectin polypeptides. Type I or type II modules contain disulfide bonds that cross-link beta-strands of the module. There is no disulfide bond in a type III module. These modules form protein-binding domains which allow fibronectin to interact with other molecules. There are four fibronectin binding domains and a cell attachment domain that contains an Arg-Gly-Asp (RGD) sequence. The fibronectin binding domains allow fibronectin to bind to other fibronectin molecules and the cell attachment domain allows cells to attach to the matrix through $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins on the cell surface (Ruoslhti and Pierschacher, 1987).
There are over 20 isoforms of fibronectin, but all come from a single gene (Pankov and Yamada, 2002). It can bind to collagen and cell surface receptors to stabilize the attachment of cells to the extracellular matrix, maintain cell shape by organizing the cytoskeleton, and create a cross-linked network in the extracellular matrix. Fibronectin also plays a critical role during embryonic development, blood clotting, tissue repair, cell adhesion, and migration.

c. Elastin

Elastin is an elastic, insoluble protein in connective tissue. It allows tissues to stretch and return to their original state. It mainly exists in the aorta, lung parenchyma, ligaments, skin, and cartilage (Keeley et al., 2002).

Elastin is synthesized as tropoelastin. Monomer tropoelastin is subsequently assembled into a stable polymeric structure (Vrhovski and Weiss, 1998). Tropoelastin has 10 rigid α-helical regions which are rich of proline and aniline and flexible domains that consist of extended and folded structures. Tropoelastins interact with each other to form polymeric elastin through the binding of side chains of lysine residues. Polymeric elastin is very stable and insoluble.

1.4.2 Proteoglycan

Proteoglycans are a major component of the extracellular matrix. They are glycosylated proteins which have covalently linked glycosaminoglycan (GAG) chains (Hardingham and Fosang, 1992) and N-linked glycosylated chains (N-glycosylated chain) attached to a center core protein. Glycosaminoglycan chains are long unbranched polysacharides consisting of a repeating disacharide unit which are covalently attached to
the core protein at Ser-Gly repeats. Glycosaminoglycan chains can be divided into four different types: heparan sulphate, chondroitin sulphate, dermatan sulphate, and keratan sulphate. N-linked glycosylated chains are polysaccharide chains attached to the core protein at Asn residues at the sequences Asn-Xaa-Ser/Thr. Xaa can be any amino acids except proline (Kornfeld and Kornfeld, 1985).

Proteoglycans can also be categorized by size: small leucine-rich proteoglycans (SLRPs), and the modular proteoglycans (Iozzo and Murdoch, 1996). The SLRPs are compact proteins with alternating hydrophobic and hydrophilic amino acid residues, and they all contain conserved leucine residues. The SLRPs include decorin, biglycan, fibromodulin and lumican. Examples of modular proteoglycans are aggrecan and versican. Aggrecan is the major proteoglycan in cartilage, and versican is present in many adult tissues including blood vessels and skin.

Proteoglycans have a net negative charge that can attract water and keep cells hydrated. Proteoglycans are involved in a variety of biological processes by modulating growth factor activity, organizing the extracellular environment, and maintaining tissue structure and function.

a. Hyaluronic acid

Hyaluronic acid is also called hyaluronan or hyaluronate. It is a non-sulfated GAG. It consists of 2,000 to 25,000 repeating units of the disaccharide, D-glucuronic acid and N-acetylglucosamine. The molecular mass of hyaluronic acid can be up to 10,000 kDa, and it can extend to 2-25 mm in length.
Hyaluronic acid is found primarily in connective, epithelial, and nerve tissues. The functions of hyaluronic acid are, in part, dependent on its hydrophilic and hydrodynamic properties which allow it to hold water and play a structural role. Hyaluronic acid absorbs significant amounts of water and lubricates and cushions body tissue. And it interacts with extracellular matrix macromolecules and constitutes a scaffold on which large aggregating proteoglycans, including aggrecan and versican are ionically bound to increase water holding capacity. Aggrecan and versican are both large chondroitin sulfate proteoglycans.

Hyaluronic acid is recognized by the cell membrane receptor, CD44. The binding to CD44 triggers several intracellular signaling pathways regulating cell proliferation, migration, and regeneration. Hyaluronic acid creates spaces for cells to divide and migrate during the embryonic period (Day and Prestwich, 2002), and decreases cell-cell interactions and increases cell migration in post-embryonic development (Stern, 2003).

b. Aggrecan and versican

Versican and aggrecan are both large chondroitin sulfate proteoglycans. Chondroitin sulfate is an unbranched sulfated polysaccharide. Versican and aggrecan are composed of two alternative residues of D-glucuronic acid and N-acetyl-D-galactosamine. The size of the chondroitin sulfate chains is variable, and they can be sulfated in variable positions and quantities. Chondroitin sulfate chains are attached to the proteins on the hydroxyl groups of serine resides at the sequence of Ser-Gly. Chondroitin sulfates are important structural components of cartilage, tendons, and aorta walls. The net negative charge of these molecules forms ionic interactions and provides
resistance to compression to the tissues. Chondroitin sulfates are major components of the extracellular matrix. They contribute to tissue structural integrity and interact with proteins in the extracellular matrix to regulate cellular activities.

Versican is expressed in the extracellular matrix of a variety of tissues and organs. It has one N-terminal and one C-terminal globular domain with a GAG binding region in the middle. The N-terminal globular domain has an immunoglobulin-like motif and a hyaluronan binding region. The C-terminal globular domain has a lectin-like domain, a carbohydrate recognition domain, and two epidermal growth factor domains. The GAG binding region attracts positively charged molecules including growth factors and cytokines. Versican plays important roles in cell adhesion, migration and proliferation (Zhang et al., 1998; Ang et al., 1999).

Aggrecan is a major structural proteoglycan in cartilage, especially articular cartilage. It is important in mediating chondrocyte-chondrocyte and chondrocyte-matrix interactions. It has three globular domains with two located near the N-terminal end and one at the C-terminal end that are separated by a large GAG attachment domain (Doege et al., 1991). The N-terminal globular domains interact with hyaluronic acid and link proteins, and form stable complexes in the extracellular matrix (Fosang and Hardingham, 1989; Watanabe et al., 1997). The C-terminal globular domain is critical for posttranslational processing of the core protein and aggrecan secretion (Zheng et al., 1998). The GAG attachment domain contributes to the formation of the large mass aggregate. The GAG attachment domain interacts with hyaluronic acid and link proteins.
that contribute to the load-bearing properties of cartilage. The GAG chains attached to the aggrecan core protein can be both chondroitin sulfate and keratan sulfate.

Keratan sulfates are linear sulfated polysaccharides. They are composed of a repeating disaccharide unit of $N$-acetylglucosamine and galactose. Keratan sulfates are mainly found in cornea, cartilage, and bone. Keratan sulfates are large molecules and absorb large amounts of water which can act as a cushion in joints to absorb mechanical stress and as a buffer of corneal hydration.

Keratan sulfates can be divided into different groups according to the way they link to the core protein: keratan I, II, and III. Keratan sulfate I is linked to the core protein through an Asn residue, keratan sulfate II is linked to the core protein through GalNAc-O-Ser/Thr, and keratan sulfate III is attached to the core protein through Man-O-Ser (Krusius, et al., 1986). To date, there are no keratan sulfates have been found in skeletal muscle.

c. Glypican and syndecan

Heparan sulfates are linear polysaccharides that are composed of two alternative residues of glucuronic acid or iduronic acid attached to a glucosamine. Heparan sulfate can interact with growth factors, chemokines, cytokines, extracellular matrix molecules, morphogens, and clotting factors (Esko and Selleck, 2002), and regulate a wide variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumor metastasis. Heparan sulfate proteoglycans are also very important regulators of muscle growth and development. Total heparan sulfate proteoglycans in growth
selected turkey breast muscle are expressed at a significantly higher level than those from turkeys in non-growth selected turkeys (Liu et al., 2002).

Heparan sulfate proteoglycans include glypicans, syndecans, and perlecan. The glypicans are attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor. Six glypicans have been identified, but only glypican-1 is expressed in skeletal muscle (Campos et al., 1993). The syndecans have four family members, and are found in skeletal muscle (Brandan and Larraín, 1998; Larraín et al., 1998; Fuentealba et al., 1999; Cornelison et al., 2001; Liu et al., 2004, 2006). They are type I membrane glycoproteins that are usually substituted with heparan sulfate chains, and some also have chondroitin or dermatan sulfate chains. Perlecan is mainly expressed in the basement membrane (Murdoch et al., 1994). It can bear both heparan sulfate and/or chondroitin sulfate chains. Perlecan has the function to regulate basement membrane assembly, cartilage and vascular development, and tumor growth (Mathiak et al., 1997; Iozzo, 1998; Iozzo and San Antonio, 2001; Hassell et al., 2002). Perlecan can bind to the extracellular matrix and cell surface molecules (Iozzo, 1994). The heparan sulfate chains of perlecan and the protein core act as key regulators for several growth factor signaling pathways and lipid metabolism (Fuki et al., 2000; Iozzo, 2005; Lindner et al., 2007).

Glypican-1 has both membrane-associated and shed forms. The membrane-associated form is attached to the cell membrane by a GPI anchor and has been hypothesized to increase fibroblast growth factor 2 (FGF2) binding to its signaling receptor (Steinfeld et al., 1996; Brandan and Larraín, 1998). The shed form of glypican-1 may be incorporated into the extracellular matrix during muscle differentiation and
sequester FGF2 away from its receptor, permitting differentiation (Brandan and Larraín, 1998). Both forms of turkey glypican-1 are composed of a Cys-rich core protein with three covalently attached GAG chains and three N-glycosylated chains. The GAG chains are attached to the core protein at Ser residues (Ser\textsubscript{483}, Ser\textsubscript{485}, and Ser\textsubscript{487}) near the C termini. Zhang et al. (2007) reported that glypican-1 GAG chains are required for its function to regulate turkey myogenic satellite cell differentiation and response to FGF2 during proliferation. The N-glycosylated chains are attached to the core protein at the Asn residue of the sequence of Asn-Xaa-Ser/Thr; Xaa can be any amino acid except Pro (Kornfeld and Kornfeld, 1985). Glypican-1 N-glycosylated chains are attached to the core protein at Asn\textsubscript{76}, Asn\textsubscript{113}, and Asn\textsubscript{382}. The N-glycosylated chains have been reported to function in many biological processes. For example, they are involved in proper folding of proteins (Parodi, 2000; Helenius and Aebi, 2001) and localization of membrane proteins to the cell surface (Martinez-Maza et al., 2001; Yan et al., 2002). Song et al. (2010) demonstrated that glypican-1 N-glycosylated chains and GAG chains are critical in regulating turkey satellite cell proliferation, differentiation, and responsiveness to FGF2.

The expression pattern of the syndecans is highly regulated during development and in tissues. Syndecan-1 is the predominant form in epithelial cells; syndecan-2 and syndecan-3 are predominantly expressed in fibroblasts and neural tissue, respectively. Syndecan-4 is expressed ubiquitously in most adult tissues and all stages of embryonic development (Kim et al., 1994). All of the syndecans have an extracellular domain, a transmembrane domain, and a short cytoplasmic domain. Syndecans have a divergent extracellular domain, but all of the four members share a highly conserved
transmembrane domain and cytoplasmic domain. The cytoplasmic domain has two constant regions separated by a unique variable region. At the C terminus of the cytoplasmic domain, the conserved amino acid sequence (EFYA) provides a binding region for postsynaptic density protein, disc-large, zonulin-1 (PDZ) domain containing proteins.

Syndecan-4 has several unique attributes compared to other syndecans. The extracellular domain of syndecan-4 can be shed from the cell surface and function as an effector in maintaining the proteolytic balance during inflammation (Kainulainen et al., 1998). The cytoplasmic domain of syndecan-4 can bind specifically to protein kinase C alpha (PKCα) and phosphatidylinositol 4,5-bisphosphate (PIP2) (Oh et al., 1997a, b, 1998). Protein kinase C alpha binds to the variable region of the syndecan-4 cytoplasmic domain through the intermediate PIP2 (Horowitz et al., 1999; Oh et al., 1997a, b, 1998).

Syndecan-4 functions to promote focal adhesion formation (Saoncella et al., 1999), regulates cell migration (Echtermeyer et al., 2001), and protects cells from apoptosis (Jeong et al., 2001). Focal adhesions are the points of strong attachment of cells to the matrix. The number, size, and stability of focal adhesions influence cell migration. Syndecan-4 also plays an important role in muscle maintaince and regeneration (Cornelison et al., 2001, 2004). During muscle development, more syndecan-4 is expressed in turkey embryonic pectoralis major muscle in growth selected turkeys compared to unselected turkeys (Liu et al., 2006). Furthermore, syndecan-4 knockout mice lose the ability to regenerate damaged muscle (Cornelison et al., 2004). Syndecan-4 has three GAG chains that are attached to the core protein at Ser^{38}, Ser^{65}, and
Ser^{67} and two N-glycosylated chains attached at Asn^{124} and Asn^{136}. Both N-glycosylated and GAG chains are required for syndecan-4 regulation of satellite cell proliferation, but not differentiation (Song et al., 2011).

1.5 Signaling pathways affected by syndecan-4

Syndecan-4 participates in signaling transduction by transmitting signals from the extracellular matrix into the cell as a co-receptor and by activating PKCα in complex with PIP2. The core protein, especially the cytoplasmic domain of syndecan-4 core protein, is involved in signal transduction. Volk et al. (1999) reported that the expression of the syndecan-4 cytoplasmic domain influences FGF2-induced cell proliferation and migration. In this process, syndecan-4 GAG chains bind to FGF2 and present FGF2 to its high affinity receptor, fibroblast growth factor receptor (FGFR). The binding of FGF2 phosphorylates FGFR, and subsequently activates Raf-1 and mitogen-activated protein (MAP) kinase which enters the nucleus to regulate gene transcription. (Figure 1.7)

In the presence of PIP2, the cytoplasmic domain of syndecan-4 can translocate PKCα to the cell membrane and activate it. The activated PKCα activates downstream signaling pathways leading to the activation of RhoA and its target, Rho kinase, to regulate gene expression (Figure 1.7; Dovas et al., 2006).
1.5.1 Protein Kinase C α signaling pathway

Syndecan-4 is involved in actin associated cytoskeleton formation and promotion of cell survival (Clark and Brugge, 1995; Schwartz et al., 1995). The binding of PIP$_2$ to the V region of syndecan-4 cytoplasmic domain promotes and stabilizes the oligomerization of syndecan-4 (Oh et al., 1997b, 1998; Lee et al., 1998). The oligomerization of syndecan-4 promotes the binding and activation of PKCα to its cytoplasmic domain (Horowitz and Simons 1998a, b), whereas the monomeric syndecan-4 is not capable of potentiating PKC-α activity. The phosphorylation status of Ser$^{183}$ in the cytoplasmic domain is critical in regulating the affinity to PIP$_2$ and the oligomerization status of syndecan-4. Fibroblast growth factor 2 can be a regulator in this process. Without FGF2, Ser$^{183}$ can be phosphorylated, which will block oligomerization of the syndecan-4 cytoplasmic domain, thus decreasing the binding and activation of PKCα (Horowitz and Simons, 1998b). When FGF2 is added, it may bind to the syndecan-4 GAG chain, and dephosphorylate Ser$^{183}$ (Horowitz and Simons, 1998a), form oligomers and activate PKCα activity and downstream signaling pathways (Rybin et al., 1999). The activation of PKCα can regulate both cell migration (Harrington et al., 1997) and proliferation (Schonwasser et al., 1998; Besson and Yong, 2000).

1.5.2 Fibroblast growth factor 2 signaling transduction

Heparan sulfate proteoglycans can bind to heparin-binding growth factors including FGF, HGF, and vascular endothelial growth factor. The binding to growth factors can help the binding of growth factors to their primary receptors and initiate downstream physiological responses (Sternfeld et al., 1996; Sperinde and Nugent, 1998;
Hartmann et al., 1998; Kan et al., 1999; Lin et al., 1999; Clayton et al., 2001). Syndecan-4 binds FGF-2 directly and increases the affinity of FGF-2 for FGF tyrosine kinase receptors (Kojima et al., 1996; Steinfeld et al., 1996). Volk et al. (1999) reported that the over-expression of syndecan-4 and syndecan-4 mutants with cytoplasmic domain increase the binding of FGF-2 to the endothelial cell surface and cause enhanced growth and migration. The growth selected turkey has been shown to have higher FGF2 expression during satellite cell proliferation at embryonic stages of development compared to unselected turkeys (Liu et al., 2003). It has been hypothesized that syndecan-4 regulates FGF2 activity by binding FGF2 to its GAG chains and presenting FGF2 to its high affinity receptors, which will initiate intracellular signaling events (reviewed by Wilcox-Adelman et al., 2002). In contrast, Zhang et al. (2008) showed that syndecan-4 GAG chains are not needed for syndecan-4 function in turkey satellite cell proliferation and differentiation. However, whether the cytoplasmic domain of the core protein and the N-glycosylated chain deletion will influence turkey satellite cell response to FGF2 is still unknown.

1.6 Purpose of the study

Syndecan-4 core protein has been studied for its function in skeletal muscle growth and development. The function of syndecan-4 and glypican-1 GAG chains has been addressed in turkey myogenic satellite cell proliferation, differentiation, and FGF2 responsiveness (Zhang et al., 2007, 2008). However, there are no publications addressing the function of syndecan-4 and glypican-1 N-glycosylated chains in regulating turkey
skeletal muscle growth and development. The overall hypotheses of this study were: glypican-1 may regulate muscle growth, development and responsiveness to FGF2 through its GAG and N-chains; and syndecan-4 may function in muscle development by modulating focal adhesion formation through its central core protein cytoplasmic domain and the attached GAG and N-chains, and FGF2 signal transduction and the PKCα pathway may be involved in this process.

The first aim of the study was to illuminate the role of glypican-1 N-glycosylated chains in turkey skeletal muscle satellite cell proliferation, differentiation, and responsiveness to FGF2 to test the hypothesis that the N-glycosylated chains of glypican-1 can regulate satellite cell proliferation, differentiation and responsiveness to FGF2. In order to test our hypothesis, satellite cells isolated from the pectoralis major muscle of 7-week-old male randombred control 2 (RBC2) line turkeys (Velleman et al., 2000) were used. The turkey RBC2 line is a representative of a 1967 turkey and is maintained at the Ohio Agricultural Research and Development Center without selection for any traits (Nestor, 1977). Only satellite cells isolated from male turkey were used in this project to avoid sex effects. Site-directed mutagenesis was used to generate glypican-1 and syndecan-4 GAG and N-glycosylated chain mutants. After transfecting cells with different glypican-1 mutants, cell proliferation, differentiation, and cellular responsiveness to FGF2 were measured to study how the glypican-1 N-glycosylated chains influence cell growth and development.

Aim 2 studied the role of syndecan-4 N-glycosylated chains and GAG chains in turkey satellite cell proliferation, differentiation, and FGF2 responsiveness. Furthermore,
it is important to study the mechanism of how syndecan-4 influences satellite cell growth and development. Thus, the second objective of the study was to explore the mechanism of how syndecan-4 regulates turkey skeletal muscle satellite cell growth and development to test the hypothesis that syndecan-4 may function in muscle growth and development through FGF2 signal transduction and the PKCα pathway.

Previous studies showed that syndecan-4 regulates cell growth through FGF2 signaling pathways (Simons and Horowitz, 2001). By binding FGF2 to its GAG chains, syndecan-4 presents FGF2 to its high affinity tyrosine kinase receptor and then initiates the MAP kinase pathway to regulate cell growth. Upon activation of the MAP kinase pathway, extracellular signal-regulated kinase (ERK) is phosphorylated. However, Zhang et al. (2008) showed that the expression of modified syndecan-4 did not influence the expression of phosphorylated ERK after exposure to FGF2, which suggests that syndecan-4 regulates satellite cell growth through other pathways instead of the MAP kinase pathway.

Syndecan-4 participates in signal transduction by transmitting signals from the extracellular matrix into the cell and by activating PKCα in complex with PIP2 (Oh et al., 1997b, 1998; Lee et al., 1998). The activated PKCα activates the downstream pathway leading to the activation of RhoA and its target, Rho kinase to regulate cell activities (Dovas et al., 2006).

Aim 3 tested the hypothesis that syndecan-4 functions in turkey skeletal muscle satellite cell focal adhesion formation and cell apoptosis. Syndecan-4 is involved in focal adhesion formation (Echtermeyer et al., 2001), and protects cells from apoptosis (Jeong
et al., 2001). The syndecan-4 cytoplasmic domain is critical in focal adhesion formation. Thus, a syndecan-4 mutant without the cytoplasmic domain was generated. Syndecan-4 functions as a dimer or oligomer. Syndecan-4 core protein, GAG chains, and N-glycosylated chains are involved in oligomer formation. Syndecan-4 mutants in the cytoplasmic domain and GAG and/or N-glycosylated chains were generated, and their role in focal adhesion formation was studied. Also, whether the syndecan-4 cytoplasmic domain, GAG chains, and N-glycosylated chains can regulate cell apoptosis was also studied to illustrate whether syndecan-4 influences cell growth and development through apoptosis.

Accordingly, three specific aims were studied:

Aim 1 was to study the function of glypican-1 in skeletal muscle growth and development;

Aim 2 was to explore the role of syndecan-4 in skeletal muscle growth and development, and the signaling pathways affected by syndecan-4;

Aim 3 was to study the role of syndecan-4 in focal adhesion formation and apoptosis.
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Figure 1.1 Muscle types and their activity.
Figure 1.2 Structure of skeletal muscle connective tissue layers.
Figure 1.3. Satellite cell myogenesis and cell membrane markers at different stages.
Figure 1.4 Mechanisms for satellite cell self-renew. a) Symmetric division results in the activation and proliferation of both daughter cells. The cells maintain Pax7 expression and down-regulating MyoD will allow the cells to self-renew and return to the quiescent state; and b) Asymmetric division generates two daughters, one of them differentiating and the other one returns to the quiescent state.
Figure 1.5. An overview of the macromolecular organization of the extracellular matrix.
Figure 1.6. Formation of collagen fibers.
Figure 1.7. Syndecan-4 signaling pathways. Syndecan-4 can regulate cell behavior either through a mitogen-activated protein (MAP) kinase pathway, by regulating fibroblast growth factor 2 (FGF2) activity, or through a Rho kinase pathway, by interacting with protein kinase C alpha (PKCα).
CHAPTER 2: EFFECT OF GLYPCAN-1 COVALENTLY ATTACHED CHAINS ON TURKEY MYOGENIC SATELLITE CELL PROLIFERATION, DIFFERENTIATION, AND FIBROBLAST GROWTH FACTOR 2 RESPONSIVENESS*

*This chapter has been published in Poultry Science 2010; 89: 123-134, and reformatted for the dissertation.

Abstract

Glypican-1 is a cell membrane heparan sulfate proteoglycan that is composed of a core protein and covalently attached glycosaminoglycan (GAG) chains and N-linked glycosylated (N-glycosylated) chains. The glypican-1 GAG chains are required for cell differentiation and responsiveness to fibroblast growth factor 2 (FGF2). The role of glypican-1 N-glycosylated chains in regulating cell activities has not been reported. The objective of the current study was to investigate the role of glypican-1 N-glycosylated chains and the interaction between N-glycosylated and GAG chains in turkey myogenic satellite cell proliferation, differentiation, and FGF2 responsiveness. The wild-type turkey glypican-1 and turkey glypican-1 with mutated GAG chain attachment sites were cloned into the pCMS-EGFP mammalian expression vector and were used as templates to
generate glypican-1 N-glycosylated 1-chain and no-chain mutants with or without GAG chains by site-directed mutagenesis. The wild-type glypican-1 and all glypican-1 N-glycosylated 1-chain and no-chain mutants with or without GAG chains were transfected into turkey myogenic satellite cells. Cell proliferation, differentiation, and FGF2 responsiveness were measured. The overexpression of glypican-1 N-glycosylated 1-chain and no-chain mutants without GAG chains increased cell proliferation and differentiation compared with the wild-type glypican-1 but not the glypican-1 N-glycosylated mutants with GAG chains attached. Cells overexpressing glypican-1 N-glycosylated mutants with or without GAG chains increased cell responsiveness to FGF2 compared with wild-type glypican-1. These data suggest that glypican-1 N-glycosylated chains and GAG chains are critical in regulating turkey myogenic satellite cell proliferation, differentiation, and responsivness to FGF2.

2.1 Introduction

The consumption of poultry meat products has increased significantly during the past decade. To continue to produce high-quality poultry meat products, it is critical to understand the mechanisms of how skeletal muscle growth and development are regulated. Muscle development can be divided into 2 periods, hyperplasia and hypertrophy. Hyperplasia occurs during the embryonic period and is characterized by an increase in muscle fiber number. Posthatch muscle growth is termed hypertrophy and is characterized by the enlargement of existing muscle fibers. Satellite cells are myogenic stem cells that play an important role in the process of muscle fiber enlargement by
proliferating, differentiating, and fusing with adjacent muscle fibers (Moss and Leblond, 1971). The increased number of nuclei in muscle fibers coincides with increased protein synthesis and muscle fiber size enlargement.

Satellite cells were first discovered or recognized by their location between the sarcolemma and basement membrane (Mauro, 1961). Satellite cell activity can be regulated by growth factors such as fibroblast growth factor 2 (FGF2), a strong stimulator of cell proliferation and inhibitor of cell differentiation (Dollenmeier et al., 1981), and by their extracellular environment (Velleman, 1999). The extracellular matrix (ECM) is secreted by cells and can influence cell activity by transducing signals into the cell. The ECM is composed of proteins and polysaccharides. Proteoglycans are a major group of molecules within the ECM and are composed of a core protein and covalently attached glycosaminoglycans (GAG). Glycosaminoglycans attached to the proteoglycans include heparan sulfate (HS), dermatan sulfate, keratan sulfate, and chondroitin sulfate. The HS proteoglycans are located on the cell membrane or in the ECM. They are involved in cell-substratum adhesion (LeBaron et al., 1988; Haugen et al., 1992; Sanderson et al., 1992), cell-cell adhesion (Cole et al., 1986; Reyes et al., 1990; Stanley et al., 1995), and signaling mediated by growth factors through binding to ECM proteins, cell adhesion molecules, and growth factors. The HS chains are potentially attached to the core protein at Ser residues containing Ser-Gly repeats (Bourdon et al., 1987; Zhang et al., 1995). Rapraeger et al. (1991) reported that HS is required for the stable binding of FGF2 to its high-affinity receptor.
Syndecans and glypicans are 2 important families of HS proteoglycans involved in FGF2 signal transduction. The syndecans are composed of a transmembrane core protein and covalently attached GAG chains and N-linked glycosylated (N-glycosylated) chains. Six glypicans have been identified, but only glypican-1 has been found in skeletal muscle (Campos et al., 1993). Glypican-1 has both membrane-associated and shed forms. The membrane-associated form is attached to the cell membrane by a glycosylphosphatidylinositol anchor and has been hypothesized to increase FGF2 binding to its signaling receptor (Steinfeld et al., 1996; Brandan and Larrain, 1998). The shed form of glypican-1 may be incorporated into the ECM during muscle differentiation and sequester FGF2 away from its receptor, permitting differentiation (Brandan and Larrain, 1998). Both forms of turkey glypican-1 are composed of a Cys-rich core protein with 3 covalently attached GAG chains and 3 N-glycosylated chains. The GAG chains are attached to the core protein at Ser residues (Ser^{483}, Ser^{485}, and Ser^{487}) near the C terminal. Zhang et al. (2007) reported that glypican-1 GAG chains are required for its function to regulate turkey myogenic satellite cell differentiation and response to FGF2 during proliferation.

The N-glycosylated chains are attached to the core protein at the Asn residue of the sequence of Asn-Xaa-Ser/Thr; Xaa can be any amino acid except Pro (Kornfeld and Kornfeld, 1985). The N-glycosylated chains have been reported to function in many biological processes. For example, they are involved in proper folding of proteins (Parodi, 2000; Helenius and Aebi, 2001) and localization of membrane proteins to the cell surface (Martinez-Maza et al., 2001; Yan et al., 2002). However, more research effort has been placed on the function of proteoglycan GAG chains, whereas the role of
N-glycosylated chains has received limited research attention. Decorin is the only proteoglycan in which the function of the N-glycosylated chains has been investigated (Seo et al., 2005). Glypican-1 has 3 N-glycosylated chains attached to the core protein at Asn\textsuperscript{76}, Asn\textsuperscript{113}, and Asn\textsuperscript{382}. To investigate the function of each of the glypican-1 N-glycosylated chains, site-directed mutagenesis was used to mutate the N-glycosylated chain attachment sites.

Glypican-1 N-glycosylated 1-chain and no-chain mutants were transfected into turkey myogenic satellite cells, and then cell proliferation, differentiation, and responsiveness to FGF2 were measured. It is highly possible that the deletion of glypican-1 N-glycosylated chains will change the 3-dimensional structure of the core protein and the availability of glypican-1 GAG chains to other molecules. Thus, the glypican-1 N-glycosylated 1-chain and no-chain mutants without GAG chains were also generated and transfected into turkey myogenic satellite cells to study the interaction of glypican-1 N-glycosylated chains and GAG chains in cell proliferation, differentiation, and FGF2 responsiveness. The results from the current study provide new information about the role of glypican-1 N-glycosylated chains and the interaction between glypican-1 N-glycosylated chain and GAG chain in turkey myogenic satellite cell myogenesis.

2.2 Materials and Methods

2.2.1 Clone generation

The N-glycosylated chains are attached to the core protein at Asn residues at the sequence of Asn-Xaa-Ser/Thr (Kornfeld and Kornfeld, 1985). The glypican-1 N-
glycosylated chains are attached to the core protein at Asn\textsuperscript{76}, Asn\textsuperscript{113}, and Asn\textsuperscript{382}. Full-length wild-type turkey glypican-1 cDNA (GenBank accession number AY551002) has been cloned into the mammalian expression vector pCMS-EGFP (BD Biosciences, Clontech, Palo Alto, CA) (Velleman et al., 2006). Glypican-1 without GAG chains (G1-S0) had been generated by Zhang et al. (2007). These 2 clones were used as templates for site-directed mutagenesis using the Quick Change Multi Site-Directed Mutagenesis Kit according to the directions of the manufacturer (Stratagene Corporation, La Jolla, CA) to generate glypican-1 N-glycosylated 1-chain and no-chain mutants with or without GAG chains. Figure 2.1 illustrates the site-directed mutagenesis strategy and the primers used to generate glypican-1 with all possible N-glycosylated 1-chain and no-chain mutations. The N-glycosylated chains attached to Asn\textsuperscript{76}, Asn\textsuperscript{113}, and Asn\textsuperscript{382} are defined as N1, N2, and N3 chains, respectively. The glypican-1 mutants with only N1, N2, or N3 chains without changing the GAG chain attachment site are referred to as G1-N1, G1-N2, and G1-N3, respectively. The glypican-1 mutants with only N1, N2, or N3 chains with the GAG chain attachment sites eliminated are termed as G1-S0-N1, G1-S0-N2, and G1-S0-N3, respectively. The glypican-1 mutants without N-glycosylated chains attached to the core protein with or without GAG chains are defined as G1-N0 and G1-S0-N0. All of the mutations were confirmed by DNA sequencing on an ABI Prism 3100 x l genetic analyzer (Applied Biosystems, Foster City, CA).

2.2.2 Satellite cell culture and transfection

Satellite cells were isolated from the pectoralis major muscle of 7-wk-old male randomed control 2 (RBC2) line turkeys (Velleman et al., 2000). The third passage
satellite cells were used in the current study. The turkey RBC2 line is a representative of a 1967 turkey and is maintained at the Ohio Agricultural Research and Development Center without selecting for any traits (Nestor, 1977). Only satellite cells isolated from male turkey were used in this project to avoid sex effects.

Satellite cells were plated in gelatin-coated cell culture plates (Greiner Bio-One, Monroe, NC) in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% chicken serum (Invitrogen), 5% horse serum (Invitrogen), 1% antibiotic-antimycotic (Invitrogen), and 0.1% gentamicin (Invitrogen) in a 37.5°C 5% CO₂/95% air incubator. After 24 h of attachment, the cells were transfected with glypican-1 N-glycosylated 1-chain and no-chain mutants with or without GAG chains, wild-type glypican-1, or pCMS-EGFP empty vector using the Optifect transfection system (Invitrogen) with 1 µg (for 24-well cell culture plate) or 0.3 µg (for 96-well cell culture plate) of plasmid DNA according to the protocol of the manufacturer. After 6 h of transfection, the medium was changed to feeding medium containing McCoy’s 5A medium (Sigma-Aldrich, St. Louis, MO), 10% chicken serum, 5% horse serum, 1% antibiotic-antimycotic, and 0.1% gentamicin. Feeding medium was changed daily until 72 h posttransfection or cells reached 60% confluency for differentiation assay.

Differentiation was induced by changing the feeding medium to a low serum medium containing DMEM, 3% horse serum, 1% antibiotic-antimycotic, 0.01 mg/mL of porcine gelatin (Sigma-Aldrich), 0.1% gentamicin, and 1.0 mg/mL of BSA (Sigma-Aldrich). The differentiation medium was changed every 24 h until 96 h of differentiation. At 0, 24, 48, and 72 h posttransfection and at 0, 24, 48, 72, and 96 h of differentiation, cell cultures
were removed from the incubator, rinsed 3 times with sterile PBS, air-dried, and then stored at -70°C until analysis.

2.2.3 Total RNA extraction and cDNA synthesis

Total RNA was extracted from the cell cultures at 48 h posttransfection using Trizol (Invitrogen) according to the protocol of the manufacturer. The cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). In brief, the RNA-primer mix consisting of 1 µg of total RNA, 1 µL of 50 µM Oligo d(T)20 (Operon, Huntsville, AL), and nuclease-free water up to 13.5 µL was incubated at 80°C for 5 min and then cooled on ice. The reaction mix containing 5 µL of 5x First-Strand buffer (Promega), 1.25 µL of 10 mM deoxynucleoside triphosphate mix, 0.5 µL of RNasin (40 U/µL), 1 µL of Moloney murine leukemia virus reverse transcriptase (200 U/µL), and nuclease-free water up to 11.5 µL was then added to the cooled RNA-primer mix. The mixture was incubated at 55°C for 60 min and was then heated at 90°C for 10 min to stop the reaction. Twenty-five microliters of nuclease-free water was then added to the cDNA.

2.2.4 Real-time quantitative PCR

Real-time quantitative PCR was performed with a DNA Engine Opticon 2 real-time system (MJ Research, Waltham, MA) using the DyNAmo Hot Start SYBR Green qPCR kit (Finnzymes, Ipswich, MA). The PCR reaction consisted of 2 µL of cDNA, 10 µL of 2x master mix, 250 nM of each of the forward and reverse primers, and nuclease-free water up to 20 µL. The primers for glypican-1 were as follows: forward primer 5'-CTTGTGCTCTCTGGCAGATCGG-3' and reverse primer 5'-
CTGCTGGAGCCTTTTGCTGA-3'. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: forward primer 5'-GAGGGTAGTGAAGGCTGCTG-3' and reverse primer 5'-CCACAACACGTTGCTGTAT-3'. The primer sequences were designed from the following GenBank accession numbers: turkey glypican-1, AY551002 [GenBank], and turkey GAPDH, U94327. For both of the genes, the cycling parameters were denaturation at 95°C for 15 min, followed by 34 cycles of 94°C for 30 s, 58°C for 30 s, and at 72°C for 30 s with a final elongation of 72°C for 5 min. The melting curve program was 52 to 95°C, 0.2°C per read, and a 1 s hold. The PCR products were analyzed on a 1% agarose gel to check the amplification specificity. Standard curves were constructed with serial dilutions of purified PCR products from each gene. The PCR products were purified by agarose gel electrophoresis using the QIAquick Gel Extraction Kit according to the directions of the manufacturer (Qiagen, Valencia, CA). The amount of sample cDNA for each gene was determined by comparing the results to the glypican-1 standard curve and then normalizing to GAPDH expression. The PCR products were verified by DNA sequencing for specificity.

2.2.5 Immunohistochemistry

Satellite cells were cultured and transfected in 24-well cell culture plates. After 24 h of transfection, the cell cultures were fixed with 3% paraformaldehyde for 30 min at room temperature. The cells were then incubated with goat anti-glypican-1 antibody (Sc-33923, Santa Cruz Biotechnology Inc., Santa Cruz, CA) 1:100 dilution in Blotto (5% nonfat milk dissolved in 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl) for 1 h. The
second antibody used was donkey anti-goat antibody conjugated with rhodamine (Sc-2904, Santa Cruz Biotechnology Inc.) 1:200 dilution in Blotto. An Olympus XI 70 microscope (Olympus, Hauppauge, NY) and an Optronics digital camera (Optronics, Goleta, CA) were used to view and record the images.

2.2.6 Western analysis

Satellite cells were cultured and transfected in 24-well cell culture plates. At 48 h posttransfection, the cell lysates were prepared in cell lysis buffer containing 50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS; 150 mM NaCl; 1 mM EDTA; 1 mM Na$_3$VO$_4$; and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min on ice. After centrifugation at 16,000 x g at 4°C for 15 min, the concentration of protein in the supernatant was measured by the method of Bradford (1976). Equal amounts of proteins were loaded on an 8% SDS polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) with a semi-dry electrophoretic transfer device (EC140 Mini Blot Module, Thermo Electron Corporation, Milford, MA). The membranes were then blocked with 5% nonfat milk dissolved in Tris-buffered saline containing 20 mM Tris-HCl, pH 7.4; 150 mM NaCl; and 0.05% Tween 20 (TBS-T) for 1 h. The polyvinylidene difluoride membranes were incubated with goat anti-glypican-1 antibody (Sc-33923, Santa Cruz Biotechnology Inc.) 1:5,000 dilution in 5% nonfat milk dissolved in TBS-T for 1 h at room temperature. The second antibody used was donkey anti-goat antibody (Sc-A0307, Santa Cruz Biotechnology Inc.) 1:5,000 dilution in 5% nonfat milk dissolved in TBS-T. After incubating with chemiluminescent alkaline phosphatase substrate (Millipore) for 5
min at room temperature, immunoreactive bands were visualized with a Bio-Rad ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA). The intensity of the bands was quantified with Quantity One 4.6.6. Software (Bio-Rad).

2.2.7 Proliferation assay

The proliferation assay was performed as described by Velleman et al. (2006). In brief, satellite cells were plated in gelatin-coated 24-well cell culture plates at a density of 12,500 cells per well and transfected as described above. At 0, 24, 48, and 72 h posttransfection, cell culture plates were removed from the incubator and rinsed with sterile PBS 3 times. All plates were then stored at -70°C until analysis. Cell proliferation was measured by DNA content in each well by the method of McFarland et al. (1995). The Hoechst 33258 fluorochrome (Sigma-Aldrich) was used to determine DNA concentration on a Fluoroskan Ascent FL plate reader (Thermo Electron Corporation) using double-stranded calf thymus DNA as the standard.

2.2.8 Responsiveness to fibroblast growth factor 2 assay

Cell responsiveness to FGF2 was determined by measuring the DNA concentration in each well (Velleman et al., 2006). Satellite cells were plated in gelatin-coated 24-well cell culture plates at a density of 12,500 cells per well, and transfected as described above. After 6 h of transfection, the DMEM medium was removed and changed to serum-free defined media (McFarland et al., 2006) containing 0, 2.5, or 10.0 ng/mL of FGF2 (Pepro Tech, Rocky Hill, NJ). Cell responsiveness was measured at 72 h post transfection by the DNA content as described in the proliferation assay.
2.2.9 Differentiation assay

Satellite cells were plated in gelatin-coated 96-well cell culture plates at a density of 2,000 cells per well and were transfected and cultured as described above. Differentiation was induced by changing feeding medium to low serum differentiation medium when cells reached 60% confluency. Differentiation medium was changed every 24 h until 96 h of differentiation. At 0, 24, 48, 72, and 96 h of differentiation, cell cultures were removed from the incubator, rinsed with sterile PBS 3 times, air-dried, and then stored at -70°C until analysis. Differentiation was determined by measuring the muscle-specific creatine kinase level by a modified method from Yun et al. (1997). In brief, all plates were brought to room temperature for 10 min. The creatine phosphokinase (Sigma-Aldrich) was used to generate a standard curve. Two hundred microliters of creatine kinase assay buffer consisting of 20 mM glucose (Fisher Scientific, Pittsburgh, PA), 10 mM Mg acetate (Fisher Scientific), 1.0 mM adenosine diphosphate (Sigma-Aldrich), 10 mM adenosine monophosphate (Sigma-Aldrich), 20 mM phosphocreatine (Calbiochem, San Diego, CA), 0.5 U/mL of hexokinase (Worthington Biochemical, Lakewood, NJ), 1 U/mL of glyceraldehyde-6-phosphodihydrogenase (Worthington Biochemical), 0.4 mM thio-NAD (Sigma-Aldrich), and 1 mg/mL of BSA prepared in 0.1 M glycylglycine (pH 7.5, Sigma-Aldrich) was added to each well. After a 20-min incubation at room temperature, cell differentiation was determined by measuring the rate of thio-NAD reduction with a Dynex MRX Revelation Microtiter Plate Reader (Dynex Technologies Inc., Chantilly, VA) at 405 nm at 5 min intervals.
2.2.10 Nuclei number counting

Satellite cells were plated in gelatin-coated 24-well cell culture plates at a density of 12,500 cells per well and were transfected and cultured as described above. At 48 h of fusion, the cells were fixed with 3% paraformaldehyde for 30 min at room temperature. The dye, 4'-6-diamidino-2-phenylindole (Invitrogen) at a 1:10,000 dilution in PBS was then added to the cell cultures to stain the cell nuclei. Images were viewed with an Olympus XI 70 microscope and recorded with an Optronics digital camera. For each of the glypican-1 mutants, wild-type glypican-1, and the empty pCMS-EGFP vector, the number of nuclei in 25 individual myotubes was counted and the average number of nuclei per myotube was calculated.

2.2.11 Statistical analysis

The SAS PROC GLM (SAS Institute Inc., Cary, NC) was used for statistical analyses. Differences among means were detected using the Fisher’s least significance method. The expression of glypican-1 in satellite cells transfected with wild-type glypican-1 and glypican-1 mutants were compared with the control. For the FGF2 responsiveness assay, the statistical model included the effect of FGF2 treatment, mutants, and their interaction. Two-sided $P < 0.05$ was considered statistically significant.
2.3 Results

2.3.1 Site-directed mutagenesis and the expression in satellite cells

The expression of glypican-1 in satellite cells was analyzed by real-time PCR at 48 h posttransfection. All of the glypican-1 mutants and wild-type glypican-1 were overexpressed 60 to 140 times compared with the empty pCMS-EGFP vector (Figure 2.2). The overexpression of glypican-1 mutants was also confirmed at the protein level by immunohistochemistry at 24 h posttransfection (Figure 2.3) and by Western analysis at 48 h posttransfection (Figure 2.4). Figures 2.3A, B, and C show the turkey satellite cells transfected with empty pCMS-EGFP vector. Figures 2.3D, E, and F are the turkey satellite cells transfected with wild-type glypican-1. Figures 2.3A and D are bright field images to show the cell morphology. Figures 2.3B and E show the enhanced green fluorescence protein marker expressed from the pCMS-EGFP vector indicating transfection of the cells. Figures 2.3C and F were cells stained with glypican-1 antibody. The cells transfected with glypican-1 and all glypican-1 mutants had a much higher protein level of glypican-1 compared with the cells transfected with the empty pCMS-EGFP vector as assayed by immunohistochemistry (data not shown for the glypican-1 mutants). Western analysis also demonstrated that the cells transfected with glypican-1 and glypican-1 mutants had a higher level of protein expression compared with the cells transfected with the empty pCMS-EGFP vector (Figure 2.4).
2.3.2 The effect of glypican-1 N-glycosylated chain mutants with glycosaminoglycan chains attached to the core protein on turkey satellite cell proliferation, cell responsiveness to fibroblast growth factor 2, and differentiation

The overexpression of glypican-1 N-glycosylated 1-chain and no-chain mutants with GAG chains attached did not affect the proliferation of the satellite cells compared with the wild-type glypican-1 (Figure 2.5A). Glypican-1 and all of the glypican-1 mutants decreased proliferation in relation to the control pCMS-EGFP transfected satellite cells. At 2.5 and 10 ng/mL of FGF2, cells transfected with G1-N2 and G1-N3 had a higher proliferation level at 72 h posttransfection compared with the cells transfected with wild-type glypican-1 and G1-N0 at 10 ng/mL (Figure 2.5B).

At 0, 24, 48, and 72 h of differentiation, there was no significant difference in differentiation between the wild-type glypican-1 and glypican-1 N-glycosylated chain mutants containing the GAG chains (Figure 2.6A). However, at 48 h of differentiation, cells transfected with G1-N3 had significantly higher differentiation compared with those transfected with G1-N1 and G1-N2 constructs. At 96 h of differentiation, cells transfected with G1-N3 had a higher differentiation level compared with the cells transfected with wild-type glypican-1, G1-N0, and G1-N1. Cells transfected with G1-N3 had more nuclei in the myotubes at 48 h of fusion than the other glypican-1 mutants (Figure 2.6B). The average number of nuclei per myotube in the G1-N3 transfected cells was 4.48 compared with those transfected with wild-type glypican-1, which contained an average of 4.12 nuclei, G1-N0 had 3.84, G1-N1 had 3.2, and G1-N2 had 3.16.
2.3.3 The effect of glypican-1 N-glycosylated chain mutants without glycosaminoglycan chains on turkey satellite cell proliferation, responsiveness to fibroblast growth factor 2, and differentiation

Starting at 24 h posttransfection, satellite cells transfected with glypican-1 N-glycosylated 1-chain and no-chain mutants without the GAG chains had a higher level of proliferation compared with the cells transfected with wild-type glypican-1 (Figure 2.7A). At 72 h posttransfection, all of the glypican-1 N-glycosylated 1-chain and no-chain mutants without the GAG chains had increased cell proliferation compared with the wild-type glypican-1. At 2.5 ng/mL of FGF2, cells transfected with G1-S0-N1 had a higher level of proliferation compared with the cells transfected with wild-type glypican-1, G1-S0-N0, G1-S0-N2, and G1-S0-N3 (Figure 2.7B). At 10 ng/mL of FGF2, cells transfected with glypican-1 N-glycosylated 1-chain mutants without the GAG chains had a higher proliferation level compared with wild-type glypican-1 and G1-S0-N0.

At 72 h of differentiation, cells transfected with glypican-1 N-glycosylated 1-chain mutants without the GAG chains had a significantly higher differentiation level compared with the wild-type glypican-1, G1-S0, and G1-S0-N0 (Figure 2.8A). The average nuclei number per myotube at 48 h of differentiation further supported the differentiation assay data (Figure 2.8B). The average number of nuclei per myotube transfected with pCMS-EGFP was 3.68, wild-type glypican-1 was 4.12, G1-S0 was 3.32, G1-S0-N0 was 3.4, G1-S0-N1 was 3.28, G1-S0-N2 was 3.72, and G1-S0-N3 was 3.8.
2.4 Discussion

In the current study, focus was placed on the role of glypican-1 N-glycosylated chains during turkey satellite cell proliferation, differentiation, and responsiveness to FGF2. Site-directed mutagenesis was used to generate glypican-1 N-glycosylated chain 1-chain and no-chain mutants with or without GAG chains. Glypican-1 mutants were transfected into RBC2 line turkey myogenic satellite cells, and then cell proliferation, differentiation, and responsiveness to FGF2 during proliferation were measured. Wild-type glypican-1 decreased turkey myogenic satellite cell proliferation, which confirmed previous data (Velleman et al., 2007). With the GAG chains attached, the glypican-1 N-glycosylated 1-chain and no-chain mutants did not affect turkey satellite cell proliferation compared with the wild-type glypican-1. Zhang et al. (2007) also reported that the glypican-1 GAG chains had no effect on turkey satellite cell proliferation. However, when both GAG chains and N-glycosylated chains were deleted, turkey myogenic satellite cell proliferation was increased. These data suggest that both glypican-1 GAG chains and N-glycosylated chains are required for glypican-1 regulation of turkey satellite cell proliferation.

The responsiveness of satellite cells to FGF2 at 72 h posttransfection was measured by adding different concentrations of FGF2 to serum-free medium. Zhang et al. (2007) reported that cells transfected with glypican-1 GAG chain 1-chain and no-chain mutants have lower responsiveness to FGF2 compared with wild-type glypican-1. In the current study, cells transfected with G1-N2 and G1-N3 had higher FGF2 responsiveness compared with the wild-type glypican-1 at 72 h posttransfection. Cellular responsiveness
to FGF2 at 2.5 ng/mL generally was not influenced by glypican-1 N-glycosylated chain mutants without the GAG chains (except G1-S0-N1). However, when 10 ng/mL of FGF2 was added to the culture, all of the cells transfected with glypican-1 N-glycosylated 1-chain mutants without GAG chains had increased responsiveness to FGF2. The difference between the proliferation and cell responsiveness to FGF2 data was likely due to the culture conditions. In the proliferation assay, cells were cultured in serum-rich medium that contains a variety of growth factors that influence cell growth, whereas in the FGF2 responsiveness assay, cells were cultured in serum-free medium only containing different concentrations of FGF2. These data suggest that GAG chains and N-glycosylated chains play an important role in regulating satellite cell responsiveness to FGF2.

One possibility to explain these results is that glypican-1 GAG chains and N-glycosylated chains interact with each other and influence the shedding of glypican-1. Compared with the cells transfected with wild-type glypican-1 (Figure 2.9A), the deletion of the GAG chains may decrease the binding and presentation of FGF2 to its Tyr kinase receptors (Figure 2.9B), thus the cells will have lower responsiveness to FGF2. With the GAG chains attached to the core protein and N-glycosylated chains deleted, the core protein 3-dimensional structure may be changed, but glypican-1 can still be shed normally. In this situation, the glypican-1 core protein directly presents FGF2 to its receptor (Figure 2.9C). Seo et al. (2005) reported that without both GAG chains and N-glycosylated chains, the secretion of decorin was inhibited. When both the glypican-1 GAG chains and N-glycosylated chains were deleted, the 3-dimensional structure of the core protein may change and inhibit the shedding of glypican-1, thus more glypican-1
core protein will remain on the cell surface (Figure 2.9D). The increased glypican-1 core protein can directly bind to FGF2 and present it to its Tyr kinase receptor (Kirkpatrick et al., 2006). As a result, the satellite cells will be more responsive to FGF2 compared with the cells transfected with wild-type glypican-1.

Another possibility is that the deletion of N-glycosylated chains changes the 3-dimensional structure of glypican-1 core protein and changes the availability of its GAG chains and its core protein to FGF2. The core protein of syndecans and glypicans can directly interact with growth factors including insulin-like growth factor 2, transforming growth factor-β, and FGF2 (Pilia et al., 1996; Chen et al., 2004; Kirkpatrick et al., 2006). Kirkpatrick et al. (2006) reported that the function of glypican does not depend entirely on HS chains but also on the glypican core protein. When the N-glycosylated chains were deleted, the 3-dimensional change in the glypican-1 core protein increased its affinity to FGF2 (Figures 2.9C and D). Thus, cells transfected with glypican-1 N-glycosylated chain mutants with or without GAG chains were more responsive to FGF2.

At the time of differentiation, turkey myogenic satellite cells transfected with wild-type glypican-1 had higher levels of differentiation compared with those transfected with glypican-1 GAG chain 1-chain and no-chain mutants (Zhang et al., 2007). The cells had increased differentiation when transfected with glypican-1 N-glycosylated chain mutants without GAG chains compared with the wild-type glypican-1 but not with the GAG chains attached (except for G1-N3 at 96 h of differentiation).

The deletion of glypican-1 GAG chains likely reduced the amount of FGF2 sequestered by glypican-1 resulting in a decrease in differentiation compared with the
cells transfected with wild-type glypican-1. With the GAG chains, the deletion of N-glycosylated chains did not influence the shedding of glypican-1; thus, there was no change in cell differentiation compared with the cells transfected with wild-type glypican-1. The differences observed with the transfection of G1-N3 may be due to the location of the N3 chain. The glypican-1 N3 chain is located at the C terminus of the core protein and near the GAG chains. If the core protein 3-dimensional structure is altered by the deletion of the other 2 N-glycosylated chains, glypican-1 N3 chain may interact with the GAG chains, modify the availability of the GAG chains to bind to FGF2, and subsequently affect cell differentiation.

Glypican-1 can be released or shed from the cell membrane through the action of phospholipases (Mythreye and Blobe, 2009). Notum is a lipase that is involved in the release of glypicans by cleaving the glycosylphosphatidylinositol anchor (Traister et al., 2008). When glypicans are cleaved by notum and shed into the ECM, they can function as an inhibitor of their ligands. The shedding of glypican-1 N-glycosylated mutants without GAG chains into the ECM will decrease the binding of FGF2 to its Tyr kinase receptor and result in an increase in cell differentiation.

In addition to FGF2 signaling, glypicans can regulate other signaling pathways, such as Wnts, hedgehogs, and bone morphogenetic proteins (Jackson et al., 1997; Lin and Perrimon, 1999; Paine-Saunders et al., 2000; Topczewski et al., 2001; Lum et al., 2003; Ohkawara et al., 2003; Song et al., 2005). Thus, glypican-1 may regulate cell signaling in a complex manner including the FGF2 pathway. The current data show the involvement of both glypican-1 GAG chains and N-glycosylated chains in the regulation of FGF2
signal transduction and during turkey myogenic satellite cell proliferation and differentiation
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Figure 2.1 Site-directed mutagenesis strategy and primers used to generate glypican-1 N-linked glycosylated (N-glycosylated) chain 1-chain and no-chain mutants with or without glycosaminoglycan (GAG) chains. Glypican-1 has 3 GAG chains attached to the core protein at Ser\(^{483}\) (S1), Ser\(^{485}\) (S2), and Ser\(^{487}\) (S3). The N-glycosylated chains are attached to the core protein at Asn\(^{76}\), Asn\(^{113}\), and Asn\(^{382}\). The Asn\(^{76}\) is referred to as the N1 chain, Asn\(^{113}\) is the N2 chain, and Asn\(^{382}\) is the N3 chain. The following nomenclature was developed to distinguish the different forms of glypican-1: G1 is wild-type glypican-1; the number after the S means the potential GAG sites are unchanged, for example, S3 means glypican-1 has the S3 chain unchanged. The number after N means the N-glycosylated chain not altered, for example, N1 means the glypican-1 has the N1 chain site unaltered. S0 and N0 indicate that all of the GAG chain attachment sites or all of the N-glycosylated chain attachment sites are altered, respectively. Primers used are indicated as primer A, B, C, D, E, and F.

A = N1, 5'-GAGATGGAGGAGGAACTTTGCAGCCAAAAGCCGAAGTGAATTTGA-3';
B = N2, 5'-CCAGGATTTGTTGGCAAGTCAGAAAAAGCCC-3';
C = N3, 5'-GGTTTTGGATGCCAAAGGGCTCTGACAGCTCTCAAGTCT-3';
D = S1, 5'-GAGTGACGACATGACCGGCTCAGGCAG-3';
E = S2, 5'-GACATGACCGGATGACGGCTCAGGCAG-3';
F = S3, 5'-ACCGGCACAGGGCCGAAGTGAATTTGA-3'.
Figure 2.2 Real-time quantitative PCR analysis of mRNA expression at 48 h posttransfection. Randombred control 2 line male turkey myogenic satellite cells were transfected with empty pCMS-EGFP vector (control), wild-type glypican-1 (G1), glypican-1 N-glycosylated chain no-chain mutant (G1-N0), glypican-1 N-glycosylated chain 1-chain mutants (G1-N1, G1-N2, and G1-N3), glypican-1 glycosaminoglycan (GAG) no-chain mutant (G1-S0), glypican-1 N-glycosylated chain no-chain mutant without GAG chains (G1-S0-N0), and glypican-1 N-glycosylated chain 1-chain mutants without GAG chains (G1-S0-N1, G1-S0-N2, and G1-S0-N3). Three replicates were used for each sample. The error bars represent SEM. An asterisk indicates a significant difference from the control ($P < 0.05$). The overexpression assay was repeated 3 times.
Figure 2.3  Overexpression of glypican-1 in randombred control 2 line male turkey myogenic satellite cells transfected with the pCMS-EGFP empty vector, wild-type glypican-1, and glypican-1 mutants at 24 h posttransfection. Panels A to C are the same cells transfected with pCMS-EGFP. Panels D to F are the same cells transfected with glypican-1. Panels A and D are the bright field images. Panels B and E illustrate the cells expressing enhanced green fluorescent protein marker in the pCMS-EGFP vector. Panels C and F are the cells expressing glypican-1 in the control cell (C) and glypican-1 transfected cells (F). Panels C and F are stained with anti-glypican-1 antibody with a rhodamine secondary antibody. The scale bar represents 50 µM.
Figure 2.4 Western analysis of glypican-1 expression in randombred control 2 line male turkey myogenic satellite cells transfected with wild-type glypican-1, glypican-1 mutants [glypican-1 N-glycosylated chain no-chain mutant (G1-N0), glypican-1 N-glycosylated chain 1-chain mutants (G1-N1, G1-N2, and G1-N3), glypican-1 glycosaminoglycan no-chain mutant (G1-S0), glypican-1 N-glycosylated chain no-chain mutant without glycosaminoglycan chains (G1-S0-N0), and glypican-1 N-glycosylated chain 1-chain mutants without glycosaminoglycan chains (G1-S0-N1, G1-S0-N2, and G1-S0-N3)], and the pCMS-EGFP empty vector at 48 h posttransfection. A) Relative densities of the Western blot. B) Western blot. Each lane contains 10 µg of protein and was hybridized to an anti-glypican-1 antibody. An asterisk indicates a significant difference from the control ($P < 0.05$).
Figure 2.5 Proliferation analysis and fibroblast growth factor 2 (FGF2) responsiveness of the randombred control 2 line turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild-type glypican-1 (G1), glypican-1 N-glycosylated chain no-chain mutant (G1-N0), and glypican-1 N-glycosylated chain 1-chain mutants (G1-N1, G1-N2, and G1-N3). A) The proliferation assay measured DNA concentration in the cultured cells at 24-h intervals after transfection for 72 h. B) Cellular response to FGF2 at concentrations of 0, 2.5, and 10 ng/mL at 72 h posttransfection. Cell responsiveness to FGF2 was measured by DNA concentration in the cultured cells. Four replicates were used for each sample. The error bars represents SEM. Bars without a common letter within times or FGF2 concentration were significantly different ($P < 0.05$). Each experiment was repeated at least 3 times.
Figure 2.6 Differentiation assay of the randombred control 2 line turkey myogenic satellite cells after transfection with pCMS-EGFP empty vector (pCMS), wild-type glypican-1 (G1), glypican-1 N-glycosylated chain no-chain mutant (G1-N0), and glypican-1 N-glycosylated chain 1-chain mutants (G1-N1, G1-N2, and G1-N3). A) Differentiation of cultured cells at 24-h intervals starting at 0 h until 96 h of differentiation. Six replicates were used for each sample. B) The average number of nuclei counted in 25 individual myofibers at 48 h of differentiation after staining with 4′-6-diamidino-2-phenylindole. Bars without common letters are significantly different ($P < 0.05$). The error bars represent SEM. The differentiation assay was repeated 3 times.
Figure 2.7 Proliferation analysis and fibroblast growth factor 2 (FGF2) responsiveness of the randombred control 2 line turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild-type glypican-1 (G1), glypican-1 N-glycosylated chain no-chain mutant without glycosaminoglycan (GAG) chains (G1-S0-N0), and glypican-1 N-glycosylated chain 1-chain mutants without GAG chains (G1-S0-N1, G1-S0-N2, and G1-S0-N3). A) Cellular proliferation at 24-h intervals after transfection for 72 h. B) Cellular response to FGF2 at concentrations of 0, 2.5, and 10 ng/mL at 72 h posttransfection. Cellular responsiveness to FGF2 was determined by measuring the DNA concentration. Four replicates were used for each sample. Bars without common letters within times or FGF2 concentration were significantly different (P < 0.05). The error bars represent SEM. Each experiment was repeated at least 3 times.
Figure 2.8 Differentiation of the randombred control 2 line turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild-type glypican-1 (G1), glypican-1 without glycosaminoglycan (GAG) chains (G1-S0), glypican-1 N-glycosylated chain no-chain mutant without GAG chains (G1-S0-N0), and glypican-1 N-glycosylated chain 1-chain mutants without GAG chains (G1-S0-N1, G1-S0-N2, and G1-S0-N3). A) Differentiation of the satellite cell cultures was measured at 24-h intervals from 0 h of differentiation through 96 h. Six replicates were used for each sample. B) The average nuclei numbers counted in 25 individual myofibers at 48 h of differentiation after staining with 4'-6-diamidino-2-phenylindole. Bars without common letters indicate significant difference ($P < 0.05$). The error bars represent SEM. The differentiation assay was repeated 3 times.
Figure 2.9  Function of glypican-1 N-linked glycosylated (N-glycosylated) chains and glycosaminoglycan (GAG) chains in fibroblast growth factor 2 (FGF2) signaling. A) Glypican-1 has both membrane-associated and shed forms. The membrane-associated form is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. Glypican-1 has 3 N-glycosylated chains and 3 GAG chains. The N-glycosylated chains are attached to the core protein at Asn$^{76}$, Asn$^{113}$, and Asn$^{382}$. Asn$^{76}$ is referred to as the N1 chain, Asn$^{113}$ is the N2 chain, and Asn$^{382}$ is the N3 chain. Both glypican-1 core protein and GAG chains can bind and present FGF2 to its Tyr kinase receptor and initiate cell signaling into the nuclei to stimulate gene transcription that influences cell proliferation and differentiation. B) When GAG chains are deleted, glypican-1 core protein can still bind and present FGF2 to its Tyr kinase receptor and initiate cell signaling. The cell has lower responsiveness to FGF2. C) The deletion of glypican-1 N-glycosylated chains will change the 3-dimensional structure of the glypican-1 core protein, which may influence glypican-1 localization on the cell membrane. Fibroblast growth factor 2 signaling will be initiated normally in the presence of the glypican-1 core protein and GAG chains. The protein without folding properly may be degraded. D) Glypican-1 N-glycosylated mutants without GAG chains cannot be released from the cell membrane normally. The increased number of glypican-1 core proteins bound to the cell surface will increase cell responsiveness to FGF2. Transcription levels are indicated by the density of the arrows (a thin black arrow indicates normal transcription level, a dashed arrow indicates reduced transcription level, and a thick black arrow indicates increased transcription level).
CHAPTER 3: ROLE OF SYNDECAN-4 SIDE CHAINS IN TURKEY

SATELLITE CELL GROWTH AND DEVELOPMENT*

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Abstract

Syndecan-4 is a cell membrane heparan sulfate proteoglycan that is composed of a core protein and covalently attached glycosaminoglycans (GAG) and N-linked glycosylated (N-glycosylated) chains. Syndecan-4 has been shown to function independent of its GAG chains. Syndecan-4 may derive its biological function from the N-glycosylated chains due to the biological role of N-glycosylated chains in protein folding and cell membrane localization. The objective of the current study was to investigate the role of syndecan-4 N-glycosylated chains and the interaction between GAG and N-glycosylated chains in turkey myogenic satellite cell proliferation, differentiation, and fibroblast growth factor 2 (FGF2) responsiveness. The wild type turkey syndecan-4 and the syndecan-4 without GAG chains were cloned into the expression vector pCMS-EGFP and used as templates to generate syndecan-4 N-glycosylated one-chain and no-chain mutants with or without GAG chains. The wild...
type syndecan-4, all of the syndecan-4 N-glycosylated chain mutants were transfected into turkey myogenic satellite cells. Cell proliferation, differentiation, and responsiveness to FGF2 were measured. The overexpression of syndecan-4 N-glycosylated mutants with or without GAG chains did not change cell proliferation, differentiation, and responsiveness to FGF2 compared to the wild type syndecan-4 except that the overexpression of syndecan-4 N-glycosylated mutants without GAG chains increased cell proliferation at 48 and 72 h post-transfection. These data suggest that syndecan-4 functions in an FGF2-independent manner, and the N-glycosylated and GAG chains are required for syndecan-4 to regulate turkey myogenic satellite cell proliferation, but not differentiation.

3.1 Introduction

Satellite cells are undifferentiated mononuclear myogenic cells that are located between the sarcolemma and basement membrane (Mauro, 1961). They play a critical role in posthatch or postnatal muscle growth by proliferating, differentiating, and donating their nuclei to adjacent muscle fibers (Moss and Leblond, 1971). In adult skeletal muscle, satellite cells are normally quiescent and can be activated in response to stimuli to proliferate and fuse to form new fibers or repair damaged fibers (Schultz and Jaryszak, 1985). For example, the activities of satellite cells can be regulated by growth factors like fibroblast growth factor 2 (FGF2; Dollenmeier et al., 1981), transforming growth factor β (Florini et al., 1986), and insulin-like growth factor (Schmid et al., 1983), and changes in their extracellular environment (Velleman, 2004).
The extracellular matrix (ECM) is a dynamic structure that is secreted by cells and regulates cell activity by transducing signals into the cell. The ECM is composed of fibrous and nonfibrous proteins and polysaccharides. The heparan sulfate proteoglycans (HSPG) are an important group of macromolecules in the ECM that have been shown to be important regulators of muscle growth and development (Brandan and Larrain, 1998; Velleman et al., 2007). In addition, they are involved in cell-substratum adhesion (LeBaron et al., 1988; Haugen et al., 1992; Sanderson et al., 1992), cell–cell adhesion (Cole et al., 1986; Reyes et al., 1990; Stanley et al., 1995), and signaling mediated by growth factors, which are properties critical in the formation of tissues.

Syndecan-4 is a HSPG that plays an essential role in muscle maintenance and regeneration (Cornelison et al., 2001, 2004; Tanaka et al., 2009). Syndecan-4 has been reported to be a marker for regenerating satellite cells (Tanaka et al., 2009). This is supported by the observation that syndecan-4 knockout mice lose their ability to regenerate damaged muscle (Cornelison et al., 2004).

Syndecan-4 is composed of a transmembrane core protein and covalently attached glycosaminoglycans (GAG) chains and N-linked glycosylated (N-glycosylated) chains. It has been reported that HSPG GAG chains are required for stable binding of FGF2 to its high affinity receptor (Yayon et al., 1991; Aviezer et al., 1994). The GAG chains are attached to the core protein at Ser residues containing Ser-Gly repeats. Turkey syndecan-4 GAG chains are attached to the core protein at Ser\(^{38}\), Ser\(^{65}\), and Ser\(^{67}\), but they are not required for syndecan-4 regulated turkey myogenic satellite cell proliferation,
differentiation, and responsiveness to FGF2 during proliferation (Velleman et al., 2007; Zhang et al., 2008).

The N-glycosylated chains are attached to the core protein at the Asn residue at the sequence of Asn-Xaa-Ser/Thr. The Xaa can be any amino acids except proline (Kornfeld and Kornfeld, 1985). N-linked glycosylated chains have been reported to function in many biological processes. For example, they are involved in the proper folding of proteins (Parodi, 2000; Helenius and Aebi, 2001), and localization of membrane proteins to the cell surface (Martinez-Maza et al., 2001; Yan et al., 2002). However, more research efforts have been placed on proteoglycan GAG chains, while the role of N-glycosylated chains has received limited attention. Decorin and glypican-1 are the only two proteoglycans for which studies on the function of their N-glycosylated chains have been reported (Seo et al., 2005; Song et al., 2010). Seo et al. (2005) found that the normal secretion of decorin into the ECM requires at least one GAG chain or N-glycosylated chain attached to the core protein. Glypican-1 N-glycosylated chains are critical in turkey myogenic satellite cell proliferation, differentiation, and responsiveness to FGF2 (Song et al., 2010). Syndecan-4 has two N-glycosylated chains attached to the core protein at Asn$^{124}$ and Asn$^{136}$. The deletion of syndecan-4 N-glycosylated chains may lead to the improper folding of the syndecan-4 protein and alter its cell membrane spatial distribution. To investigate the function of each syndecan-4 N-glycosylated chain, site-directed mutagenesis was used to change the Asn that is used in the attachment of N-glycosylated chains to Ala.
Syndecan-4 N-glycosylated one-chain and no-chain mutants were transfected into turkey myogenic satellite cells, following which the proliferation, differentiation, and responsiveness to FGF2 were measured. Based on N-glycosylated chains affecting protein secondary structure, the deletion of the syndecan-4 N-glycosylated chains may lead to the improper folding of the syndecan-4 core protein and change the interaction of syndecan-4 GAG chains with other molecules. Thus, the syndecan-4 N-glycosylated one-chain and no-chain mutants without GAG chains were also generated and transfected into turkey myogenic satellite cells to study the interaction between syndecan-4 N-glycosylated chains and GAG chains on cell proliferation, differentiation, and FGF2 responsiveness.

The results from the current study provide new information about the function of syndecan-4 N-glycosylated chains and the interaction between syndecan-4 GAG chains and N-glycosylated chains during myogenesis.

3.2 Materials and Methods

3.2.1 Clone Generation

A full length wild type turkey syndecan-4 cDNA (GenBank accession number AY852251; Velleman et al., 2006) and syndecan-4 without GAG chains have been previously cloned into the mammalian expression vector pCMS-EGFP (BD Biosciences Clontech) (Velleman et al., 2006). These two clones were used as templates to generate syndecan-4 N-glycosylated one-chain and no-chain mutants with or without GAG chains using the Quick Change Multi Site-Directed Mutagenesis kit (Stratagene Corporation). Figure 3.1 illustrates the site-directed mutagenesis strategy used to generate syndecan-4
all possible N-glycosylated one-chain and no-chain mutations. The primers used to change N1 and N2 chains were: N1, 5′-AGC TTC ACC TGT TGA AGA GCC CCT GTC CAA CAA GAT CTC CAT GGC-3′ and N2, 5′-TCC ATG GCA AGC ACA GCC GCC AGC AGC ATC TTT GAA AG-3′. All of the mutations were confirmed by DNA sequencing.

3.2.2 Satellite Cell Culture and Transfection

Satellite cells were isolated from the pectoralis major muscle of 7-week-old male randombred control 2 (RBC2) line turkeys (Velleman et al., 2000). The turkey RBC2 line is representative of a 1967 turkey and maintained at the Ohio Agricultural Research and Development Center without selection for any growth traits (Nestor 1977).

Satellite cells were plated and transfected with syndecan-4 N-glycosylated one-chain and no-chain mutants with or without GAG chains, wild type syndecan-4, or pCMS-EGFP empty vector as described by Song et al. (2010). At 0, 24, 48, and 72 h post-transfection and at 0, 24, 48, 72, and 96 h of differentiation, cell cultures were removed and stored at -70°C until analysis.

3.2.3 Real-time quantitative PCR

Total RNA was extracted from the cell cultures at 48 h post-transfection using TRIzol (Invitrogen) according to the manufacturer’s protocol (Song et al., 2010). Real-time quantitative polymerase chain reaction (PCR) was performed with a DNA Engine Opticon 2 real-time system (MJ Research) using the DyNAmo Hot Start SYBR Green qPCR kit (Finnzymes). The PCR reaction consisted of 2 μL cDNA, 10 μL 2× master mix, 250 nmol/L of each of the forward and reverse primers, and nuclease-free water up
to 20 μL. The primers for syndecan-4 were: forward primer 5′-CTACCCTGGCTCTGGAGACCT-3′ and reverse primer 5′-TCATTGTCCAGCATGGGTCTTT-3′. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: forward primer 5′-GAGGGTAGTGAGGCTGCTG-3′ and reverse primer 5′-CCACAACACGGTTGCTGAT-3′. For both of the genes, the cycling parameters were denaturation at 95°C for 15 min, followed by 34 cycles of 94°C for 30 s, 58°C for 30 s and at 72°C for 30 s with a final elongation of 72°C for 5 min. The melting curve program was 52-95°C, 0.2°C per read, and a 1 s hold. The PCR products were analyzed on a 1% agarose gel to check the amplification specificity. Standard curves were constructed with serial dilutions of purified PCR products from each gene. The PCR products were purified by agarose gel electrophoresis using the QIAquick Gel Extraction Kit (Qiagen). The amount of sample cDNA for each gene was determined by comparing the results to the syndecan-4 standard curve, and then normalized to GAPDH expression. The PCR products were verified by DNA sequencing for specificity.

3.2.4 Immunohistochemistry

Satellite cells were cultured and transfected in 24 well cell culture plates. After 24 h of transfection, the cell cultures were fixed with 3% paraformaldehyde for 30 min at room temperature. The cells were then incubated with goat anti-syndecan-4 antibody (Santa Cruz, Sc-33913) 1:100 dilution in blotto (5% nonfat milk dissolved in 20 mmol/L Tris-HCl, pH 7.4, and 150 mmol/L NaCl) for 1 h. The secondary antibody used was donkey anti-goat antibody conjugated with rhodamine (Santa Cruz, SC-2904) 1:200
dilution in 5% nonfat milk dissolved in blotto. An Olympus XI 70 microscope and an Optronics digital camera (Optronics) were used to view and record the images.

3.2.5 Proliferation Assay

The proliferation assay was performed as described by Velleman et al. (2006). Cell proliferation was measured by the DNA content in each well by the method of McFarland et al. (1995). The Hoechst 33258 fluorochrome (Sigma-Aldrich) was used to determine DNA concentration on a Fluoroskan Ascent FL plate reader (ThermoElectron Co.) using double-stranded calf thymus DNA as the standard.

3.2.6 Cell Count

Satellite cells were plated and transfected as described in the proliferation assay. At 48 h post-transfection, plates were removed from the incubator and rinsed three times with phosphate-buffered saline (PBS). Cells were treated with 0.1% trypsin in PBS, scraped off the well and then transferred into 1.5 mL centrifuges. Cell pellets were washed with PBS, and then resuspended in 100 μL of PBS. Ten microliters of suspension was stained with equal amounts of trypan blue. The cell count measurement was repeated three times for each sample using a Countess Automated Cell Counter (Invitrogen).

3.2.7 Differentiation Assay

Satellite cells were cultured and transfected for differentiation as described in Zhang et al. (2008). Differentiation was determined by measuring the muscle specific creatine kinase level by a modified method from Yun et al. (1997). In brief, all plates
were brought to room temperature for 10 min. Creatine phosphokinase (Sigma-Aldrich) was used to generate a standard curve.

Two hundred microliters of creatine kinase assay buffer consisting of 20 mmol/L glucose (Fisher Scientific), 10 mmol/L Mg acetate (Fisher Scientific), 1.0 mmol/L adenosine diphosphate (Sigma-Aldrich), 10 mmol/L adenosine monophosphate (Sigma-Aldrich), 20 mmol/L phosphocreatine (Calbiochem), 0.5 U/mL of hexokinase (Worthington Biochemical), 1 U/mL of glucose-6-PO₄ dehydrogenase (Worthington Biochemical), 0.4 mmol/L thio-nicotinamide adenine dinucleotide (Sigma-Aldrich), and 1 mg/mL of bovine serum albumin prepared in 0.1 mol/L glycylglycine (Sigma-Aldrich, pH 7.5) was added to each well. After a 20 min incubation at room temperature, cell differentiation was determined by measuring the rate of thio-nicotinamide adenine dinucleotide reduction with a Dynex MRX Revelation Microtiter Plate Reader (Dynex Technologies Inc.) at 405 nm at 5 min intervals.

3.2.8 Nuclei Number Counting

Satellite cells were plated in gelatin-coated 24 well cell culture plates at a density of 12,500 cells per well, and were transfected and cultured as described above. At 72 h of fusion, the cells were fixed with 3% paraformaldehyde for 30 min at room temperature. The dye, 4’, 6-diamidino-2-phenylindole (Invitrogen) at a 1:10,000 dilution in PBS was then added to the cell cultures to stain the cell nuclei. Images were viewed with an Olympus XI 70 microscope and recorded with an Optronics digital camera. For each of the syndecan-4 mutants, wild type syndecan-4, and the empty pCMS-EGFP
vector, the number of nuclei in 25 individual myotubes was counted and the average number of nuclei per myotube was calculated.

### 3.2.9 Responsiveness to Fibroblast Growth Factor 2 Assay

Cellular responsiveness to FGF2 during proliferation was determined by measuring the DNA concentration in each well (Velleman et al., 2006). Satellite cells were plated in gelatin-coated 24 well cell culture plates at a density of 12,500 cells per well, and transfected as described above. After 6 h of transfection, the Dulbecco’s Modified Eagle Medium (DMEM) was removed and changed to serum-free defined media (McFarland et al., 2006) containing 0, 2.5, or 10.0 ng/mL of FGF2 (Pepro Tech). Cell responsiveness was measured at 72 h post-transfection by the DNA content as described above in the proliferation assay.

Cellular responsiveness to FGF2 was also measured at 0, 48, and 72 h of differentiation. Cells were plated and cultured as described in the differentiation assay except at the beginning of differentiation FGF2 was added at 0, 2.5 or 10 ng/mL. Cell responsiveness to FGF2 was determined by measuring the creatine kinase level as described above in the differentiation assay.

### 3.2.10 Syndecan-4 fusion protein analysis

Full length syndecan-4 was amplified from wild type syndecan-4 and syndecan-4 without N-glycosylated chains in pCMS-EGFP vector. The following primers were used for PCR reaction. The forward primer was 5′-ATA ATG CCG CTG CTC CGC G-3′ (Operon), and the reverse primer was 5′-AGC GTA GAA CTC ATT TGT AGG GGC-3′ (Operon). The PCR condition was denaturation at 95°C for 15 min, and 35 cycles of 30 s
at 94°C, 30 s at 60°C, 30 s at 72°C followed by 15 min at 72°C. The ligation reaction contained 3 μL of fresh PCR product from the above reaction, 1 μL of salt solution (1.2 mol/L NaCl and 0.06 mol/L MgCl2), and 1 μL of pcDNA3.1/V5-TOPO Vector (Invitrogen). After being incubated for 5 min at room temperature, 2 μL of the reaction was used to transform TOP10 Chemically Competent Escherichia coli (Invitrogen) according to the manufacturer’s instruction. Luria-Bertani-ampicillin agar plates were used to select positive clones. The positive clones were grown overnight at 37°C in LB broth containing 100 μg/mL ampicillin. The plasmids were isolated using QIAquick Miniprep kit (Qiagen) and confirmed by DNA sequencing.

Wild type syndecan-4 and syndecan-4 without N-glycosylated chains in pcDNA3.1/V5-TOPO Vector were then transfected into turkey satellite cells as described above. Control cells were treated through the transfection process but without any plasmid. At 72 h post-transfection, proteins were extracted from cells using cell lysis buffer A containing 50 mmol/L Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 1 mmol/L Na3VO4, and freshly prepared protease inhibitor cocktail (Roche Applied Science). Protein concentration in the supernatant was determined by the Bradford assay (Bradford 1976). Equal amounts of protein were mixed with reducing sample buffer (0.125 mol/L Tris–HCl, pH6.8, 4.1% SDS, 20% glycerol, 2%β-mercaptoethanol, and 0.001% bromphenol blue), boiled for 5 min and then separated on an 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Millipore). After being blocked for 2 h with 5% nonfat milk
dissolved in tris-buffered saline containing 20 mmol/L Tris–HCl, pH7.4, 150 mmol/L NaCl, and 0.05% Tween 20 (TBS-T), the PVDF membrane was incubated with the indicated antibodies in blocking buffer for 2 h at room temperature. Antibodies used for immunoblotting were mouse anti-V5 conjugated with alkaline phosphatase (1:5000 dilution; Invitrogen) and mouse monoclonal β-actin (1:10,000 dilution; Sigma). Secondary antibody used for β-actin was goat anti-mouse IgG alkaline phosphatase conjugated (1:10,000 dilution; Santa Cruz). After incubating with chemiluminescent alkaline phosphatase substrate (Millipore) for 5 min at room temperature, immunoreactive bands were visualized with a Bio-Rad ChemiDoc XRS imaging system (Bio-Rad). The size of the bands was determined with Quantity One 4.6.6 Software (Bio-Rad).

3.2.11 Western analysis

The pCMS-EGFP empty vector, wild type syndecan-4 and all of the syndecan-4 N-glycosylated chain mutants were transfected into turkey satellite cells. At 48 h post-transfection, proteins were extracted from cells using cell lysis buffer A. Protein concentration in the supernatant was determined by the Bradford assay. Equal amounts of protein were loaded on an 8% SDS-PAGE. Proteins were transferred to a PVDF membrane and screened with goat anti-human syndecan-4 antibody (1:2000 dilution; Santa Cruz) and mouse monoclonal β-actin antibody (1:10,000 dilution; Sigma). The size and quantity of the bands was determined with Quantity One 4.6.6 Software (Bio-Rad).
3.2.12 Statistical Analysis

Cell proliferation, differentiation, and FGF2 responsiveness assays were independently repeated at least three times. Within each experiment, there were four replicates of each treatment for proliferation and FGF2 responsiveness measurements during proliferation, and six replicates for differentiation and FGF2 responsiveness during differentiation. For the real time PCR, two replicates for each treatment in each assay were performed. The data from each repeat were used to calculate a mean and the standard error of the mean (SEM). Data are graphed as the mean ± SEM. The SAS PROC GLM (SAS Institute Inc.) was used for statistical analyses. Differences among means were detected using the Fisher’s least significance method. The cell proliferation assays, differentiation assays, and real-time quantitative PCR data were analyzed using SAS Proc GLM procedures. For the FGF2 responsiveness assays, the statistical model included the effect of FGF2 treatment, mutant transfection, and their interaction. The main factors of FGF2 treatment and variant mutant transfection and the interaction between two-factors were analyzed using SAS Proc GLM procedures. Differences among means in each experiment were evaluated using an anova and detected using Fisher’s least-significant-difference. Two-sided $P$-values of $P < 0.05$ were considered statistically significantly.

3.3 Results

3.3.1 Site-Directed Mutagenesis and the Over-Expression in Satellite Cells

Six syndecan-4 N-glycosylated chain mutants were generated by site-directed mutagenesis. The N-glycosylated chains attached to Asn$^{124}$ and Asn$^{136}$ are defined as N1
and N2 chain, respectively. The syndecan-4 mutants with N1 or N2 chains without changing the GAG chain attachment site are referred to as S4-N1 and S4-N2, respectively. The syndecan-4 mutants with the N1 or N2 chains with the GAG chain attachment sites eliminated are termed as S4-S0-N1 and S4-S0-N2, respectively. The syndecan-4 mutants without N-glycosylated chains attached to the core protein with or without GAG chains are defined as S4-N0 and S4-S0-N0, respectively. Immunoblotting of the fusion protein confirmed the deletion of syndecan-4 N-glycosylated chains (Figure 3.2). The wild type syndecan-4 had a molecular mass of 32 kDa, whereas S4-N0 had a molecular mass of 31 kDa.

All of the syndecan-4 N-glycosylated one chain and no-chain mutants with or without GAG chains and wild type syndecan-4 had significantly higher expression of syndecan-4 compared to the empty pCMS-EGFP vector (Figure 3.3). The overexpression of syndecan-4 was also confirmed at the protein level by immunohistochemistry at 24 h post-transfection (Figure 3.4). Figure 3.4a–c illustrates turkey satellite cells transfected with the pCMS-EGFP empty vector. Figure 3.4d–f illustrates turkey satellite cells transfected with wild type syndecan-4. Figure 3.4a,d shows brightfield images to indicate the cell morphology. Figure 3.4b,e shows the enhanced green fluorescence protein marker expressed from the pCMS-EGFP vector indicating the transfection of the cells. Figure 3.4c,f shows the cells stained with syndecan-4 antibody. The cells transfected with syndecan-4 and all of the syndecan-4 mutants had higher protein levels of syndecan-4 compared to the cells transfected with pCMS-EGFP empty vector as assayed by immunohistochemistry and western blot (Figure 3.5).
3.3.2 The effect of syndecan-4 N-glycosylated chains with glycosaminoglycan chains attached to the core protein on turkey satellite cell proliferation, responsiveness to fibroblast growth factor 2, and differentiation

Cells transfected with wild type syndecan-4 and all of the syndecan-4 N-glycosylated chain mutants with GAG chains had decreased proliferation at 72 h post-transfection compared to the cells transfected with the control pCMS-EGFP empty vector. There was no difference in proliferation between the cells transfected with syndecan-4 N-glycosylated one-chain and no-chain mutants with the GAG chains attached compared to the wild type syndecan-4 (Figure 3.6a). As shown in Figure 3.6b, cell replication was not affected by the transfection at 48 h post-transfection. Cell number was not significantly different among wild type syndecan-4, syndecan-4 N-glycosylated chain mutants with GAG chains, and pCMS-EGFP empty vector transfection, ranging from 80.0 to 92.3 cells/μL. At 72 h post-transfection, with 0, 2.5, or 10 ng/mL of FGF2, the cells transfected with S4-N2 had a significantly higher proliferation rate compared to the cells transfected with wild type syndecan-4, and other N-glycosylated chain mutants with GAG chains except for S4-N1 at 2.5 ng/mL of FGF2 (Figure 3.6c). Without FGF2 treatment, compared to the wild type syndecan-4 transfection, the S4-N2 transfected cells had significantly higher proliferation; with 2.5 and 10 ng/mL FGF2 the S4-N2 group also had significantly higher proliferation compared to the wild type syndecan-4 transfection. Statistical analysis was used to analyze the effect of FGF2 treatment, the effect of mutant transfection, and their interaction. Based on this analysis, the higher proliferation of the cells transfected with S4-N2 was due to a gene effect not the FGF2 treatment. These data suggested that
syndecan-4 N-glycosylated chains have no effect on cell responsiveness to FGF2 during proliferation.

At 48 and 72 h of differentiation, cells transfected with wild type syndecan-4 and syndecan-4 N-glycosylated mutants had decreased cell differentiation compared to those transfected with pCMS-EGFP empty vector, except for S4-N1 at 72 h of differentiation (Figure 3.7a). There was no significant difference in differentiation from 0 to 96 h between the cells transfected with syndecan-4 N-glycosylated chain mutants containing GAG chains and the wild type syndecan-4. The number of nuclei in the myotubes at 72 h of differentiation was similar in trend to the differentiation assay results (Figure 3.7b). At 72 h of differentiation, the average number of nuclei per myotube in the empty vector transfected cells was 27 compared to those transfected with wild type syndecan-4, S4-N0, S4-N1 and S4-N2 with 19.7, 22.9, 23.4, and 21.4, respectively.

3.3.3 The Effect of Syndecan-4 N-Glycosylated Chain Mutants with the Deletion of Glycosaminoglycan Chains on Turkey Satellite Cell Proliferation, Responsiveness to Fibroblast Growth Factor 2, and Differentiation

At 48 h post-transfection, satellite cells transfected with syndecan-4 N-glycosylated one-chain and no-chain mutants without the GAG chains had a higher level of proliferation compared to the cells transfected with wild type syndecan-4 (Figure 3.8a). At 72 h post-transfection, all the syndecan-4 N-glycosylated one-chain and no-chain mutants without the GAG chains also had a higher proliferation compared to the wild type syndecan-4. Transfection with S4-S0 did not influence cell proliferation compared to the cells transfected with wild type syndecan-4 at all sampling times.
number at 48 h post-transfection was not significantly affected by the overexpression of the syndecan-4 constructs (Figure 3.8b). At 0, 2.5, or 10 ng/mL of FGF2, cells transfected with syndecan-4 N-glycosylated one-chain and no-chain mutants without the GAG chains had no significant difference in proliferation ($P < 0.05$) compared to those transfected with wild type syndecan-4 (Figure 3.8c).

At 0, 24, 48, 72, and 96 h of differentiation, cells transfected with syndecan-4 N-glycosylated one-chain and no-chain mutants without GAG chains showed no difference in differentiation compared to the cells transfected with wild type syndecan-4 (Figure 3.9a). The average number of nuclei per myotube at 72 h of differentiation was similar to the differentiation assay data except the differentiation of S4-S0-N0 and S4-S0-N1 was significantly higher than the wild type syndecan-4 (Figure 3.9b). The average number of nuclei per myotube in cells transfected with pCMS-EGFP was 27.0, wild type syndecan-4 was 19.7, S4-S0 was 22.2, S4-S0-N0 was 24.7, S4-S0-N1 was 23.3, and S4-S0-N2 was 23.1.

### 3.3.4 The Effect of Syndecan-4 Glycosaminoglycan and N-Glycosylated Chains on Turkey Satellite Cell Responsiveness to Fibroblast Growth Factor 2 during Differentiation

Cell responsiveness to FGF2 was measured at 0, 48 and 72 h of differentiation (Figure 3.10, respectively). Cells treated with FGF2 showed decreased differentiation after 48 h in differentiation medium, but was not significantly decreased at 72 h of differentiation compared to the cells without FGF2 treatment. At 0 and 48 h of differentiation, the differentiation of the cells transfected with wild type syndecan-4, S4-
S0, S4-N0, and S4-S0-N0 was not significantly different compared to those transfected with the pCMS-EGFP empty vector within the same treatment of FGF2. Without FGF2 treatment, compared to the wild type syndecan-4 and pCMS-EGFP empty vector transfection, the cells transfected with S4-S0-N0 had significantly higher differentiation; with 2.5 and 10 ng/mL FGF2 treatment cells transfected with S4-S0-N0 also having significantly higher differentiation compared to the wild type syndecan-4 and pCMS-EGFP empty vector transfections. Statistical analysis was used to analyze the effect of FGF2 treatment, effect of mutant transfection, and their interaction. The analysis showed that the higher differentiation of the cells transfected with S4-S0-N0 was due to a gene effect, not the FGF2 treatment. These data indicated that syndecan-4 N-glycosylated chains and GAG chains are not required for cell responsiveness to FGF2 during differentiation.

3.4 Discussion

Syndecan-4 is a membrane associated HSPG that plays an important role in satellite cell maintenance, activation, proliferation, and differentiation (Cornelison et al., 2001, 2004; Tanaka et al., 2009). However, the mechanism of how syndecan-4 functions in these processes is still not well understood. Zhang et al. (2008) reported that syndecan-4 GAG chains are not required for syndecan-4 to affect turkey satellite cell proliferation, differentiation, and FGF2 responsiveness during proliferation. In the present study, focus was placed on the function of syndecan-4 N-glycosylated chains and the interaction between syndecan-4 N-glycosylated chains and GAG chains on turkey satellite cell proliferation, differentiation, and responsiveness to FGF2.
Syndecan-4 GAG chains and N-glycosylated chains may affect cell proliferation through regulating focal adhesion formation. Focal adhesions form where the cells attach to the substrate. Focal adhesions are composed of a large number of proteins including but not limited to integrins (Hynes, 1992; Schwartz et al., 1995), cadherins (Takeichi, 1988, 1990, 1991), and selectins (Bevilacqua and Nelson, 1993). They act not only as anchorage points for the cells, but also function in signal transduction pathways. Integrins are involved in cell proliferation, adhesion, and apoptosis in muscle cells (Liu et al., 2008). Saoncella et al. (1999) reported that syndecan-4 is required for integrin mediated fibronectin-induced focal adhesion formation. Syndecan-4 is an important regulator of the composition of focal adhesions (Woods and Couchman, 1994). The overexpression of syndecan-4 in Chinese hamster ovary K1 cells resulted in increased focal adhesion formation and decreased cell migration (Longley et al., 1999). It is possible that when syndecan-4 is overexpressed, more focal adhesions form and cell migration is decreased. Because migration is important for satellite cells to interact with each other to receive signals to promote proliferation, the increased number of focal adhesions may inhibit cell proliferation.

Oligomerization of syndecan-4 plays a critical role in the regulation of focal adhesion formation (Choi et al., 2005). Syndecan-4 has the tendency to form SDS-resistant dimers or oligomers (Rapraeger, 2000). Except for the transmembrane domain, syndecan-4 cytoplasmic domain and extracellular domain participate in syndecan-4 dimer formation (Carey, 1997; Oh et al., 1997). Whether syndecan-4 GAG chains and N-glycosylated chains are involved in dimer formation is not well understood at the present time. Sareneva et al. (1994) reported that the N-glycosylated chains are required for
proteins to form dimers. Syndecan-4 N-glycosylated chains may influence syndecan-4 dimer formation. Syndecan-4 GAG chains have been reported to play an important role in the initial binding of syndecan-4 to focal adhesions (Woods and Couchman, 2001). It is possible that when both GAG chains and N-glycosylated chains are deleted, syndecan-4 regulated focal adhesion formation is decreased, thus, the cells may have increased migration and a higher rate of proliferation. When only the GAG chains or N-glycosylated chains are deleted, syndecan-4 still has the ability to increase focal adhesion formation. As a result, cells will have decreased migration and proliferation.

Liu et al. (2006) found that syndecan-4 expression is higher during satellite cell proliferation but not during differentiation, which suggested that syndecan-4 may play an important role in regulating satellite cell proliferation but not differentiation. Zhang et al. (2008) reported that syndecan-4 GAG chains are not required for the initial differentiation. In the current study, syndecan-4 N-glycosylated chain mutants with or without GAG chains did not influence turkey myogenic satellite cell differentiation compared to wild type syndecan-4, which suggests that both GAG chains and N-glycosylated chains are not required for syndecan-4 to regulate turkey myogenic satellite cell differentiation.

The addition of exogenous FGF2 to satellite cell cultures increased proliferation and decreased differentiation reflecting its stimulating effect on proliferation and inhibitory effect on differentiation. However, the cellular responsiveness to FGF2 during both proliferation and differentiation suggest that syndecan-4 GAG and N-glycosylated chains are not required for the cellular response to FGF2. In other studies, syndecan-4
has been shown to function in an FGF2-independent manner (Velleman et al., 2007; Zhang et al., 2008). Syndecan-4 may regulate satellite cell growth and development through other signaling pathways like protein kinase C alpha (PKCα). In the presence of phosphatidylinositol 4,5-bisphosphate, the syndecan-4 cytoplasmic tail can bind to PKCα and activate PKCα (Horowitz and Simons, 1998; Oh et al., 1998). The activated PKCα can stimulate downstream signaling pathways to regulate cell growth and development. In summary, the data from the current study suggested that syndecan-4 N-glycosylated chains and GAG chains are required for satellite cell proliferation, but not for differentiation and cellular FGF2 responsiveness during proliferation and differentiation.
Acknowledgements

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References


Figure 3.1 Site-directed mutagenesis strategy used to generate syndecan-4 N-linked glycosylated (N-glycosylated) chain one-chain and no-chain mutants with or without glycosaminoglycan (GAG) chains. Syndecan-4 has three GAG chains attached to the core protein at Ser38, Ser65, and Ser67. The two N-glycosylated chains are attached to the core protein at Asn124 and Asn139. The Asn124 is referred to as N1 chain, and Asn139 is N2 chain. To distinguish different forms of syndecan-4, the following nomenclature was developed: S4 is syndecan-4; the number after N means the potential N-glycosylated chain attachment sites unaltered, for example: N1 means the syndecan-4 has the N1 chain site unaltered. S0 and N0 indicate all of the GAG chain attachment sites or all of the N chain attachment sites were altered.
Figure 3.2  Syndecan-4 fusion protein analysis. Proteins were extracted from randombred control 2 line male turkey skeletal muscle satellite cells that were transfected with wild type syndecan-4 (S4), syndecan-4 without N-glycosylated chains (S4N0) in pcDNA3.1/V5-His-TOPO vector or without any plasmid (NC). The anti-V5 antibody was used to detect the V5 tag expressed from the pcDNA3.1/V5-His-TOPO vector. The lanes are NC, S4, and S4-N0. Molecular mass is highlighted with an arrow. b-actin was used to confirm protein migration and had a molecular mass of 42 kDa.
Figure 3.3  Real-time quantitative PCR analysis of mRNA expression at 48 h post transfection. Randombred control 2 line male turkey myogenic satellite cells were transfected with the empty pCMS-EGFP vector (control), wild type syndecan-4 (S4), syndecan-4 N-glycosylated chain no-chain mutant (G1-N0), syndecan-4 N-glycosylated chain one-chain mutants (S4-N1 and S4-N2), syndecan-4 GAG no-chain mutant (S4-S0), syndecan-4 N-glycosylated chain no-chain mutant without GAG chains (S4-S0-N0), and syndecan-4 N-glycosylated chain one-chain mutants without GAG chains (S4-S0-N1 and S4-S0-N2). The error bars represent standard error of the mean. * Indicates a significant difference from the control ($P < 0.05$).
Figure 3.4 Syndecan-4 expression in randombred control 2 line male turkey myogenic satellite cells transfected with the pCMS-EGFP empty vector, wild type syndecan-4, and syndecan-4 mutants at 24 h post-transfection. Panel (a) cells transfected with pCMS-EGFP. Panel (b) cells transfected with syndecan-4. Panel (c) cells transfected with syndecan-4 N-glycosylated chain no-chain mutant. Panels (d) and (e) cells transfected with syndecan-4 N-glycosylated chain one-chain mutants attached to core protein at the Asn124 and Asn139, respectively. Panel (f) cells transfected with syndecan-4 glycosaminoglycan (GAG) no-chain mutant. Panel (g) cells transfected with syndecan-4 N-glycosylated chain no-chain mutant without GAG chains, and panels (h) and (i) cells transfected with syndecan-4 Nglycosylated chain one-chain mutants attached to the core protein at the Asn124 and Asn139 without GAG chains, respectively. Black and white images were bright field (BF) views of the cell (left column). Green fluorescence indicates the cells expressing enhanced green fluorescent protein (EGFP) from the pCMS-EGFP vector (middle column). The red fluorescent images were stained with goat anti-human syndecan-4 antibody and a donkey anti-goat IgG rhodamine conjugated secondary antibody (right column). The scale bar represents 50µmol/L.
Figure 3.5 Western analysis of syndecan-4 expression in randombred control 2 line male turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (control), wild-type syndecan-4 (S4), syndecan-4 mutants (syndecan-4 Nglycosylated chain no-chain mutant [S4-N0], syndecan-4 Nglycosylated chain one-chain mutants attached to core protein at the Asn124 [S4-N1] and Asn139 [S4-N2], respectively, syndecan-4 glycosaminoglycan (GAG) no-chain mutant [S4-S0], syndecan-4 N-glycosylated chain no-chain mutant without GAG chains [S4S0-N0], syndecan-4 N-glycosylated chain one-chain mutants attached to the core protein at the Asn124 and Asn139 without GAG chains [S4-S0-N1 and S4-S0-N2, respectively] at 48 h post-transfection. Each lane contains 50μg of protein and was hybridized to anti-human syndecan-4 and anti-mouse β-actin antibodies. A ratio was calculated with the average density of syndecan-4 and its mutants relative to the control after normalization with β-actin.
Figure 3.6 Proliferation analysis and fibroblast growth factor 2 (FGF2) responsiveness of the randombred control 2 line male turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), syndecan-4 N-glycosylated chain no-chain mutant (S4-N0), and syndecan-4 N-glycosylated chain one-chain mutants (S4-N1 and S4-N2). A) The proliferation assay measured the DNA concentration in the wells of cultured cells at 24 h intervals after transfection for 72 h. B) Cell concentration at 48 h post transfection. C) Cellular response to FGF2 at concentrations of 0, 2.5, and 10 ng/mL at 72 h post transfection. The error bars represent standard error of the mean. Bars without a common letter within times or FGF2 concentration were significantly different ($P<0.05$).
Figure 3.7 Differentiation assay of the randombred control 2 line male turkey myogenic satellite cells after transfection with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), syndecan-4 N-glycosylated chain no-chain mutant (S4-N0), and syndecan-4 N-glycosylated chain one-chain mutants (S4-N1 and S4-N2). A) The differentiation assay in cultured cells at 24 h intervals starting at 0 h until 96 h of differentiation. B) The average number of nuclei counted in 25 individual myofibers at 72 h differentiation after being stained with 4′-6-diamidino-2-phenylindole. The error bars represent the standard error of the mean. Bars without common letters are significantly different ($P < 0.05$).
Figure 3.8 Proliferation analysis and fibroblast growth factor 2 (FGF2) responsiveness of the randombred control 2 line male turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), syndecan-4 without GAG chains (S4-S0), syndecan-4 N-glycosylated chain no-chain mutant without GAG chains (S4-S0-N0), and syndecan-4 N-glycosylated chain one-chain mutants without GAG chains (S4-S0-N1 and S4-S0-N2). A) Cell proliferation at 24 h intervals until 72 h post transfection. B) Cell concentration at 48 h post transfection. C) Cellular response to FGF2 at concentrations of 0, 2.5, and 10 ng/mL at 72 h post transfection. Cell responsiveness to FGF2 was measured by the DNA concentration in the wells of cultured cells. The error bars represent standard error of the mean. Bars without common letter within times or FGF2 concentration were significantly different ($P < 0.05$).
Figure 3.9  Differentiation of the randombred control 2 line male turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), syndecan-4 without GAG chains (S4-S0), Syndecan-4 N-glycosylated chain no-chain mutant without GAG chains (S4-S0-N0), and syndecan-4 N-glycosylated chain one-chain mutants without GAG chains (S4-S0-N1 and S4-S0-N2).  A) The rate of differentiation was measured at 24 h intervals starting from 0 h differentiation through 96 h.  B) The average number of nuclei counted in 25 individual myofibers at 72 h differentiation.  Cells were stained with 4'-6-diamidino-2-phenylindole.  The error bars represent standard error of the mean.  Bars without common letters indicate a significant difference ($P < 0.05$).
Figure 3.10 Fibroblast growth factor 2 (FGF2) responsiveness of the randombred control 2 line male turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), syndecan-4 N-glycosylated chain no-chain mutant with or without GAG chains (S4-N0 and S4-S0-N0), and syndecan-4 without GAG chains (S4-S0).  A) Cellular response to FGF2 at concentrations of 0, 2.5, and 10 ng/mL at 0 h of differentiation.  B) Cellular response to FGF2 at concentrations of 0, 2.5, and 10 ng/mL at 48 h of differentiation.  C) Cellular response to FGF2 at concentrations of 0, 2.5, and 10 ng/mL at 72 h of differentiation.  The error bars represent standard error of the mean.  Bars without common letter within times or FGF2 concentration were significantly different (P < 0.05).
CHAPTER 4: FIBROBLAST GROWTH FACTOR 2 AND PROTEIN KINASE C ARE INVOLVED IN SYNDECAN-4 CYTOPLASMIC DOMAIN MODULATED SATELLITE CELL PROLIFERATION

Abstract

The core protein of syndecan-4 is composed of extracellular, transmembrane, and cytoplasmic domains. The cytoplasmic domain functions in signal transduction into the cell through protein kinase C alpha (PKCα) pathway. The current study investigated the function of the syndecan-4 cytoplasmic domain in turkey muscle cell proliferation, differentiation, fibroblast growth factor 2 (FGF2) responsiveness, and PKCα activity. Turkey syndecan-4, syndecan-4 without the cytoplasmic domain with or without the glycosaminoglycan (GAG) and N-linked glycosylated (N-glycosylated) chains were cloned into the pCMS-EGFP and the pcDNA3.1/V5-His TOPO expression vectors. Syndecan-4 and syndecan-4 mutants were transfected or co-transfected with a small interfering RNA targeting the cytoplasmic domain into turkey muscle satellite cells. After transfection, cell proliferation, differentiation, FGF2 responsiveness, and PKCα activity (cell membrane localization) were measured. The overexpression of syndecan-4 mutants increased cell proliferation but did not change cell differentiation. Syndecan-4 without the cytoplasmic domain with or without the GAG chains and N-glycosylated
chains had increased cellular responsiveness to FGF2 during proliferation. Syndecan-4 increased cell membrane localization of PKCα, whereas syndecan-4 mutants had decreased PKCα activity compared to syndecan-4. These data indicated that syndecan-4 cytoplasmic domain and the GAG and N-glycosylated chains are critical in syndecan-4 regulating satellite cell proliferation, responsiveness to FGF2, and PKCα activity.

4.1 Introduction

Syndecans are cell surface proteoglycans. There are four syndecans expressed in mammals, syndecan-1 through -4. Syndecan-1 and syndecan-3 belong to one subfamily whereas syndecan-2 and syndecan-4 belong to another based on sequence homology (Bernfield et al., 1999; Couchman et al., 2001). All syndecans have a core protein and covalently attached glycosaminoglycan (GAG) chains. Syndecans are always substituted with heparan sulfate chains, and can also be attached with dermatan or chondroitin sulfate chains (Bernfield et al., 1999; Deepa et al., 2004). The heparan sulfate chains play important roles in interacting with extracellular matrix molecules, growth factors, chemokines, and cytokines (Esko and Selleck 2002).

Syndecan-4 functions as a coreceptor for fibroblast growth factor 2 (FGF2) by binding FGF2 to its heparan sulfate chains, presenting FGF2 to its high affinity tyrosine kinase receptor and then initiating downstream signaling pathways (Volk et al., 1999). The chimera experiments by Volk et al. (1999) and Horowitz et al. (2002) suggested that the cytoplasmic domain of syndecan-4 core protein is also important in mediating FGF2 signaling. The syndecan-4 cytoplasmic domain of syndecan-4 functions by regulating
FGF2 induced cell proliferation (Volk et al., 1999). Syndecan-4 core proteins have three domains: extracellular, transmembrane, and cytoplasmic domains. The extracellular domain has low homology with other syndecans, whereas the transmembrane and cytoplasmic domains have highly conserved sequences. The cytoplasmic domain has three regions, one variable region (V) and two highly conserved regions (C1 and C2). The V region is presumed to be unique for each syndecan family member.

The V region of the syndecan-4 cytoplasmic domain interacts with each other to form a homodimer structure (Lee et al., 1998; Shin et al., 2001). The dimer structure is further stabilized by the binding of phosphatidylinositol 4, 5 bisphosphate (PIP2) to the V region (Lee et al., 1998). The oligomerization of the syndecan-4 cytoplasmic domain stimulates the cell membrane localization (activity) of protein kinase Ca (PKCa) (Oh et al., 1997a) by directly binding the catalytic domain of PKCa to the V region of syndecan-4.

In the current study, how the syndecan-4 cytoplasmic domain regulates turkey myogenic satellite cell proliferation, differentiation, FGF2 responsiveness, and PKCa activity was explored. Syndecan-4 mutants without cytoplasmic domains with deletion of N-linked glycosylated (N-glycosylated) chains and GAG chains were transfected into turkey myogenic satellite cells. Proliferation, differentiation, responsiveness to FGF2, and PKCa activity were then measured. The results from the current study provide new information about the function of the syndecan-4 cytoplasmic domain in myogenesis, and the pathways that are involved.
4.2 Materials and Methods

4.2.1 Clone Generation

Turkey cDNA generated from 18 day embryonic pectoralis major muscle was used as a template to generate syndecan-4 without its cytoplasmic domain (S4C). Syndecan-4 without any N-glycosylated chains (S4-N0), syndecan-4 without any GAG chains (S4-S0), and syndecan-4 without any GAG and N-glycosylated chains (S4-S0N0) cloned into the pCMS-EGFP vector (Song et al., 2011) were used as templates to generate syndecan-4 cytoplasmic domain mutants with or without N-glycosylated chains and GAG chains (S4C, S4C-N0, S4C-S0, and S4C-S0N0; Figure 4.1). The primers used for all of the mutants were: forward primer 5’- ATA CTC GAG 1ATG CCG CTG CTC CGC GCC GCC TTC 24 -3’, and reverse primer 5’- CGC TCT AGA TTA 507ATA GAC TAA GAG GAT CAG G486 -3’. Amplification consisted of an initial denaturation at 94 °C for 15 min, and 35 cycles of 30 s at 94 °C, 20 s at 55 °C, 30 s at 72 °C followed by 5 min at 72°C. The PCR product was inserted into the pCMS-EGFP vector. The ligation reaction was performed by using T4 ligase (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Clones were confirmed by DNA sequencing in the Molecular and Cellular Imaging Center (The Ohio State University, Wooster, OH, USA).

The wild type syndecan-4 (S4) was subcloned into a pcDNA3.1/V5 His TOPO vector (Invitrogen, Carlsbad, CA, USA) previously by Song et al. (2011). The S4C, S4C-N0, S4C-S0, and S4C-S0N0 were cloned into the pCMS-EGFP vector and also subcloned into the pcDNA3.1/V5 His TOPO vector. The primers for subcloning were: forward
primer 5’-ATA 1ATG CCG CTG CTC CGC G16 -3’, and reverse primer 5’-507ATA GAC TAA GAG GAG GAT CAG GAA G483 -3’. The PCR condition was denaturation at 95 °C for 15 min, and 35 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 75 °C followed by 15 min at 75 °C. The ligation reaction contained 3 µl of PCR product from the above reaction, 1 µl of pcDNA3.1/V5-His-TOPO Vector (Invitrogen) and 1 µl of salt solution (1.2 M NaCl and 0.06 M MgCl2). Clones were confirmed by DNA sequencing in the Molecular and Cellular Imaging Center.

4.2.2 Satellite Cell Culture and Co-transfection

Satellite cells were isolated from the pectoralis major muscle of 7-week-old male randombred control 2 line turkeys (Velleman et al., 2000). Satellite cells were plated in gelatin-coated cell culture plates (Greiner Bio-one, Monroe, NC, USA) in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% chicken serum (Invitrogen), 5% horse serum (Invitrogen), 1% antibiotic/antimycotic (Invitrogen), and 0.1% gentamicin (Invitrogen) in a 37.5 °C 5%CO2/ 95% air incubator. After 24 h attachment, the cells were transfected with the empty vector, or S4, or co-transfected with S4C, S4C-N0, S4C-S0, and S4C-S0N0 and a siRNA (Invitrogen) targeting syndecan-4 cytoplasmic domain which will knock down endogenous syndecan-4 and have no effect on the syndecan-4 mutants without the cytoplasmic domain (Figure 4.1). The siRNA used was, sense: 5’-544GGG AAG AAA CCA AUC UAC AAG AAA G568 -3’, and antisense: 5’-CUU UCU UGU AGA UUG GUU UCU UCC C -3’. For transfection, 0.1 µg plasmid DNA (for 48-well cell culture plate) or 0.5 µg plasmid DNA (for 24-well cell culture plate) was used to transfect cells using Optifect transfection reagent (Invitrogen).
according to the manufacturer’s protocol. For co-transfection, 0.1 µg plasmid DNA and 2 pmol siRNA (48-well cell culture plate) or 0.5 µg plasmid DNA and 20 pmol siRNA (for 24-well cell culture plate) were used to transfect cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s protocol. After 6 h of transfection, the medium was changed to feeding medium (McCoy’s 5A medium (Sigma-Aldrich, St. Louis, MO, USA), 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic, and 0.1% gentamicin). Feeding medium was changed daily until 72 h following transfection or until the cells reached 60% confluency for differentiation assays. Differentiation was induced by changing the feeding medium to fusion medium (DMEM containing 3% horse serum, 1% antibiotic/antimycotic, 0.01 mg/mL porcine gelatin (Sigma-Aldrich), 0.1% gentamicin, and 1.0 mg/mL bovine serum albumin (Sigma-Aldrich)). The differentiation medium was changed every 24 h until 72 h of differentiation. At each sampling time, cell cultures were removed from the incubator, rinsed 3 times with sterile phosphate buffered saline (PBS; pH 7.08, 137 mM NaCl, 1.47 mM KH₂PO₄, 7.81 mM Na₂HPO₄, and 2.68 mM KCl), air dried, and then stored at -70 °C until analysis.

4.2.3 Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the cell cultures at 48 h post-transfection using TRIzol (Invitrogen) according to the manufacturer’s protocol. The cDNA was synthesized using moloney murine leukemia virus reverse transcriptase (M-MLV; Promega) according to the protocol by Song et al. (2011).
4.2.4 Real-Time Quantitative PCR

Real-time quantitative PCR was performed using the DyNAmo Hot Start SYBR Green qPCR kit (Finnzymes, Ipswich, MA, USA). The PCR reaction consisted of 2 μL cDNA, 10 μL 2X master mix, 250 nM of each of the forward and reverse primers, and nuclease-free water up to 20 μL. The primers targeting the extracellular domain to measure total syndecan-4 (syndecan-4 with and without cytoplasmic domain) expression were: forward primer 5’-102CTACCCTGGCTCTGGAGACCT-3’ and reverse primer 5’-335TCATTGTCCAGCATGGGTGTTT315-3’. The primers targeting the cytoplasmic domain to determine endogenous syndecan-4 (syndecan-4 with cytoplasmic domain) expression were: forward primer 5’-382AAGATCTCCATGGCAAGC401-3’ and reverse primer 5’-539TCGTAGCTGCCTCATCCTT520-3’. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: forward primer 5’-504GAGGGTAGTGGAAGGCTG523-3’ and reverse primer 5’-703CCACAACACGGTTCTGTAT684-3’. For all of the genes, the cycling parameters were denaturation at 95 °C for 15 min, followed by 34 cycles of 94 °C for 30 s, 60 °C for 30 s and at 72 °C for 30 s with a final elongation of 72 °C for 5 min. The melting curve program was 52 °C to 95 °C, 0.2 °C per read, and a 1 s hold. Standard curves were constructed with serial dilutions of purified PCR products from each gene. The amount of sample cDNA for endogenous and total syndecan-4 was determined by comparing the results to the standard curve respectively, and then normalized to GAPDH expression. The PCR products were verified by DNA sequencing for specificity.
4.2.5 Proliferation Assay

The proliferation assay was performed as described by Velleman et al. (2006). In brief, satellite cells were plated in gelatin-coated 24-well cell culture plates at a density of 12,500 cells per well, and transfected or cotransfected as described above. At 0, 24, 48, and 72 h following transfection, cell culture plates were removed from the incubator and rinsed with sterile PBS 3 times. All plates were then stored at -70 °C until analysis. Cell proliferation was measured by DNA content in each well by the method of McFarland et al. (1995). The Hoechst 33258 fluorochrome (Sigma-Aldrich) was used to measure DNA concentration on a Fluoroskan Ascent FL plate reader (ThermoElectron Co., Waltham, MA) using double-stranded calf thymus DNA as the standard.

4.2.6 Differentiation Assay

Satellite cells were plated in gelatin-coated 48-well cell culture plates at a density of 10,000 cells per well, and were transfected or co-transfected as described above. Differentiation was induced by changing the feeding medium to low serum differentiation medium when the cells reached 60% confluency. At 0, 24, 48, and 72 h of differentiation, cell cultures were removed from the incubator, rinsed with sterile PBS 3 times, air dried, and then stored at -70 °C until analysis. Differentiation was determined by measuring the muscle specific creatine kinase level by a method modified from Yun et al. (1997). In brief, all plates were brought to room temperature for 10 min. Creatine phosphokinase (Sigma-Aldrich) was used to generate a standard curve. Five hundred microliters of creatine kinase assay buffer consisting of 20 mM glucose (Thermo Fisher Scientific, Hudson, NH, USA), 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 of hexokinase (Worthington Biochemical, Lakewood, NJ, USA), 1 U/mL of glucose-6-PO4 dehydrogenase (Worthington Biochemical), 0.4 mM thio-nicotinamide adenine dinucleotide (Sigma-Aldrich), and 1 mg/mL of bovine serum albumin prepared in 0.1 M glycylglycine (Sigma-Aldrich, pH 7.5) was added to each well. After a 5 min incubation at room temperature, cell differentiation was determined by measuring the rate of thio-nicotinamide adenine dinucleotide reduction with a BioTek ELx800 plate reader (Biotek, Winooski, VT, USA) at 405 nm at 5 min intervals.

4.2.7 Responsiveness to Fibroblast Growth Factor 2 Assay

Cellular responsiveness to FGF2 during proliferation was determined by measuring the DNA concentration in each well (Velleman et al., 2006). Satellite cells were plated and transfected as described in the proliferation assay. After 6 h of transfection, the DMEM medium was removed and changed to serum-free defined media (McFarland et al., 2006) containing 0, 2.5, or 10.0 ng/mL of FGF2 (Pepro Tech, Rocky Hill, NJ, USA). Cell responsiveness was measured at 72 h post-transfection by the DNA content as described above in the proliferation assay.

4.2.8 Western analysis

The pCMS-EGFP empty vector, S4, S4C, S4C-N0, S4C-S0, and S4C-S0N0 were transfected or co-transfected as described above into turkey satellite cells. Cell membrane proteins were extracted using a hypo-osmotic solution (20 mM Tris/HCl (pH 7.5), 2 mM 2-mercaptoethanol, 5 mM ethylene glycol tetraacetic acid, and 2 mM
Ethlenediaminetetraacetic acid) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) (Keum et al., 2004). Briefly, 150 µl of hypo-osmotic solution was added to the cell culture, cells were then scraped off the plate and homogenized on ice for 10 min. The cell membrane proteins were collected by centrifuging the cell lysate at 13,000 x g for 15 min at 4 °C. The cell pellet was then resuspended in RIPA buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 10 mM NaF, and 2 mM Na3VO4, and protease inhibitor cocktail) and centrifuged at 15,000 x g for 15 min at 4 °C. The supernatant containing cell membrane proteins was collected.

Ten microgram of protein was mixed with reducing sample buffer (0.125 M Tris-HCl, pH6.8, 4.1% SDS, 20% glycerol, 2% β-mercaptoethanol, and 0.001% bromphenol blue), boiled for 5 min, and then separated on an 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) by the method of Laemmli (1970). Proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA). After being blocked with 5% nonfat milk dissolved in Tris-buffered saline containing 20 mM Tris–HCl, pH7.4, 150 mM NaCl, and 0.05% Tween 20 (TBS-T) for 1 h, the PVDF membrane was incubated with the indicated antibodies in blocking buffer for 2 h at room temperature or overnight at 4 °C. Primary antibodies used were mouse anti-human PKCα (BD Biosciences, Franklin Lakes, NJ, USA; 1:5,000) and mouse monoclonal β-actin (Sigma-Aldrich, 1:10,000). Secondary antibody used was rabbit anti-mouse IgG alkaline phosphatase conjugated (Santa Cruz, Santa Cruz, CA, USA; 1:10,000). After incubating with chemiluminescent alkaline phosphatase substrate (Millipore) for 5 min at room temperature, immunoreactive bands were detected with a
Bio Rad ChemiDoc XRS imaging system (Bio Rad, Hercules, CA, USA). The relative quantity of the bands was measured with Quantity One 4.6.6 Software (Bio Rad).

4.2.9 Confocal Immunofluorescence Microscopy

Cells were seeded on gelatin coated polystyrene slides (Electron Microscopy Sciences, Hatfield, PA, USA) and transfected with S4 and the appropriate syndecan-4 mutants in pcDNA3.1/V5 His TOPO vector. At 48 h following transfection, cells were rinsed with PBS 3 times and fixed with 3% paraformaldehyde for 30 min at room temperature. After 30 min of blocking in 1% BSA in TBS-T, cells were incubated with the indicated primary antibody overnight at 4 °C for 2 h at room temperature. Cells were then rinsed 3 X with 1% BSA in TBS-T and incubated with the appropriate secondary antibody for 1 h. Primary antibodies were sheep anti-V5 tag (Abcam, Cambridge, MA, USA; 1: 2,000) and mouse anti-human PKCα (BD Biosciences, 1:5,000). The secondary antibodies were Alexa Fluor 488 donkey anti-sheep (Invitrogen, 1:1,000), and Alexa Fluor 568 rabbit anti-mouse (Invitrogen, 1:1,000), respectively. Samples were mounted with gel/mount media and covered with microscope glass cover slips No. 1.5 (VWR, Radnor, PA, USA). The cell cultures were viewed and recorded with a Leica TCS SP confocal scanning microscope system (Leica, Allendale, NJ, USA).

4.2.10 Statistical Analysis

Cell proliferation, differentiation, and FGF2 responsiveness assays were independently repeated at least three times. Within each experiment, there were four replicates of each treatment for proliferation and FGF2 responsiveness measurements during proliferation, and five replicates for differentiation. For real time quantitative
PCR, two replicates for each treatment in each assay were performed. The data from each repeat were used to calculate a mean and the standard error of the mean (SEM). Data are graphed as the mean ± SEM. The SAS PROC GLM (SAS Institute Inc. 2002, Cary, NC, USA) was used for statistical analyses. Differences among means were detected using the Fisher’s least significant difference method. The cell proliferation assays, differentiation assays, and real-time quantitative PCR data were analyzed using SAS Proc GLM procedures. For the FGF2 responsiveness assays, the statistical model included the effect of FGF2 treatment, mutant transfection, and their interaction. The main factors of FGF2 treatment and variant mutant transfection and the interaction between two-factors were analyzed using SAS Proc GLM procedures. Differences among means in each experiment were evaluated using an ANOVA and detected using Fisher's least-significant-difference. Two-sided $P$ values of $P < 0.05$ were considered statistically significantly.

4.3 Results

4.3.1 Satellite Cell Co-transfection

The cotransfection of the syndecan-4 mutants significantly increased the expression of total syndecan-4 contained both endogenous and transfected exogenous syndecan-4 (Figure 4.2a). The expression of endogenous syndecan-4 (Figure 4.2b) was decreased by the cotransfection of the syndecan-4 siRNA compared to the empty pCMS-EGFP vector.
4.3.2 Syndecan-4 Cytoplasmic Domain Modulation of Turkey Satellite Cell Proliferation

At 0 and 24 h following transfection, cells transfected with syndecan-4 cytoplasmic domain mutants had no significant difference in proliferation compared to the cells transfected with S4 (Figure 4.3). Cells transfected with S4C-N0, S4C-S0, and S4C-S0N0 had significantly higher proliferation at 48 h following transfection compared to the cells transfected with the S4 and pCMS-EGFP empty vector. At 72 h following transfection, cells transfected with S4C, S4C-N0, S4C-S0, and S4C-S0N0 had increased cell proliferation compared to the cells transfected with the S4 and pCMS-EGFP empty vector. These data suggest that the cytoplasmic domain, the N-glycosylated chains and GAG chains in combination with the cytoplasmic domain are critical in regulating satellite cell proliferation.

4.3.3 Syndecan-4 Cytoplasmic Domain Regulation of Satellite Cell Responsiveness to Fibroblast Growth Factor 2

At 72 h following transfection, without FGF2 treatment, cells transfected with S4, S4C, S4C-N0, S4C-S0, and S4C-S0N0 had significantly higher proliferation compared to the cells transfected with empty pCMS-EGFP vector (Figure 4.4). With 2.5 and 10 ng/ml FGF2, the cells transfected with S4C, S4C-S0, and S4C-S0N0 had significantly higher proliferation compared to the cells transfected with S4 and the empty pCMS-EGFP vector. However, S4C-N0 had no significant effect on cell proliferation in response to FGF2 compared to the S4 and the empty pCMS-EGFP vector. Statistical analysis was used to analyze the effect of FGF2 treatment, effect of mutant transfection, and their
interaction, and indicated that the syndecan-4 cytoplasmic domain, the GAG and N-glycosylated chains in combination with the cytoplasmic domain are critical for the cells to respond to FGF2 during proliferation.

4.3.4 Syndecan-4 Cytoplasmic Domain Regulation of Turkey Satellite Cell Differentiation

At 0, 24, 48, and 72 h differentiation, cells transfected with S4C, S4C-N0, S4C-S0, and S4C-S0N0 had no significant effect on cell differentiation compared to the cells transfected with the S4 (Figure 4.5). These data suggested that syndecan-4 cytoplasmic domain, the N-glycosylated chains, and the GAG chains have no significant effect on satellite cell differentiation.

4.3.5 Protein Kinase C Alpha Signal Transduction is Regulated by the Syndecan-4 Cytoplasmic Domain

At 48 h following transfection, cells transfected with S4 had increased membrane localization of PKCα compared to the cells transfected with pCMS-EGFP empty vector (Figure 4.6). Compared to the cells transfected with S4, cells transfected with S4C, S4C-N0, S4C-S0, and S4C-S0N0 had decreased levels of PKCα localized on the cell membrane. These data were supported by confocal immunofluorescence microscopy (Figure 4.7). Cells transfected with S4 and S4-S0N0 had increased membrane localization of PKCα compared to the cells without transfection (Figure 4.7a and e, respectively). Cells transfected with S4C, S4C-N0, and S4C-S0 also had increased cell membrane localization of PKCα compared to the cells without transfection (Figure 4.7b-d), but relatively lower than the cells transfected with S4 and S4C-S0N0. These data
indicated that the cytoplasmic domain of syndecan-4, GAG chains, and N-glycosylated chains are important in syndecan-4 regulation of PKCα activity.

4.4 Discussion

Syndecan-4 is a cell membrane associated heparan sulfate proteoglycan. It plays an important role in satellite cell maintenance, activation, proliferation, and differentiation (Cornelison et al., 2001; Cornelison et al., 2004; Tanaka et al., 2009). However, the mechanism of how syndecan-4 functions in these processes is still not well understood. In a previous study by Zhang et al. (2008), it was reported that the deletion of one or more syndecan-4 GAG chains did not influence turkey satellite cell proliferation, differentiation, and FGF2 responsiveness during proliferation, and the deletion of GAG chains in combination with N-glycosylated chains modulated cell proliferation, but not differentiation and cellular responsiveness to FGF2 (Song et al., 2011). It is possible the deletion of both GAG chains and N-glycosylated chains changes the conformation of the core protein and leads to the functional changes in cellular responsiveness to FGF2. Not only the GAG and N-glycosylated chains, but also syndecan-4 core protein are critical in its biological functions. The core protein of syndecan-4 is divided into an extracellular, transmembrane, and cytoplasmic domain. The cytoplasmic domain of syndecan-4 is important in transferring signals into the cell. In the current study, focus was placed on the function of the syndecan-4 cytoplasmic domain and the interaction between the cytoplasmic domain and side chains (GAG and
N-glycosylated chains) on turkey satellite cell proliferation, differentiation, responsiveness to FGF2, and PKCα activity.

The transfection of S4C, S4C-N0, S4C-S0, and S4C-S0N0 increased cell proliferation compared to S4. Song et al. (2011) showed at 72 h following transfection, syndecan-4 without N-glycosylated chains (S4-N0), without GAG chains (S4-S0), and without both N-glycosylated and GAG chains (S4-S0N0) had no affect on cell proliferation compared to S4. These data indicated that the cytoplasmic domain is critical for syndecan-4 regulating turkey satellite cell proliferation. The cytoplasmic domain of syndecan-4 plays important roles in focal adhesion formation and signal transduction (Echtermeyer et al., 1999; Saoncella et al., 1999; Woods et al., 2000; Woods and Couchman, 2001). Syndecan-4 activates PKCα by binding the catalytic domain of the PKCα to the V region of the syndecan-4 cytoplasmic domain (Oh et al., 1997 a, b). The deletion of the syndecan-4 cytoplasmic domain may decrease the activity of PKCα. Data from the current study indeed showed that the overexpression of S4 increased PKCα activity. The deletion of the cytoplasmic domain decreased PKCα activity compared to S4. Not only syndecan-4 but also PKCα can affect the formation of focal adhesions (Haller et al., 1998). It is possible that the deletion of the cytoplasmic domain of syndecan-4 decreased focal adhesion formation both directly and through decreased PKCα membrane localization, which causes cells to have more freedom to migrate and proliferate.

In low serum media containing 2.5 or 10 ng/ml FGF2, cells transfected with S4C, S4C-S0, and S4C-S0N0 had increased cell proliferation compared to the cells transfected
with S4 and S4C-N0. Volk et al. (1999) reported that the cytoplasmic domain of syndecan-4 is important in FGF2-induced proliferation in immortalized ECV304 cells. Data in the current study suggested that not only the cytoplasmic domain, but also the N-glycosylated chains and GAG chains play important roles in FGF2-induced cell proliferation. When comparing S4 to S4C cells, the latter were more responsive to FGF2, indicating that the cytoplasmic domain is critical in FGF2-induced cell proliferation. When comparing S4C with S4C-N0, S4C cells lost the proliferation responsiveness to FGF2, indicating that the N-glycosylated chains have an important effect on cellular responsiveness to this growth factor. Furthermore, when comparing S4C-N0 and S4C-S0N0, only S4C-N0 cells retained proliferation responsiveness to FGF2, suggesting an important role of the GAG chains in the FGF2 signaling pathway.

The mechanism whereby the deletion of the syndecan-4 cytoplasmic domain increases cellular responsiveness to FGF2 may involve the Ser residue in the C1 region of the syndecan-4 cytoplasmic domain (Horowitz and Simons 1998). Horowitz and Simons (1998) reported that the Ser residue in the C1 region of syndecan-4 cytoplasmic domain can be phosphorylated by growth inhibition and dephosphorylated by the addition of FGF2. The mutation or deletion of the Ser residue in the syndecan-4 cytoplasmic domain may increase syndecan-4 binding of FGF2 and presentation of FGF2 to its high affinity tyrosine kinase receptor, thus increasing the cellular responsiveness to FGF2 (Figure 4.8a,b).

It has been reported that the core protein of syndecans and glypicans can directly bind to growth factors including transforming growth factor-β, insulin-like growth factor
2, and FGF2 (Pilia et al., 1996; Chen et al., 2004; Kirkpatrick et al., 2006). Syndecan-4 interaction with FGF2 may not entirely depend on GAG chains but also on the core protein. Based on the fact that N-linked glycosylated chains are involved in proper folding of proteins (Parodi 2000; Helenius and Aebi 2001), and localization of membrane proteins to the cell surface (Martínez-Maza et al., 2001; Yan et al., 2002), the deletion of N-glycosylated chains from S4C may change the three dimensional structure of the core protein leading to decreased binding of FGF2 to the GAG chains and the core protein (Figure 4.8c) causing decreased cellular responsiveness to FGF2. The deletion of GAG chains from S4C may increase core protein binding of FGF2 by exposing more space of the core protein to FGF2 (Figure 4.8d). The deletion of both GAG chains and N-glycosylated chains from S4C may change core protein three dimensional structure, however, without GAG chains attached to the core protein, there may be more available spatially for the core protein to bind to FGF2 (Figure 4.8e) or direct interactions between the GAG and N-glycosylated chains, as hypothesized by Song et al. (2011).

The expression of syndecan-4 is higher during satellite cell proliferation, suggesting that syndecan-4 may play an important role in regulating satellite cell proliferation but not differentiation (Liu et al., 2006). Song et al. (2011) reported that S4-N0, S4-S0, and S4-S0N0 had no effect on turkey satellite cell differentiation compared to S4. In the present study, satellite cells transfected with S4C, S4C-N0, S4C-S0, and S4C-S0N0 displayed differentiation rates similar to the cells transfected with S4. These data together indicated that the cytoplasmic domain of syndecan-4 has no major role in mediating turkey satellite cell differentiation.
In summary, the data from the current study supported that syndecan-4 cytoplasmic domain, N-glycosylated chains, and GAG chains have no effect on satellite cell differentiation, but are important in regulating turkey satellite cell proliferation, and FGF2 and PKCα are involved in this process.
Acknowledgments

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References


Figure 4.1 Syndecan-4 cytoplasmic domain mutagenesis strategy and the small interfering RNA (siRNA) targeting the cytoplasmic domain used to knockdown endogenous syndecan-4. S4, wild type syndecan-4; S4C, syndecan-4 without cytoplasmic domain; S4C-N0, syndecan-4 without cytoplasmic domain and N-linked glycosylated chains; S4C-S0, syndecan-4 without cytoplasmic domain and glycosaminoglycan chains; and S4C-S0N0, syndecan-4 without cytoplasmic domain, N-linked glycosylated chains, and glycosaminoglycan chains.
Figure 4.2  Real-time quantitative PCR analysis of mRNA expression at 48 h following transfection. Randombred control 2 line male turkey myogenic satellite cells were either transfected with empty pCMS-EGFP vector (pCMS) or co-transfected with siRNA targeting syndecan-4 cytoplasmic domain and syndecan-4 cytoplasmic domain mutants (syndecan-4 without cytoplasmic domain, S4C; syndecan-4 without cytoplasmic domain and N-linked glycosylated chains, S4C-N0; syndecan-4 without cytoplasmic domain and glycosaminoglycan chains, S4C-S0; and syndecan-4 without cytoplasmic domain, N-linked glycosylated chains, and glycosaminoglycan chains, S4C-S0N0). a) overexpression of transfected syndecan-4 cytoplasmic domain mutants; and b) knockdown of endogenous syndecan-4. Cells transfected with pCMS indicates the expression of endogenous syndecan-4. The error bars represent standard error of the mean. * Indicates a significantly difference from the control ($P < 0.05$).
Figure 4.3  Proliferation analysis of the randombred control 2 line male turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), syndecan-4 without cytoplasmic domain (S4C), syndecan-4 without cytoplasmic domain and N-linked glycosylated chains (S4C-N0), syndecan-4 without cytoplasmic domain and glycosaminoglycan chains (S4C-S0), and syndecan-4 without cytoplasmic domain, N-linked glycosylated chains, and glycosaminoglycan chains (S4C-S0N0). The bars represent standard error of the mean. Bars without a common letter within times were significantly different ($P<0.05$).
Figure 4.4 Fibroblast growth factor 2 (FGF2) responsiveness of the randombred control 2 line male turkey myogenic satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), syndecan-4 without cytoplasmic domain (S4C), syndecan-4 without cytoplasmic domain and N-linked glycosylated chains (S4C-N0), syndecan-4 without cytoplasmic domain and glycosaminoglycan chains (S4C-S0), and syndecan-4 without cytoplasmic domain, N-linked glycosylated chains, and glycosaminoglycan chains (S4C-S0N0) at 72 h following transfection. The bars represent standard error of the mean. Bars without a common letter within the same FGF2 treatment were significantly different ($P< 0.05$).
Figure 4.5  Differentiation of the randombred control 2 line male turkey myogenic satellite cells after transfection with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), syndecan-4 without cytoplasmic domain (S4C), syndecan-4 without cytoplasmic domain and N-linked glycosylated chains (S4C-N0), syndecan-4 without cytoplasmic domain and glycosaminoglycan chains (S4C-S0), and syndecan-4 without cytoplasmic domain, N-linked glycosylated chains, and glycosaminoglycan chains (S4C-S0N0). The bars represent the standard error of the mean. Bars without common letters within time are significantly different ($P < 0.05$).
Figure 4.6 Western blot analysis of protein kinase C alpha (PKCα) activity. Randombred control 2 line male turkey myogenic satellite cells were transfected with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), syndecan-4 without cytoplasmic domain (S4C), syndecan-4 without cytoplasmic domain and N-linked glycosylated chains (S4C-N0), syndecan-4 without cytoplasmic domain and glycosaminoglycan chains (S4C-S0), and syndecan-4 without cytoplasmic domain, N-linked glycosylated chains, and glycosaminoglycan chains (S4C-S0N0) at 48 h following transfection. Each lane contains 10 µg of protein and was hybridized to mouse anti-human PKCα antibody and mouse monoclonal β-actin antibody. A ratio was calculated by the average density of PKCα of samples transfected with syndecan-4 and its mutants relative to the pCMS after normalization with β-actin.
Protein kinase C alpha (PKCα) expression in syndecan-4 and its mutants (in pcDNA3.1/V5-His-TOPO vector) transfected randombred control 2 line turkey satellite cells at 48 h following transfection. a) cells were transfected with wild type syndecan-4 (S4); b) cells were transfected with syndecan-4 without cytoplasmic domain (S4C); c) cells were transfected with syndecan-4 without cytoplasmic domain and N-linked glycosylated chains (S4C-N0); d) cells were transfected with syndecan-4 without cytoplasmic domain and glycosaminoglycan chains (S4C-S0); and e) cells were transfected with syndecan-4 without cytoplasmic domain, N-linked glycosylated chains, and glycosaminoglycan chains (S4C-S0N0). Sheep anti-V5 antibody was used to detect the syndecan-4 core protein (green fluorescence); mouse anti-human PKCα antibody was used to detect PKCα (red fluorescence); and BF contains the bright field images for each construct. Arrows highlight the co-localization of syndecan-4 and PKCα. Scale bars in the BF images represent the size in µm for each construct.
Figure 4.8  Syndecan-4 cytoplasmic domain regulation of cellular responsiveness to fibroblast growth factor 2 (FGF2).  a) Syndecan-4 can bind FGF2 to its core protein and glycosaminoglycan (GAG) chains, and then present FGF2 to its receptor to initiate cellular responsiveness to FGF2; b) The deletion of syndecan-4 cytoplasmic domain may cause increased binding of FGF2 to syndecan-4 (Ser residue in the conserved 1 region is critical in this process).  c) The deletion of syndecan-4 N-linked glycosylated (N-glycosylated) chains changes the three dimensional structure of the core protein which may lead to the decreased interaction with FGF2; d) The deletion of syndecan-4 GAG chains gives more spaces for the core protein to bind to FGF2; and e) The deletion of both syndecan-4 N-glycosylated chains and GAG chains changes the three dimensional structure of the core protein which decreases FGF2 binding to syndecan-4, however, the deletion of the GAG chains give more spaces for the core protein to bind to FGF2 which rescues the interaction between syndecan-4 and FGF2.  S4, wild type syndecan-4; S4C, syndecan-4 without cytoplasmic domain; S4C-N0, syndecan-4 without cytoplasmic domain and N-glycosylated chains; S4C-S0, syndecan-4 without cytoplasmic domain and GAG chains; and S4C-S0N0, syndecan-4 without cytoplasmic domain, N-glycosylated chains, and GAG chains.
CHAPTER 5: CRITICAL AMINO ACIDS IN SYNDECAN-4 CYTOPLASMIC
DOMAIN MODULATION OF TURKEY SATELLITE CELL GROWTH AND
DEVELOPMENT

Abstract

Syndecan-4 is a cell membrane heparan sulfate proteoglycan that is composed of a core protein and covalently attached glycosaminoglycan (GAG) and N-linked glycosylated (N-glycosylated) chains. The core protein is divided into extracellular, transmembrane, and cytoplasmic domains. The cytoplasmic domain has two conserved regions and a variable region in the middle. The Ser residue in the conserved region 1 close to the cell membrane and the Tyr residue in the variable region are important in regulating protein kinase C alpha (PKCα) activity and focal adhesion formation which is critical for cell migration and proliferation. The objective of the current study was to investigate the role of syndecan-4 Ser and Tyr residues in combination with the GAG and N-glycosylated chains in turkey satellite cell proliferation, differentiation, fibroblast growth factor 2 (FGF2) responsiveness, and PKCα activity. Site-directed mutagenesis was used to generate Ser and Tyr mutants with or without GAG and N-glycosylated chains. The wild type syndecan-4 and the Ser and Tyr mutants were transfected into turkey satellite cells, and cell proliferation, differentiation, responsiveness to FGF2, and PKCα activity (cell membrane localization) were then measured. Over-expression of Ser
and Tyr mutants increased cell proliferation and differentiation and decreased cell membrane localization of PKCα, compared to the cells transfected with wild type syndecan-4. Furthermore, Ser mutants enhanced cellular responsiveness to FGF2. These data suggested that the Ser and Tyr residues are critical in regulating turkey satellite cell proliferation, differentiation, and PKCα activity and the Ser residue is essential in modulating cellular responsiveness to FGF2.

5.1 Introduction

Syndecans are a group of cell membrane proteins that comprise four members, syndecan-1 to -4. All syndecans are composed of a transmembrane core protein and substituted heparan sulfate chains. The core proteins are composed of extracellular, transmembrane, and cytoplasmic domains. The extracellular domains have low homology within the family (Bernfield et al., 1999; Couchman et al., 2001), whereas the transmembrane and cytoplasmic domains have highly conserved sequences. The cytoplasmic domains are divided into three regions (Couchman et al., 2001), two conserved regions (C1 and C2) with a variable region (V) in the middle.

The expression of syndecan-1 to -3 is tissue-specific, with syndecan-1 mainly expressed in epithelial cells; syndecan-2 in fibroblasts, and syndecan-3 in neuronal tissue (Kim et al., 1994). Syndecan-4 is expressed ubiquitously in focal adhesions of adherent cells (Woods and Couchman, 1994). Syndecan-4 has three glycosaminoglycan (GAG) chains and two N-linked glycosylated (N-glycosylated) chains attached to the extracellular domain. It has been reported that syndecan-4 GAG chains and N-
glycosylated chains are required during turkey satellite cell proliferation (Song et al., 2011), and the cytoplasmic domain is critical in turkey satellite cell proliferation, fibroblast growth factor 2 (FGF2) responsiveness, and protein kinase C alpha (PKCα) activity (Song unpublished data). The cytoplasmic domain of syndecan-4 binds to phosphatidylinositol (4,5) bisphosphate (PIP2) at the sequence of KKPIYKK (Oh et al., 1998; Horowitz et al., 1999; Couchman et al., 2002) and then binds and strongly activates PKCα (Oh et al., 1997; Horowitz and Simons, 1998a; Oh et al., 1998; Couchman et al., 2002). The Ser residue in the C1 region of syndecan-4 cytoplasmic domain can be phosphorylated by growth inhibition and dephosphorylated by the addition of FGF2 (Horowitz and Simons, 1998a, b). The phosphorylation of the Ser residue causes the decreased affinity of the PIP2 binding to the V region of syndecan-4, thus decreasing PKCα activity. In the current study, the role of the Ser residue in the C1 region of syndecan-4 (Ser178) and the Tyr in the sequence of KKPIYKK in the V region of the syndecan-4 (Tyr187) in combination with the N-glycosylated and GAG chains in turkey satellite cell proliferation, differentiation, cellular responsiveness to FGF2, and PKCα activity (PKCα cell membrane localization) was studied. The results from the present study provide new information about the function of syndecan-4 Ser178 and Tyr187 together with N-glycosylated chains and GAG chains during myogenesis.
5.2 Materials and Methods

5.2.1 Clone Generation

The Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants were generated using the Quick Change Multi Site-Directed Mutagenesis kit (Stratagene Corporation) according to the manufacturer’s protocol. The mutant with the amino acid Ser\textsuperscript{178} changed to Ala is referred to as S4SA. The S4SA without N-glycosylated chains or GAG chains or both chains are termed: S4SA-N0, S4SA-S0, and S4SA-S0N0, respectively. The mutant with amino acid Tyr\textsuperscript{187} changed to Phe is termed S4YF. The S4YF without N-glycosylated chains or GAG chains or both chains are: S4YF-N0, S4YF-S0, and S4YF-S0N0, respectively. The templates used were wild type syndecan-4 (S4), syndecan-4 without N-glycosylated chains, syndecan-4 without GAG chains, and syndecan-4 without both N-glycosylated chains and GAG chains cloned into the pCMS-EGFP vector (Velleman et al., 2006; Song et al., 2011). The primers used for Ser\textsuperscript{178} mutants were 5’-GAA GAA AAA GGA TGA AGG CGC CTA CGA CCT TGG GAA GAA A\textsubscript{552}-3’ and for Tyr\textsuperscript{187} mutants were 5’-CTT GGG AAG AAA CCA ATC TTC AAG AAA GCC CCT ACA AA\textsubscript{579}-3’. The Ser residue was mutated to Ala, and the Tyr residue was mutated to Phe (highlighted in the primer sequences). All of the mutations were confirmed by DNA sequencing in the Molecular and Cellular Imaging Center (The Ohio State University).

The S4 had been subcloned into a pcDNA3.1/V5 His TOPO vector (Invitrogen; Song et al., 2011). The Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants were also subcloned into the pcDNA3.1/V5 His TOPO vector. The primers for subcloning were: forward primer 5’-ATA ATG CCG CTG CTC CGC G\textsubscript{16} -3’, and reverse primer 5’-AGC GTA GAA
CTC ATT TGT AGG GGC<sub>568</sub>-3’. The PCR conditions were denaturation at 95 °C for 15 min, and 35 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 75 °C followed by 15 min at 75 °C. The ligation reaction contained 3 µl of PCR product from the above reaction, 1 µl of pcDNA3.1/V5-His-TOPO Vector (Invitrogen) and 1 µl of salt solution (1.2 M NaCl and 0.06 M MgCl₂). All of the mutations were confirmed by DNA sequencing in the Molecular and Cellular Imaging Center.

5.2.2 Satellite Cell Culture and Transfection

Randombred control 2 line turkey satellite cells were used in the current study. Satellite cells were plated in gelatin-coated cell culture plates (Greiner Bio-one) in plating medium [Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) containing 10% chicken serum (Invitrogen), 5% horse serum (Invitrogen), 1% antibiotic/antimycotic (Invitrogen), and 0.1% gentamicin (Invitrogen)] in a 37.5 °C 5%CO₂/ 95% air incubator. After a 24 h attachment period the cells were transfected with pCMS-EGFP empty vector, S4, or Ser<sup>178</sup> and Tyr<sup>187</sup> mutants using the Optifect transfection system (Invitrogen) with 0.5 µg (for 24-well cell culture plate) or 0.2 µg (for 48-well cell culture plate) of plasmid DNA according to the manufacturer’s protocol. After 6 h of transfection, the medium was changed to feeding medium [McCoy’s 5A medium (Sigma-Aldrich) containing 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic, and 0.1% gentamicin]. Feeding medium was changed daily until 72 h following transfection or until the cells reached 60% confluency for differentiation assays. Differentiation was induced using a low serum medium [DMEM, 3% horse serum, 1% antibiotic/antimycotic, 0.01 mg/ml porcine gelatin (Sigma-Aldrich), 0.1% gentamicin, and 1.0 mg/ml bovine
serum albumin (Sigma-Aldrich)]. The differentiation medium was changed every 24 h until 72 h of differentiation. At 0, 24, 48, and 72 h following transfection and at 0, 48, and 72 h of differentiation, cell cultures were removed from the incubator, rinsed 3 times with sterile phosphate buffered saline (PBS, pH 7.08; 137 mM NaCl, 1.47 mM KH$_2$PO$_4$, 7.81 mM Na$_2$HPO$_4$, and 2.68 mM KCL), air dried, and then stored at -70 °C until analysis.

5.2.3 Total RNA Extraction and cDNA Synthesis

At 48 h following transfection, total RNA was extracted from the cell cultures using TRIzol (Invitrogen) according to the manufacturer’s protocol. The cDNA was synthesized using moloney murine leukemia virus reverse transcriptase (M-MLV; Promega). In brief, 1 μg of total RNA, 1 μl of 50 μM Oligo d (T)$_{20}$ (Operon), and nuclease-free water up to 13.5 μl was incubated at 80 °C for 5 min, and then cooled on ice. The reaction mixture containing 5 μl of 5X First-Strand buffer, 1.25 μl 10 mM deoxynucleoside triphosphate mix, 0.5 μl Rnasin (40 U/μl), 1 μl M-MLV (200 U/μl), and nuclease-free water up to 11.5 μl was then added. The mixture was incubated at 55 °C for 60 min, and then heated at 90 °C for 10 min to stop the reaction.

5.2.4 Real-Time Quantitative PCR

Real-time quantitative PCR was performed using the DyNAmo Hot Start SYBR Green qPCR kit (Finnzymes) with a DNA Engine Opticon 2 real-time system (MJ Research). The PCR reaction consisted of 2 μl cDNA, 10 μl 2X master mix, 250 nM of each of the forward and reverse primers, and nuclease-free water up to 20 μl. Syndecan-4 primers were: forward primer 5’- CTA CCC TGG CTC TGG AGA CCT$_{122}$ -3’ and
reverse primer 5’-335TCA TTG TCC AGC ATG GTG TTT315-3’. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were: forward primer 5’-504GAG GGT AGT GAA GGC TGC TG523-3’ and reverse primer 5’-703CCA CAA CAC GGT TGC TGT AT684-3’. The cycling parameters were denaturation at 95 °C for 15 min, followed by 34 cycles of 94 °C for 30 s, 58 °C for 30 s, and at 72 °C for 30 s with a final elongation at 72 °C for 5 min. The melting curve program was 52 °C to 95 °C, 0.2 °C per read, and a 1 s hold. Amplification specificity was checked on a 1% agarose gel. Standard curves were constructed with serial dilutions of purified PCR products from each gene. The amount of sample cDNA for S4 and Ser178 and Tyr187 mutants was determined by comparing the results to the syndecan-4 standard curve, and then normalized to GAPDH expression.

5.2.5 Immunocytochemistry

Satellite cells were cultured and transfected in 24-well cell culture plates. At 24 h following transfection, the cell cultures were fixed with 3% paraformaldehyde for 30 min at room temperature. The cells were then incubated with goat anti-syndecan-4 antibody (Santa Cruz) 1:100 dilution in blotto (5% nonfat milk dissolved in 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl) for 1 h. The donkey anti-goat antibody conjugated with rhodamine (Santa Cruz) 1:200 dilution in 5% nonfat milk dissolved in blotto was used as the secondary antibody. An Olympus XI 70 microscope and an Optronics digital camera (Optronics) were used to view and record the images.
5.2.6 Proliferation Assay

Satellite cells were plated in gelatin-coated 24-well cell culture plates at a density of 12,500 cells per well, and transfected as described above. At 0, 24, 48, and 72 h following transfection, cell culture plates were removed from the incubator, rinsed with PBS 3 times, air dried, and then stored at -70 °C until analysis. Cell proliferation was measured by DNA content in each well by the method of McFarland et al. (1995). The Hoechst 33258 fluorochrome (Sigma-Aldrich) was utilized to determine DNA concentration on a Fluoroskan Ascent FL plate reader (ThermoElectron Co.) using double-stranded calf thymus DNA as the standard.

5.2.7 Responsiveness to Fibroblast Growth Factor 2 Assay

Satellite cells were plated and transfected as described above. After 6 h of transfection, the DMEM medium was removed and changed to serum-free defined media (McFarland et al., 2006) containing 0, 2.5, or 10.0 ng/ml of FGF2 (Pepro Tech). Cellular responsiveness to FGF2 during proliferation was determined at 72 h following transfection by measuring the DNA concentration in each well (Velleman et al., 2006).

5.2.8 Differentiation Assay

Satellite cells were plated in gelatin-coated 48-well cell culture plates at a density of 9,000 cells per well, and were transfected and cultured as described above. Differentiation was induced when the cells reached 60% confluency by using a low serum media containing DMEM, 3% horse serum, 1% antibiotics-antimycotics, 0.1% gentamicin, 1% gelatin, and 1% BSA. At 0, 48, and 72 h of differentiation, cell cultures were removed from the incubator, rinsed with sterile PBS 3 times, air dried, and then
stored at -70 °C until analysis. Differentiation was determined by measuring the muscle specific creatine kinase level by a modified method from Yun et al. (1997). Five hundred microliters of creatine kinase assay buffer [20 mM glucose (Fisher Scientific), 10 mM Mg acetate (Fisher Scientific), 1.0 mM adenosine diphosphate (Sigma-Aldrich), 10 mM adenosine monophosphate (Sigma-Aldrich), 20 mM phosphocreatine (Calbiochem), 0.5 U/ml of hexokinase (Worthington Biochemical), 1 U/ml of glucose-6-PO₄ dehydrogenase (Worthington Biochemical), 0.4 mM thio-nicotinamide adenine dinucleotide (Sigma-Aldrich), and 1 mg/ml of bovine serum albumin prepared in 0.1 M glycylglycine (Sigma-Aldrich, pH 7.5)] was added to each well. Creatine phosphokinase (Sigma-Aldrich) was used to generate a standard curve. After a 5 min incubation at room temperature, cell differentiation was measured by the rate of thio-nicotinamide adenine dinucleotide reduction with a BioTek ELx800 plate reader (Biotek) at 405 nm at 5 min intervals.

5.2.9 Western Analysis

The pCMS-EGFP empty vector, S4 and all of the Ser¹⁷⁸ and Tyr¹⁸⁷ mutants were transfected into turkey satellite cells. Cell membrane proteins were extracted using a hypo-osmotic solution [20 mM Tris/HCl (pH 7.5), 2 mM 2-mercaptoethanol/5 mM EGTA/2 mM EDTA] containing a protease inhibitor cocktail (Roche Applied Science; Keum et al., 2004). Briefly, 150 µl of hypo-osmotic solution was added to the cell culture and cells were then scraped off the plate and homogenized on ice for 10 min. The cell membrane proteins were extracted by centrifuging the cell lysate at 13,000 x g for 15 min at 4 °C. Cell pellets were then resuspended in RIPA buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 10 mM NaF, and 2 mM Na₃VO₄, and protease
inhibitor cocktail) and centrifuged at 15,000 x g for 15 min at 4 °C. Equal amount of supernatant proteins were mixed with reducing sample buffer (0.125 M Tris–HCl, pH 6.8, 4.1% sodium dodecyl sulfate (SDS), 20% glycerol, 2% β-mercaptoethanol, and 0.001% bromphenol blue), boiled for 5 min and then separated on an 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) by the method of Laemmli et al. (1970). Proteins were then transferred to a polyvinylidene difluoride membrane (PVDF; Millipore). After blocking with 5% nonfat milk dissolved in Tris-buffered saline [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 (TBS-T)] for 1 h, the PVDF membrane was incubated with the indicated antibodies in blocking buffer for 2 h at room temperature. Primary antibodies used were mouse anti-human PKCα (BD Biosciences, 1:5,000) and mouse monoclonal β-actin antibodies (Sigma, 1:10,000). The secondary antibody used was rabbit anti-mouse IgG alkaline phosphatase conjugated (Santa Cruz, 1:10,000). After incubating with chemiluminescent alkaline phosphatase substrate (Millipore) for 5 min at room temperature, immunoreactive bands were detected with a Bio Rad ChemiDoc XRS imaging system (Bio Rad). The quality of the bands were determined with Quantity One 4.6.6 Software (Bio Rad).

5.2.10 Confocal Immunofluorescence Microscopy

Cells were seeded on gelatin coated polystyrene slides (Electron Microscopy Sciences) and transfected with S4 and all of the Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants in pcDNA3.1/V5 His TOPO vector. At 48 h following transfection, cells were rinsed with PBS 3 times and fixed with 3% paraformaldehyde for 30 min at room temperature. After blocking with 1% BSA in TBS-T for 30 min, cells were incubated with the indicated
primary antibodies for 2 hs at room temperature. Cells were then rinsed 3 X with 1% BSA in TBS-T and incubated with the appropriate secondary antibodies for 1 h. Primary antibodies used were sheep anti-V5 tag (Abcam; 1:2,000) and mouse anti-human PKCα (BD Biosciences, 1:5,000). The secondary antibodies used were Alexa Fluor 488 donkey anti-sheep (Invitrogen, 1:1,000), and Alexa Fluor 568 rabbit anti-mouse (Invitrogen, 1:1,000) respectively. Samples then were mounted with gel/mount media (Biomeda) and covered with microscope cover glass No. 1.5 (VWR). The cell cultures were then viewed and recorded by a Leica TCS SP confocal scanning microscope system (Leica).

5.2.11 Statistical Analysis

All of the experiments were independently repeated at least three times. There were four replicates of each treatment for proliferation and FGF2 responsiveness, and five replicates for the differentiation assay. For the real time PCR, three replicates for each treatment were performed. The data from each repeat were used to calculate the mean and the standard error of the mean (SEM). Data were graphed as the mean ± SEM. The SAS PROC GLM (SAS Institute Inc. 2002, Cary, NC) was used for statistical analyses. Differences among means were detected using the Fisher’s least significant difference method. For the FGF2 responsiveness assays, the statistical model included the effect of FGF2 treatment, mutant transfection, and their interaction. The main factors of FGF2 treatment and variant mutant transfection and the interaction between the two factors were analyzed using SAS Proc GLM procedures. Differences among means in each experiment were evaluated using an ANOVA and detected using Fisher's least-
significant-difference. Two-sided $P$ values of $P < 0.05$ were considered statistically significant.

5.3 Results

5.3.1 Site-Directed Mutagenesis and the Over-Expression in Satellite Cells

Eight syndecan-4 mutants were generated by site-directed mutagenesis. At 48 h following transfection, all of the syndecan-4 mutants had significantly higher expression of syndecan-4 compared to the empty pCMS-EGFP vector (Figure 5.1). The over-expression of syndecan-4 was also confirmed at the protein level by immunocytochemistry at 24 h following transfection (Figure 5.2). Figure 5.2a illustrates cells transfected with the pCMS-EGFP empty vector. Figure 5.2b illustrates cells transfected with wild type syndecan-4 (S4). Figure 5.2c-j are cells transfected with S4SA, S4SA-N0, S4SA-S0, S4SA-S0N0, S4YF, S4YF-N0, S4YF-S0, and S4YF-S0N0, respectively. Brightfield (BF) images show the cell morphology. Green fluorescence images show the enhanced green fluorescence protein marker expressed by the pCMS-EGFP vector indicating the transfection of the cells. Red fluorescence images show the cells stained with syndecan-4 antibody. The cells transfected with S4 and all of the syndecan-4 mutants had higher protein levels of syndecan-4 compared to the cells transfected with pCMS-EGFP empty vector.

5.3.2 Cell Proliferation and Fibroblast Growth Factor 2 Responsiveness

At 0, 24, and 48 h following transfection, cells transfected with S4SA, S4SA-N0, S4SA-S0, and S4-S0N0 had no significant difference in proliferation compared to the
cells transfected with S4 (Figure 5.3a). At 72 h following transfection, cells transfected with S4SA-N0 had significantly higher proliferation rates compared to the cells transfected with S4. When cells were transfected with S4YF, S4YF-N0, S4YF-S0, and S4YF-S0N0, at 0, 24, and 48 h following transfection there were no differences in proliferation rates compared to S4 (Figure 5.3b). At 72 h following transfection, cells transfected with S4YF had significantly higher proliferation rates compared to S4. These data indicated that Ser\textsuperscript{178} and Tyr\textsuperscript{187} have a limited effect on cell proliferation.

Cellular responsiveness to FGF2 was measured at 72 h following transfection. Without FGF2 treatment, the syndecan-4 mutants had no effect on cell proliferation compared to S4 (Figure 5.4a, b). With 2.5 ng/ml of FGF2, cells transfected with S4SA had significantly higher proliferation compared to the cells transfected with S4 (Figure 5.4a). When cells were treated with 10 ng/ml of FGF2, S4SA, S4SA-S0, and S4SA-S0N0, cells increased proliferation compared to S4. Statistical analysis to determine the effect of FGF2 treatment, effect of mutant transfection, and their interaction indicated that the cells transfected with S4SA, S4SA-S0, and S4SA-S0N0 were more responsive to FGF2 compared to S4. However, for the cells transfected with S4YF, S4YF-N0, S4YF-S0, and S4YF-S0N0, there were no differences in proliferation rates compared to S4 cells treated with 2.5 ng/ml, 10 ng/ml, or without FGF2 (Figure 5.4b).

5.3.3 Cell Differentiation

At 0 and 48 h of differentiation, cells transfected with S4SA, S4SA-N0, S4SA-S0, and S4SA-S0N0 had significantly higher differentiation levels compared to the cells
transfected with S4 (Figure 5.5a). However, at 72 h of differentiation there was no significant difference between the S4SA mutants and S4.

Cells transfected with S4YF, S4YF-S0, and S4YF-S0N0 had significantly higher differentiation levels compared to the cells transfected with S4 at 0 and 48 h of differentiation (Figure 5.5B). Cells transfected with S4YF and S4YF-N0 also had significantly higher differentiation levels compared to the cells transfected with S4 at 72 h of differentiation. These data suggested that both the Ser\textsuperscript{178} and Tyr\textsuperscript{187} are important in regulating cell differentiation.

5.3.4 Protein Kinase C alpha activity

Confocal microscopy showed that cells transfected with S4, S4SA, S4SA-N0, S4SA-S0, S4SA-S0N0, S4YF, S4YF-N0, S4YF-S0, and S4YF-S0N0 all had increased cell membrane localization of PKCα compared to the cells not transfected (Figure 5.6). In contrast, western analysis showed that, when compared to S4, cells transfected with Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants had decreased cell membrane localization of PKCα (except for S4SA; Figure 5.7). These data suggested that Ser\textsuperscript{178} and Tyr\textsuperscript{187} are critical in regulating PKCα activity in turkey satellite cells.

5.4 Discussion

All of the syndecan core proteins have a short cytoplasmic tail. The cytoplasmic domain of syndecan-4 is specific for interacting with PIP\textsubscript{2} and then forming dimers or oligomers (Oh et al., 1997; Lee et al., 1998) which have an unusual twisted clamp motif formed at either end of the V region (Lee et al., 1998; Shin et al., 2001). The binding of
PIP₂ at the amino sequence KKPIYKK (Oh et al., 1998; Horowitz et al., 1999; Couchman et al., 2002) triggers the activation of PKCα (Oh et al., 1997; Horowitz and Simons, 1998a; Oh et al., 1998; Couchman et al., 2002). Lim et al. (2003) reported that the overexpression of syndecan-4 increased the amount of PKCα localized in focal adhesions. The activity of PKCα can also be regulated by the Ser residue in the C1 region of the syndecan-4 cytoplasmic domain. The dephosphorylation of Ser will increase the affinity of the PIP₂ binding to the V region of syndecan-4, and increase PKCα activity. In the current study, Ser₁⁷⁸ in the C1 region of syndecan-4 cytoplasmic domain and Tyr₁⁸⁷ in the KKPIYKK sequence were mutated to Ala and Phe, respectively. The Ser₁⁷⁸ mutant increased PKCα activity compared to S4. However, Ser₁⁷⁸ mutants without GAG or N-glycosylated chains or both chains decreased PKCα activity which suggested that there is a functional interaction between syndecan-4 side chains and Ser₁⁷⁸. The Tyr₁⁸⁷ mutants with or without GAG or N-linked glycosylated chains or both chains decreased PKCα activity in turkey satellite cells. These data indicated that Ser₁⁷⁸ and Tyr₁⁸⁷ are two critical amino acids in regulating PKCα function in turkey satellite cells.

Syndecan-4 and FGF2 interact with each other. The FGF2 can dephosphorylate syndecan-4 at Ser₁⁷⁸ (Horowitz and Simons, 1998a, b) whereas syndecan-4 can modulate FGF2 signaling (Volk et al., 1999; Zhang et al., 2003). However, there are many conflicted reports on syndecan-4 regulation of FGF2 signaling. Velleman et al. (2006), Zhang et al. (2008), and Song et al. (2011) reported that syndecan-4 regulates turkey satellite cell growth and development in a FGF2-independent manner. When the cytoplasmic domain of the syndecan-4 is deleted, turkey satellite cell growth triggered by FGF2 is altered (Song et al., submitted) which indicates the critical role of the
cytoplasmic domain of syndecan-4 in regulation of cellular responsiveness to FGF2. Volk et al. (1999) also supported evidence that the cytoplasmic domain of syndecan-4 is important in FGF2-dependent signal transduction. In the current study, it was further demonstrated that Ser$^{178}$, but not Tyr$^{187}$ was key in the regulation of cellular responsiveness to FGF2. The Ser$^{178}$ mutants including S4SA, S4SA-S0, and S4SA-S0N0 increased FGF2 induced cell proliferation. Tyr$^{187}$ had no effect on cellular responsiveness to FGF2. Except for S4YF, the other Tyr$^{187}$ mutants without GAG or N-glycosylated chains or both chains did not increase cell proliferation compared to S4 cells. These data suggested that S4SA-N0 and S4YF increased cell proliferation but independent of FGF2 signaling.

Cornelison et al. (2001, 2004) reported that syndecan-4 plays important roles in satellite cell proliferation and differentiation. Song et al. (submitted) showed that the syndecan-4 cytoplasmic domain had no effect on turkey satellite cell differentiation. Surprisingly, Ser$^{178}$ and Tyr$^{187}$ mutants increased cell differentiation compared to S4. Ser$^{178}$ and Tyr$^{187}$ are two critical amino acids regulating turkey satellite cell differentiation. However, the mechanism of how Ser$^{178}$ and Tyr$^{187}$ manipulate cell differentiation needs to be determined.

In summary, the data from the current study suggested that the Ser$^{178}$ and Tyr$^{187}$ residues in the cytoplasmic domain of syndecan-4 are critical in modulating turkey satellite cell proliferation, differentiation, and PKCα activity. Furthermore, Ser$^{178}$ plays important roles in regulating cellular responsiveness to FGF2. This is the first demonstration of a single syndecan-4 cytoplasmic domain amino acid playing a role in
the regulation of satellite cell growth, differentiation, and the ability to respond to mitogenic stimuli. Both Ser\textsuperscript{178} and Tyr\textsuperscript{187} residues function in regulating satellite cell growth, development, and PKC\textalpha{} activity, but only Ser\textsuperscript{178} is critical in modulating FGF2-induced cell proliferation.
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Figure 5.1 Real-time quantitative PCR analysis of mRNA expression at 48 h following transfection. Randombred control 2 line male turkey satellite cells were transfected with the empty pCMS-EGFP vector (control), wild type syndecan-4 (S4), syndecan-4 Ser<sup>178</sup> mutants with or without glycosaminoglycan or N-linked glycosylated chains or both chains (S4SA, S4SA-N0, S4SA-S0, and S4SA-S0N0, respectively), and Tyr<sup>187</sup> mutants with or without glycosaminoglycan or N-linked glycosylated chains or both chains (S4YF, S4YF-N0, S4YF-S0, and S4YF-S0N0, respectively). The bars represent the standard error of the mean. * Indicates a significant difference from the control (P < 0.05).
Figure 5.2 Over-expression of syndecan-4 in randombred control 2 line male turkey myogenic satellite cells transfected with the pCMS-EGFP empty vector, wild type syndecan-4, and syndecan-4 Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants at 24 h following transfection. Panel a) was the same cells transfected with pCMS-EGFP. Panel b) was the same cells transfected with wild type syndecan-4. Panel c) was the same cells transfected with syndecan-4 Ser\textsuperscript{178} mutants. Panels d) to f) were the same cells transfected with syndecan-4 Ser\textsuperscript{178} mutants without N-linked glycosylated chains or glycosaminoglycan chains or both chains, respectively. Panel g) was the same cells transfected with syndecan-4 Tyr\textsuperscript{187} mutants. Panels h) to j) were the same cells transfected with syndecan-4 Tyr\textsuperscript{187} mutants without N-linked glycosylated chains or glycosaminoglycan chains or both chains, respectively. Gray pictures are brightfield (BF) images. Green fluorescence images illustrate the cells expressing enhanced green fluorescent protein (EGFP) in the pCMS-EGFP vector. Red fluorescence images are the cells stained with goat anti-human syndecan-4 antibody and a donkey anti-goat IgG rhodamine conjugated secondary antibody. The scale bar represents 50 µM.
Figure 5.3 Proliferation analysis of the randombred control 2 line male turkey satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), and Ser$^{178}$ and Tyr$^{187}$ mutants. S4SA, syndecan-4 Ser$^{178}$ mutant; S4SA-N0, syndecan-4 Ser$^{178}$ mutant without N-linked glycosylated (N-glycosylated) chains; S4SA-S0, syndecan-4 Ser$^{178}$ mutant without glycosaminoglycan (GAG) chains; S4SA-S0N0, syndecan-4 Ser$^{178}$ mutant without both N-glycosylated chains and GAG chains; S4YF, syndecan-4 Tyr$^{187}$ mutant; S4YF-N0, syndecan-4 Tyr$^{187}$ mutant without N-glycosylated chains; S4YF-S0, syndecan-4 Tyr$^{187}$ mutant without GAG chains; and S4YF-S0N0, syndecan-4 Tyr$^{187}$ mutant without both N-glycosylated chains and GAG chains. The bars represent the standard error of the mean. Bars without a common letter within times were significantly different ($P<0.05$).
Figure 5.4 Cellular responsiveness to fibroblast growth factor 2 (FGF2) of the randombred control 2 line male turkey satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), and Ser$^{178}$ and Tyr$^{187}$ mutants at 72 h following transfection. S4SA, syndecan-4 Ser$^{178}$ mutant; S4SA-N0, syndecan-4 Ser$^{178}$ mutant without N-linked glycosylated (N-glycosylated) chains; S4SA-S0, syndecan-4 Ser$^{178}$ mutant without glycosaminoglycan (GAG) chains; S4SA-S0N0, syndecan-4 Ser$^{178}$ mutant without both N-glycosylated chains and GAG chains; S4YF, syndecan-4 Tyr$^{187}$ mutant; S4YF-N0, syndecan-4 Tyr$^{187}$ mutant without N-glycosylated chains; S4YF-S0, syndecan-4 Tyr$^{187}$ mutant without GAG chains; and S4YF-S0N0, syndecan-4 Tyr$^{187}$ mutant without both N-glycosylated chains and GAG chains. The bars represent the standard error of the mean. Bars without a common letter within the same FGF2 concentration were significantly different ($P<0.05$).
Figure 5.5 Differentiation of the randombred control 2 line male turkey myogenic satellite cells after transfection with pCMS-EGFP empty vector (pCMS), wild type syndecan-4 (S4), and Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants. S4SA, syndecan-4 Ser\textsuperscript{178} mutant; S4SA-N0, syndecan-4 Ser\textsuperscript{178} mutant without N-linked glycosylated (N-glycosylated) chains; S4SA-S0, syndecan-4 Ser\textsuperscript{178} mutant without glycosaminoglycan (GAG) chains; S4SA-S0N0, syndecan-4 Ser\textsuperscript{178} mutant without both N-glycosylated chains and GAG chains; S4YF, syndecan-4 Tyr\textsuperscript{187} mutant; S4YF-N0, syndecan-4 Tyr\textsuperscript{187} mutant without N-glycosylated chains; S4YF-S0, syndecan-4 Tyr\textsuperscript{187} mutant without GAG chains; and S4YF-S0N0, syndecan-4 Tyr\textsuperscript{187} mutant without both N-glycosylated chains and GAG chains. The bars represent the standard error of the mean. Bars without common letters are significantly different ($P < 0.05$).
Figure 5.6 Protein kinase C alpha (PKCα) expression in syndecan-4 and Ser$^{178}$ and Tyr$^{187}$ mutants (in pcDNA3.1/V5-His-TOPO vector) transfected randombred control 2 line turkey satellite cells at 48 h following transfection. a), cells were transfected with wild type syndecan-4 (S4); b) cells were transfected with syndecan-4 Ser$^{178}$ mutant; c) to e) cells were transfected with Ser$^{178}$ mutant without N-linked glycosylated (N-glycosylated) chains, glycosaminoglycan (GAG) chains, or both chains, respectively. f), cells were transfected with syndecan-4 Tyr$^{187}$ mutant; g) to i) cells were transfected with Tyr$^{187}$ mutant without N-glycosylated chains, GAG chains, or both chains, respectively. Green fluorescence images were stained with sheep anti-V5 antibody; red fluorescence images were stained with mouse anti-human PKCα antibody; and BF was bright field images. Arrows point out the co-localization of syndecan-4 and PKCα.
Figure 5.7 Western analysis of protein kinase C alpha (PKCα) activity in randombred control 2 line male turkey satellite cells transfected with pCMS-EGFP empty vector (pCMS), wild-type syndecan-4 (S4), and Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants at 48 h following transfection. S4SA, syndecan-4 Ser\textsuperscript{178} mutant; S4SA-N0, syndecan-4 Ser\textsuperscript{178} mutant without N-linked glycosylated (N-glycosylated) chains; S4SA-S0, syndecan-4 Ser\textsuperscript{178} mutant without glycosaminoglycan (GAG) chains; S4SA-S0N0, syndecan-4 Ser\textsuperscript{178} mutant without both N-glycosylated chains and GAG chains; S4YF, syndecan-4 Tyr\textsuperscript{187} mutant; S4YF-N0, syndecan-4 Tyr\textsuperscript{187} mutant without N-glycosylated chains; S4YF-S0, syndecan-4 Tyr\textsuperscript{187} mutant without GAG chains; and S4YF-S0N0, syndecan-4 Tyr\textsuperscript{187} mutant without both N-glycosylated chains and GAG chains. Each lane contains 10 µg of protein and was hybridized to anti-mouse PKCα and anti-mouse β-actin antibodies. β-actin was used to normalize PKCα expression.
CHAPTER 6: THE ROLE OF SYNDECAN-4 SIDE CHAINS IN TURKEY
SATELLITE CELL APOPTOSIS AND FOCAL ADHESION FORMATION

Abstract

Syndecan-4 is a cell membrane heparan sulfate proteoglycan that functions in muscle growth and development. It is composed of a central core protein and two types of side chains: glycosaminoglycans (GAG) and N-linked glycosylated (N-glycosylated) chains. It has been shown that the N-glycosylated chains and GAG chains are required for syndecan-4 to regulate turkey myogenic satellite cell proliferation. The objective of the current study was to determine whether the syndecan-4 side chains regulate cell proliferation through regulating muscle cell focal adhesion formation and apoptosis. Syndecan-4 with only one or without any N-glycosylated chain attached to the core protein with or without GAG chains were generated to study the function of N-glycosylated chains and the interaction between N-glycosylated chains and GAG chains. The wild type syndecan-4 and all of the syndecan-4 side chain mutants were transfected into turkey myogenic satellite cells. Cell apoptosis and focal adhesion formation were measured, and the protein kinase C alpha (PKCα) activity was investigated. Syndecan-4 and syndecan-4 side chain mutants did not influence cell apoptosis but syndecan-4 increased focal adhesion kinase (FAK) activity and the deletion of the side chains
decreased this function; syndecan-4 and syndecan-4 side chain mutants did not influence focal adhesion formation but enhanced β1-integrin expression and increased PKCα cell membrane localization. These results suggested that syndecan-4 and its side chains play important roles in regulating FAK and PKCα activity and β1-integrin expression but not in cell apoptosis and focal adhesion formation.

6.1 Introduction

Syndecan-4 is a type I transmembrane heparan sulfate proteoglycan. It is composed of a transmembrane core protein and two types of side chains: three glycosaminoglycan (GAG) chains and two N-linked glycosylated (N-glycosylated) chains. The GAG chains are attached to the core protein at Ser residues containing Ser-Gly repeats. Turkey syndecan-4 GAG chains are attached to the core protein at Ser$^{38}$, Ser$^{65}$, and Ser$^{67}$. The N-glycosylated chains are attached to the core protein at the Asn residue at the sequence of Asn-Xaa-Ser/Thr. The Xaa can be any amino acid except proline (Kornfeld and Kornfeld 1985). Turkey syndecan-4 has two N-glycosylated chains attached to the core protein at Asn$^{124}$ and Asn$^{139}$.

Syndecan-4 plays an important role in muscle maintenance and regeneration (Cornelison et al., 2001; Tanaka et al., 2009). This is supported by the fact that syndecan-4 knockout mice lose the ability to regenerate damaged muscle (Cornelison et al., 2004). Syndecan-4 also functions in muscle growth and development. During muscle development, more syndecan-4 is expressed in turkey embryonic pectoralis major muscle in turkeys selected for increased growth rate compared to unselected ones (Liu et al.,
Syndecan-4 is a component of focal adhesions, plays an important role in promoting focal adhesion formation (Echtermeyer et al., 1999), regulates cell migration (Echtermeyer et al., 2001), and prevents cells from undergoing apoptosis (Jeong et al., 2001). Focal adhesions are the points of strong attachment of cells to the matrix. The number, size, and stability of focal adhesions influence cell migration and survival of adhesive cells. Syndecan-4 participates in signal transduction by transmitting signals from the extracellular matrix into the cell and by activating protein kinase C alpha (PKCα) in complex with phosphatidylinositol 4,5-bisphosphate (PIP₂; Oh et al., 1998). In the presence of PIP₂, syndecan-4 can translocate PKCα to the cell membrane and activate it. The activated PKCα triggers the downstream pathway leading to the activation of RhoA and its targets Rho kinase, to regulate formation and maintenance of stress fibers (Dovas et al., 2006).

It has been reported that syndecan-4 N-glycosylated chains and GAG chains are required for skeletal muscle satellite cell proliferation in turkeys (Song et al., 2011). However, the mechanism for how syndecan-4 N-glycosylated chains and GAG chains regulate turkey skeletal muscle satellite cell proliferation is unclear. In the current study, the role of syndecan-4 GAG chains and N-glycosylated chains in turkey skeletal muscle satellite cell apoptosis, focal adhesion formations and PKCα activity (cell membrane localization) were investigated.

Syndecan-4 with only one or without N-glycosylated chain attached to the core protein with or without GAG chains were transfected into the turkey myogenic satellite cells, following which cell apoptosis, focal adhesion formation, and PKCα activity were
measured. The results from the current study provide new information about the function of syndecan-4 N-glycosylated chains and GAG chains in turkey satellite cell apoptosis, focal adhesion formation and PKCα activity.

6.2 Materials and Methods

6.2.1 Clone Generation

Turkey syndecan-4 N-glycosylated chain one-chain and no-chain mutants (Table 1) have been cloned into the pCMS-EGFP vector (Song et al., 2011). These mutants were then subcloned into the pcDNA3.1/V5-His-TOPO vector (Song et al., 2011). Briefly, turkey syndecan-4 N-glycosylated chain, one-chain, and no-chain mutants with or without GAG chains were used as templates to amplify syndecan-4 mutant PCR products.

The primers used were as follows: forward primer: 5’- ATG CCG CTG CTC CGC G16 -3’ (Operon), and the reverse primer: 5’- 591 AGC GTA GAA CTC ATT TGT AGG GGC 568 -3’ (Operon). The PCR conditions were denaturation at 95 °C for 15 min, and 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C followed by 15 min at 72 °C. The ligation reaction contained 3 µl of fresh PCR product from the above reaction, 1 µl of salt solution (1.2 M NaCl and 0.06 M MgCl2), and 1 µl of pcDNA3.1/V5-His-TOPO Vector (Invitrogen). After being incubated for 5 min at room temperature, 2 µl of the reaction mixture was used to transform TOP10 Chemically Competent E. coli (Invitrogen) according to the manufacturer’s instruction. Lauria-Bertani (LB) ampicillin agar plates were used to select positive clones. The positive clones were grown overnight
The plasmids were isolated using the QIAquick Miniprep Kit (Qiagen) and confirmed by DNA sequencing in the Molecular and Cellular Imaging Center (The Ohio State University).

6.2.2 Satellite Cell Culture and Transfection

Satellite cells isolated from the pectoralis major muscle of 7-week-old male randombred control 2 line turkeys (Velleman et al., 2000) were used. Satellite cells were plated in gelatin-coated cell culture plates (Greiner Bio-One) in plating medium [Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) containing 10% chicken serum (Invitrogen), 5% horse serum (Invitrogen), 1% antibiotic/antimycotic (Invitrogen), and 0.1% gentamicin (Invitrogen)] and incubated in a 37.5 °C 5%CO₂/95% air incubator. After 24 h attachment, the cells were transfected with syndecan-4 N-glycosylated one-chain and no-chain mutants with or without GAG chains, wild type syndecan-4 (S4), or pCMS-EGFP empty vector using the Optifect transfection system (Invitrogen) with 0.5 µg of plasmid DNA according to the manufacturer’s protocol. After 6 h of transfection, the medium was changed to feeding medium [McCoy’s 5A medium (Sigma-Aldrich) containing 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic, and 0.1% gentamicin]. Feeding medium was changed daily until 72 h following-transfection. Differentiation was induced when cells reached 60% confluency by changing the feeding medium to fusion medium [DMEM containing 3% horse serum, 1% antibiotic/antimycotic, 0.01 mg/mL porcine gelatin (Sigma-Aldrich), 0.1% gentamicin, and 1.0 mg/mL bovine serum albumin (Sigma-Aldrich)]. The fusion medium was changed every 24 h until 48 h of differentiation.
6.2.3 Caspase Assay

Satellite cells were cultured and transfected in 24-well cell culture plates. At 24 and 48 h following transfection, and 48 h of differentiation, cell culture medium was removed and the cells were washed twice with phosphate buffered saline (PBS). CaspACE-fluoroisothiocyanate-valylalanyl-aspartyl [O-methyl]-fluoromethylketone in situ marker (1:500; Promega) was added to the cell cultures and incubated in the dark for 40 min at 37 °C in a 95% air/5% CO₂ cell culture incubator. After incubation, the cell cultures were washed twice with PBS and then fixed with 3% paraformaldehyde for 30 min at room temperature in the dark. After fixation, 1µg/ml 4, 6-diamidino-2-phenylindole was added into the cell culture wells and incubated for 5 min. The cell cultures were then viewed and recorded with an Olympus XI 70 microscope and an Optronics digital camera (Optronics).

6.2.4 Guava Nexin Assay

Satellite cells were cultured and transfected in 24-well cell culture plates. Culture medium was changed daily, collected for each sample and stored at 37 °C in a 95% air/5% CO₂ cell culture incubator for further analysis. At 24 and 48 h following the transfection, and 48 h of differentiation, cells were scraped off the plates and added to each tube containing culture medium. The cells were collected by centrifuging at 350 x g for 10 min at 4 °C. After removing the supernatant, the cells were washed twice by resuspending the pellet in 1 ml cold 1X Nexin buffer (Millipore). After a second wash, the cell concentration was adjusted to 2 X 10⁶ cells/ml. Forty µl of each sample were
added to 5 µl of Annexin V conjugated with Phycoerythrin (MBL International) to detect early apoptotic cells, and 5 µl of 7-amino-actinomycin D (BD Biosciences) to detect late apoptotic cells. After 20 min of incubation on ice, 450 µl of cold 1X Nexin buffer was added to each sample and analyzed on a Guava EasyCyte system (Millipore) following the manufacturer’s Guava Nexin assay protocol.

6.2.5 Western Blot

Satellite cells were cultured and transfected with the syndecan-4 mutants in 24-well cell culture plates. At 6, 24, 48, and 72 h following the transfection, cell cultures were removed from the 95% air/5% CO₂ cell culture incubator, rinsed with PBS, air-dried and then stored at -70 °C until analysis. Total cell protein was extracted using RIPA buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 10 mM NaF, and 2 mM Na₃VO₄, and freshly prepared protease inhibitor cocktail (Roche Applied Science)). After incubating with 200 µl of radioimmunoprecipitation assay (RIPA) buffer on ice for 5 min, cells were scraped off the plates, collected and homogenized on ice for 30 min. Following centrifugation at 16,000 x g at 4 °C for 15 min, proteins in the supernatant were collected.

Cell membrane proteins were extracted using a hypo-osmotic solution [20 mM Tris/HCl (pH 7.5) / 2 mM 2-mercaptoethanol/5 mM ethylene glycol tetraacetic acid/2 mM ethylenediaminetetraacetic acid] containing a protease inhibitor cocktail (Keum et al., 2004). Briefly, after adding 150 µl of hypo-osmotic solution to the cell culture, cells were scraped off the plate and homogenized on ice for 10 min. The cell membrane proteins were collected by centrifuging the cell lysate at 13,000 x g for 15 min at 4 °C.
Cell pellets were then resuspended in RIPA buffer and centrifuged at 15,000 x g for 15 min at 4 °C. The supernatant contained cell membrane proteins.

Protein concentration was determined by the Bradford (1976) assay. Equal amount of proteins were mixed with reducing sample buffer (0.125 M Tris–HCl, pH6.8, 4.1% SDS, 20% glycerol, 2% β-mercaptoethanol, and 0.001% bromphenol blue), boiled for 5 min and then separated on an 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) by the method of Laemmli, (1970). Proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Millipore). After blocking for 1 h with 5% nonfat dry milk dissolved in Tris-buffered saline containing 20 mM Tris–HCl, pH7.4, 150 mM NaCl, and 0.05% Tween 20 (TBS-T), the PVDF membrane was incubated with the indicated antibodies in blocking buffer for 2 h at room temperature or overnight at 4 °C. Antibodies used for total cell protein immunoblotting were rabbit anti-human focal adhesion kinase (FAK) (pY\textsuperscript{397}) (Invitrogen, 1:1,000) and rabbit anti-human FAK (Santa Cruz; 1:3,000). The secondary antibody used was donkey anti-rabbit IgG alkaline phosphatase conjugated (Santa Cruz, 1:10,000). Primary antibodies used for cell membrane proteins were mouse anti-human PKCα (BD Biosciences, 1:5,000) and mouse monoclonal β-actin (Sigma, 1:10,000). Secondary antibody used was rabbit anti-mouse IgG alkaline phosphatase conjugated (Santa Cruz, 1:10,000). After incubating with chemiluminescent alkaline phosphatase substrate (Millipore) for 5 min at room temperature, immunoreactive bands were visualized with a Bio Rad ChemiDoc XRS imaging system (Bio Rad). The quality and size of the bands were determined with Quantity One 4.6.6 Software (Bio Rad).
**6.2.6 Confocal Immunofluorescence Microscopy**

Cells were seeded on gelatin coated polystyrene slides (Electron Microscopy Sciences) and transfected. At 48 h following the transfection, cells were rinsed with PBS 3 X and fixed with 3% paraformaldehyde for 30 min at room temperature. After a 30 min block with 1% BSA in TBS-T, cells were incubated with the indicated primary antibodies overnight at 4 °C or 2 h at room temperature. Cells were then rinsed 3 X with 1% BSA in TBS-T and incubated with the appropriate secondary antibodies for 1 h. Primary antibodies used were sheep anti-V5 tag (Abcam, 1:12,000), mouse anti-human PKCα (BD Biosciences, 1:5,000), mouse monoclonal vinculin (Abcam, 1:300), mouse monoclonal β-actin (Sigma, 1:300), and mouse anti-chicken β1-integrin (CSAT, 1:300) antibodies. The secondary antibodies used were Alexa Fluor 488 donkey anti-sheep (Invitrogen, 1:1,000), and Alexa Fluor 568 rabbit anti-mouse (Invitrogen, 1:1,000) respectively. Samples were then mounted with gel/mount media (Biomeda) and covered with a microscope cover glass No. 1.5 (VWR). The cell cultures were then viewed and recorded by a **Leica TCS SP** confocal scanning microscope system (Leica).

**6.2.7 Statistical Analysis**

All experiments were repeated at least three times. For the Guava Nexin assay, there were three replicates for each treatment, and the data from each repeat were used to calculate a mean and the standard error of the mean (SEM). Data are graphed as the mean ± SEM. The SAS PROC GLM (SAS Institute Inc.) was used for statistical analyses. Differences among means were detected using the Fisher’s least significance method. Differences among means in each experiment were evaluated using an ANOVA
and detected using Fisher's least-significant-difference. Two-sided $P$ values of $P < 0.05$ were considered statistically significant.

6.3 Results

6.3.1 Apoptotic effect of syndecan-4 side chains

Cell apoptosis was detected with the CaspACE marker specific for apoptotic cells. At 24 h following the transfection, satellite cells transfected with the empty vector, wild-type syndecan-4, and all of the syndecan-4 mutants had no detectable apoptotic cells when hybridized to the CaspACE marker (Figure 6.1, not all syndecan-4 mutants are shown). Satellite cells transfected with S4-N0 had more apoptotic cells relative to the cells transfected with empty vector and S4 (Figure 6.1). At 48 h of differentiation, satellite cells transfected with empty vector, S4, and all of the syndecan-4 side chain mutants had no apoptotic cells (Figure 6.2).

Cell cytometry was used to confirm the function of syndecan-4 and its side chains on apoptosis. At 24 and 48 h following the transfection and 48 h of differentiation, transfection of all of the syndecan-4 mutants had no significant effect on the number of early (Annexin V +/-7AAD-) and late (Annexin +/-7-AAD+) apoptotic cells compared to the S4 transfection (Tables 2 and 3).

6.3.2 Syndecan-4 side chain modulation of focal adhesion formation

At 48 h following the transfection, cells transfected with S4, S4-N0, S4-N1, S4-S4-S0, S4-S0N0, and S4-S0N1 had higher expression of β1-integrin compared to the
cells without transfection (Figure 6.3a-c and Figure 6.3e-g, respectively). However, cells transfected with S4-N2 and S4-S0N2 had no significant difference in β1-integrin expression compared to the cells without transfection (Figure 6.3d and h, respectively). The expression of β-actin was increased by the transfection of S4 (Figure 6.4a), but not other syndecan-4 side chain mutants (Figure 6.4b-h). Furthermore, S4 and syndecan-4 side chain mutants did not influence the expression of vinculin compared to the cells without transfection (Figure 6.5).

At 48 h following the transfection, the activity of FAK was measured by the ratio of phosphorylated FAK to total FAK. Syndecan-4 and its side chain mutants had different effects on FAK activity (Figure 6.6). Compared to the pCMS-EGFP empty vector, the transfection of S4 increased FAK activity, whereas the syndecan-4 side chain mutants decreased FAK activity. Among syndecan-4 side chain mutants, S4-N2 transfection had the lowest FAK activity with a ratio of 0.83 compared to the S4 at 1.20.

6.3.3 Syndecan-4 side chains regulation of PKCα activity

At 48 h following the transfection, except for the transfection of S4-N2 (Figure 6.7d), S4, S4-N0, S4-N1, S4-S0, S4-S0N0, S4-S0N1, and S4-S0N2 (Figure 6.7a-c and Figure 6.7e-h, respectively) increased the expression and cell membrane localization of PKCα compared to the control cells without transfection. Protein analysis by western blotting also showed that the transfection of S4 increased the cell membrane localization of PKCα compared to the pCMS-EGFP empty vector transfection (Figure 6.8). Compared to the S4, syndecan-4 side chain mutants including S4-N0, S4-N1, S4-N2, S4-S0, S4-S0N0, and S4-S0N1 had decreased PKCα associated with the cell membrane.
However, S4-S0N2 had increased amounts of PKCα in the cell membrane fraction compared to the S4.

6.4 Discussion

Syndecan-4 side chains are important in the regulation of turkey skeletal muscle satellite cell proliferation (Song et al., 2011). Syndecan-4 may modulate this process through participating in focal adhesion formation. Focal adhesions contain transmembrane receptors including integrins and syndecan-4, cytoplasmic structural proteins such as talin, vinculin, α-actinin, paxillin, and tensin, and the signaling proteins including PKC, FAK, and Src (Clark and Brugge, 1995; Burridge and Chrzanowska-Wodnicka, 1996). Syndecan-4 can either support β1-integrin mediated focal adhesion formation (Saoncella et al., 1999) through enhancing FAK activity (Guan et al., 1991; Kornberg et al., 1992) or it can function directly by activating PKC α (Oh et al., 1997; Lim et al., 2003; Keum et al., 2004). The interaction between β1-integrin and fibronectin induces phosphorylation of FAK, and then changes the number and size of the focal adhesions (Pirone et al., 2006; Israeli et al., 2010). The size and number of focal adhesions influences cell motility and shape (Goffin et al., 2006). Focal adhesions can inhibit cell rounding and shedding from the substrate, decreasing cell apoptosis (Jeong et al., 2001) and modulating cell proliferation (Wozniak et al., 2004). The appropriate amount of focal adhesions may promote cell migration and then increase cell proliferation. However, too many focal adhesions may inhibit cell migration and proliferation. Figure 6.9 illustrates syndecan-4 regulation of satellite cell proliferation.
Syndecan-4 cooperates with integrins in promoting focal adhesion formation and modulating migration (Couchman, 2003; Morgan et al., 2007). Integrins regulate cell behavior by forming adhesive receptors which are essential for cell migration. Echtermeyer et al. (1999) reported that the syndecan-4 core protein is sufficient to assemble focal adhesions and actin stress fibers. However, they only investigated syndecan-4 core protein and the GAG chains attached to the core protein, and did not study the N-glycosylated chains. In the current study, it was found that overexpression of S4 and syndecan-4 side chain mutants (except S4-N2 and S4-S0N2) increased the cell membrane localization of β1-integrin. The overexpression of S4-N2 and S4-S0N2 did not increase β1-integrin cell membrane localization compared to the cells without transfection. These data demonstrated that syndecan-4 N-glycosylated chains attached to the Asn$^{124}$ (N1 chain) are critical for syndecan-4 to interact with β1-integrin. In contrast to the function of the N1 chain in β1-integrin cell membrane localization, the deletion of the N1 chain (S4-N2) had the lowest FAK activity whereas the deletion of both the N1 chain and the entire GAG chains (S4-S0N2) had highest FAK activity in syndecan-4 mutants which is similar to that of S4. Compared to the empty vector, S4 increased FAK activity and all of the syndecan-4 mutants decreased FAK activity.

Syndecan-4 and its side chain mutants did not influence the cell membrane localization of vinculin which is used as a universal focal adhesion marker. Based on these data, it appeared that syndecan-4 and its covalently attached side chains effected the recruitment of β1-integrin to the cell membrane, but did not influence focal adhesion formation. How syndecan-4 and integrin interact with each other is still not clear. Ligand affinity can change the function of β1-integrin. Mould and Humphries (2004)
suggested that integrin activation involves the multiple intermediate conformation changes to an adhesion-competent conformation. The deletion of the syndecan-4 N1 chain may change the conformation of syndecan-4 and then influence the interaction of syndecan-4 with β1-integrin directly or indirectly. However, the localization of β1-integrin on the cell membrane did not modulate focal adhesions. Only S4 increased β-actin expression, whereas the deletion of the side chains did not affect β-actin expression. These data suggested that syndecan-4 side chains are essential for stress fiber formation.

Syndecan-4 can interact with PKCα and signal through the PKCα pathway and activate the downstream RhoA family of G proteins (Woods et al., 2000; Dovas et al., 2006). Syndecan-4 increased PKCα activity in turkey satellite cells during proliferation. However, western analysis showed that except for S4-S0N2, syndecan-4 side chain mutants decreased PKCα activity compared to the S4. Syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsuperscript{139} without any GAG chains increased PKCα activity compared to the S4. It is possible that the deletion of N1 chains that attached to the core protein at Asn\textsuperscript{124} changed the conformation of syndecan-4 core protein plus the deletion of GAG chains influenced the interaction with other molecules that enhanced syndecan-4 function with PKCα. On the other hand, immunofluorescence analysis showed that compared to the control cell, except for S4-N2, syndecan-4 side chain mutants increased cell membrane localization of PKCα, which indicated that the N1 chain and GAG chains are critical for syndecan-4 regulation of PKCα activity.

Data from the current study indicated that syndecan-4 had no effect on turkey satellite cell apoptosis. Syndecan-4 may regulate cell proliferation through modulating
cell migration. The overexpression of syndecan-4 in Chinese hamster ovary K1 cells resulted in increased focal adhesion formation and decreased cell migration (Longley et al., 1999). It is possible that when syndecan-4 is overexpressed, more focal adhesions form and cell migration is decreased. Because migration is important for satellite cells to interact with each other to receive signals to promote proliferation, the increased number of focal adhesions may inhibit cell proliferation. The results in the current study suggested that in cultured turkey satellite cells, syndecan-4 and its side chains play important roles in syndecan-4 regulated FAK and PKCα activity, and β1-integrin expression but not in cell apoptosis and focal adhesion formation.
Acknowledgements

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References


<table>
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<th>Code</th>
<th>Description</th>
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<td>S4-N0</td>
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</tr>
<tr>
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</tr>
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<td>Syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn^{139} without any GAG chains</td>
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Table 6.1 Syndecan-4 mutants and the abbreviated names.
Table 6.2 Cell apoptosis (mean ± SEM) detected by Guava Nexin assay. Cell apoptosis was measured at 24 and 48 h following the transfection and 48 h of differentiation after transfected with the empty pCMS-EGFP vector (pCMS), wild type syndecan-4 (S4), syndecan-4 mutant without any N-linked glycosylated chains attached to the core protein (S4-N0), syndecan-4 mutants with only one N-linked glycosylated chains attached to the core protein at Asn^{124} or Asn^{139} (S4-N1 or S4-N2), respectively. The percentage of early and late apoptotic cells in total cells was measured. Numbers within columns without common letters are significantly different ($P < 0.05$).

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<th>48 h following the transfection</th>
<th>48 h of differentiation</th>
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<td>Late apoptotic cell (%)</td>
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</table>
### Table 6.3 Cell apoptosis (mean ± SEM) detected by Guava Nexin assay

Cell apoptosis was measured at 24 and 48 h following the transfection and 48 h of differentiation after transfected with the empty pCMS-EGFP vector (pCMS), wild type syndecan-4 (S4), syndecan-4 mutant without any glycosaminoglycan (GAG) chains attached to the core protein (S4-S0), syndecan-4 mutant without any N-linked glycosylated chains and GAG chains attached to the core protein (S4-S0N0), syndecan-4 mutants with only one N-linked glycosylated chain attached to the core protein at Asn$^{124}$ or Asn$^{139}$ without any GAG chains attached (S4-S0N1 or S4-S0N2), respectively. The percentage of early and late apoptotic cells in total cells was measured. Numbers within columns without common letters are significantly different ($P < 0.05$).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h following the transfection</th>
<th>48 h following the transfection</th>
<th>48 h of differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early apoptotic cell (%)</td>
<td>Late apoptotic cell (%)</td>
<td>Early apoptotic cell (%)</td>
</tr>
<tr>
<td>pCMS</td>
<td>0.62 ± 0.09</td>
<td>0.97 ± 0.07 $^{ab}$</td>
<td>1.11 ± 0.10</td>
</tr>
<tr>
<td>S4</td>
<td>0.73 ± 0.07</td>
<td>0.91 ± 0.04 $^{abc}$</td>
<td>1.06 ± 0.13</td>
</tr>
<tr>
<td>S4-S0</td>
<td>0.74 ± 0.10</td>
<td>0.76 ± 0.07 $^c$</td>
<td>1.11 ± 0.04</td>
</tr>
<tr>
<td>S4-S0N0</td>
<td>0.80 ± 0.02</td>
<td>0.91 ± 0.02 $^{abc}$</td>
<td>1.19 ± 0.11</td>
</tr>
<tr>
<td>S4-S0N1</td>
<td>0.71 ± 0.04</td>
<td>0.79 ± 0.03 $^{bc}$</td>
<td>1.09 ± 0.13</td>
</tr>
<tr>
<td>S4-S0N2</td>
<td>0.77 ± 0.04</td>
<td>1.11 ± 0.12 $^a$</td>
<td>1.13 ± 0.12</td>
</tr>
</tbody>
</table>
Figure 6.1 Cell apoptosis at 24 h following the transfection. a-c, cells without transfection (Control); d-f, cells were transfected with wild type syndecan-4 (S4) in pcDNA3.1/V5-His-TOPO vector; g-i, cells were transfected with syndecan-4 without any N-linked glycosylated chains (S4-N0) in pcDNA3.1/V5-His-TOPO vector. a, d, and g, bright field images; b, e, and h, cells were stained with 4',6-diamidino-2-phenylindole; c, f, and i, cells were stained with CaspACE-fluoroisothiocyanate-valylalanly-aspartyl [O-methyl]-fluoromethylketone in situ marker. Arrow highlights the apoptotic cell. The scale bar represents 50 µM.
Figure 6.2 Cell apoptosis at 48 h of differentiation. a-c, cells without transfection (Control); d-f, cells were transfected with wild type syndecan-4 (S4) in pcDNA3.1/V5-His-TOPO vector; g-i, cells were transfected with syndecan-4 without any N-linked glycosylated chains (S4-N0) in pcDNA3.1/V5-His-TOPO vector. a, d, and g, bright field images; b, e, and h, cells were stained with 4’,6-diamidino-2-phenylindole; c, f, and i, cells were stained with CaspACE-fluoroisothiocyanate-valylalanyl-aspartyl [O-methyl]-fluoromethylketone in situ marker. The scale bar represents 50 µM.
Figure 6.3 β1-integrin expression in syndecan-4 and its side chain mutants (in pcDNA3.1/V5-His-TOPO vector) transfected randombred control 2 line turkey satellite cells at 48 h following the transfection. Cells were transfected with a) wild type syndecan-4 (S4); b) syndecan-4 without any N-linked glycosylated (N-glycosylated) chains (S4-N0); c) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsuperscript{124} (S4-N1); d) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsuperscript{139} (S4-N2); e) syndecan-4 with both N-glycosylated chains attached to the core protein without any glycosaminoglycan (GAG) chains (S4-S0); f) syndecan-4 without any N-glycosylated chains and GAG chains attached to the core protein (S4-S0N0); g) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsuperscript{124} without any GAG chains (S4-S0N1); and h) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsuperscript{139} without any GAG chains (S4-S0N2). Sheep anti-V5 antibody was used to detect the syndecan-4 core protein (green fluorescence); mouse anti-chicken β1-integrin antibody was used to detect β1-integrin (red fluorescence); and BF contains the bright field images for each construct. Scale bars in the BF images represent the size in μm for each construct.
Figure 6.4 β-actin expression in syndecan-4 and its side chain mutants (in pcDNA3.1/V5-His-TOPO vector) transfected randombred control 2 line turkey satellite cells at 48 h following the transfection. Cells were transfected with a) wild type syndecan-4 (S4); b) syndecan-4 without any N-linked glycosylated (N-glycosylated) chains (S4-N0); c) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsuperscript{124} (S4-N1); d) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsuperscript{139} (S4-N2); e) syndecan-4 with both N-glycosylated chains attached to the core protein without any glycosaminoglycan (GAG) chains (S4-S0); f) syndecan-4 without any N-glycosylated chains and GAG chains attached to the core protein (S4-S0N0); g) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsuperscript{124} without any GAG chains (S4-S0N1); and h) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsuperscript{139} without any GAG chains (S4-S0N2). Sheep anti-V5 antibody was used to detect the syndecan-4 core protein (green fluorescence); mouse monoclonal β-actin antibody was used to detect β-actin (red fluorescence); and BF contains the bright field images for each construct. Scale bars in the BF images represent the size in μm for each construct.
Figure 6.5  Vinculin expression in syndecan-4 and its side chain mutants (in pcDNA3.1/V5-His-TOPO vector) transfected randombred control 2 line turkey satellite cells at 48 h following the transfection. Cells were transfected with a) wild type syndecan-4 (S4); b) syndecan-4 without any N-linked glycosylated (N-glycosylated) chains (S4-N0); c) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{124}$ (S4-N1); d) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{139}$ (S4-N2); e) syndecan-4 with both N-glycosylated chains attached to the core protein without any glycosaminoglycan (GAG) chains (S4-S0); f) syndecan-4 without any N-glycosylated chains and GAG chains attached to the core protein (S4-S0N0); g) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{124}$ without any GAG chains (S4-S0N1); and h) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{139}$ without any GAG chains (S4-S0N2). Sheep anti-V5 antibody was used to detect the syndecan-4 core protein (green fluorescence); mouse monoclonal vinculin antibody was used to detect vinculin (red fluorescence); and BF contains the bright field images for each construct. Scale bars in the BF images represent the size in μm for each construct.
Figure 6.6 Western blot analysis of focal adhesion kinase (FAK) activity in syndecan-4 and its side chain mutants transfected randombred control 2 line turkey satellite cells at 48 h following the transfection. pCMS: pCMS-EGFP empty vector; S4: wild type syndecan-4; S4-N0: syndecan-4 without any N-linked glycosylated (N-glycosylated) chains; S4-N1: syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsubscript{124}; S4-N2: syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsubscript{139}; S4-S0: syndecan-4 with both N-glycosylated chains attached to the core protein without any glycosaminoglycan (GAG) chains; S4-S0N0: syndecan-4 without any N-glycosylated chains and GAG chains attached to the core protein; S4-S0N1: syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsubscript{124} without any GAG chains; and S4-S0N2: syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn\textsubscript{139} without any GAG chains.
Figure 6.7 PKCα expression in syndecan-4 and its side chain mutants (in pcDNA3.1/V5-His-TOPO vector) transfected randombred control 2 line turkey satellite cells at 48 h following the transfection. Cells were transfected with a) wild type syndecan-4 (S4); b) syndecan-4 without any N-linked glycosylated (N-glycosylated) chains (S4-N0); c) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn^{124} (S4-N1); d) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn^{139} (S4-N2); e) syndecan-4 with both N-glycosylated chains attached to the core protein without any glycosaminoglycan (GAG) chains (S4-S0); f) syndecan-4 without any N-glycosylated chains and GAG chains attached to the core protein (S4-S0N0); g) syndecan-4 with only one N-glycosylated chain attached to the core protein without any GAG chains (S4-S0N1); and h) syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn^{139} without any GAG chains (S4-S0N2). Sheep anti-V5 antibody was used to detect the syndecan-4 core protein (green fluorescence); mouse anti-human PKCα antibody was used to detect PKCα (red fluorescence); and BF contains the bright field images for each construct. Arrows highlight the co-localization of syndecan-4 and PKCα. Scale bars in the BF images represent the size in μm for each construct.
Figure 6.8 Western blot analysis of protein kinase C alpha (PKCα) activity in syndecan-4 and its side chain mutants transfected randombred control 2 line turkey satellite cells at 48 h following the transfection. pCMS: pCMS-EGFP empty vector; S4: wild type syndecan-4; S4-N0: syndecan-4 without any N-linked glycosylated (N-glycosylated) chains; S4-N1: syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{124}$; S4-N2: syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{139}$; S4-S0: syndecan-4 with both N-glycosylated chains attached to the core protein without any glycosaminoglycan (GAG) chains; S4-S0N0: syndecan-4 without any N-glycosylated chains and GAG chains attached to the core protein; S4-S0N1: syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{124}$ without any GAG chains; and S4-S0N2: syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{139}$ without any GAG chains.
Figure 6.9 Syndecan-4 regulation of myogenic satellite cell proliferation. Syndecan-4 regulation of cell proliferation through modulating focal adhesion formation. Syndecan-4 can either support β1-integrin mediated focal adhesion formation through enhancing focal adhesion kinase (FAK) activity or function directly by activating protein kinase C alpha (PKC α). The size and number of focal adhesions influence cell motility and shape. Focal adhesions can inhibit cell rounding and shedding from the substrate thus decreasing cell apoptosis and enhancing cell proliferation. The appropriate level of focal adhesions will promote cell migration and then increase cell proliferation. However, too many focal adhesions will inhibit cell migration and proliferation.
CHAPTER 7: SYNGECAN-4 CYTOPLASMIC DOMAIN REGULATION OF TURKEY SATELLITE CELL FOCAL ADHESIONS AND APOPTOSIS

Abstract

Syndecan-4 is a cell membrane proteoglycan that is composed of a transmembrane core protein and substituted glycosaminoglycan (GAG) and N-linked glycosylated (N-glycosylated) chains. The core protein has three domains: extracellular, transmembrane and cytoplasmic domains. The GAG chains and the cytoplasmic domain of syndecan-4 play critical roles in focal adhesion formation and apoptosis. The GAG and N-glycosylated chains and the cytoplasmic domain of syndecan-4, especially the amino acids: Ser$^{178}$ and Tyr$^{187}$ are critical in the regulation of turkey satellite cell growth and development. How these processes are regulated is still unknown. The objective of the current study was to determine whether the syndecan-4 cytoplasmic domain functions through modulating focal adhesion formation and apoptosis. Twelve mutant clones were generated: a truncated syndecan-4 without the cytoplasmic domain with or without GAG and N-glycosylated chains, and Ser$^{178}$ and Tyr$^{187}$ mutants with or without GAG and N-glycosylated chains. Wild type syndecan-4, and all of the syndecan-4 mutants were transfected into turkey myogenic satellite cells, and cell apoptosis and focal adhesion formation were measured. Syndecan-4 and syndecan-4 mutants did not influence cell
apoptosis and vinculin-containing focal adhesion formation, but increased cell membrane localization of β1-integrin. Syndecan-4 increased focal adhesion kinase (FAK) activity and the cytoplasmic domain mutation decreased the phosphorylation of FAK. These results suggested that syndecan-4 cytoplasmic domain plays an important role in regulating FAK activity and β1-integrin cell membrane localization but not in cell apoptosis and vinculin-containing focal adhesion formation.

7.1 Introduction

Syndecans are a family of heparan sulfate proteoglycans. There are four members in vertebrates, syndecan-1 through -4 (Rapraeger, 2001). All of the syndecans have a highly conserved short cytoplasmic tail, a transmembrane domain, and an extracellular domain bearing heparan sulfate glycosaminoglycan (GAG) chains and N-linked glycosylated (N-glycosylated) chains. The cytoplasmic domain has two conserved regions (C1 and C2), with an intervening sequence that is unique to each family member (V region). Syndecan-4 is unique for its ubiquitous expression in tissues (Elenius and Jalkanen 1994; Carey 1997; Bernfield et al., 1999; Rapraeger, 2001; Woods, 2001). In muscle, syndecan-4 expression is required for proper muscle maintenance, regeneration, growth and development (Cornelison et al., 2001; Liu et al., 2006; Tanaka et al., 2009; Sporer et al., 2011). In adhesive cells, syndecan-4 is primarily located in focal adhesions and it plays an important role in promoting focal adhesion formation (Echtermeyer et al., 1999), regulating cell migration (Echtermeyer et al., 2001), and protecting cells from apoptosis (Jeong et al., 2001). Focal adhesions are the areas where cells stably attach to
the matrix. Focal adhesions are composed of many proteins including extracellular matrix molecules that cells attach to, cell membrane receptors including integrins and syndecan-4, and cytoplasmic proteins including vinculin, talin, alpha actinin, paxillin, focal adhesion kinase (FAK), SrC, and protein kinase C (PKC) (Clark and Brugge, 1995; Burridge and Chrzanowska-Wodnicka, 1996). Integrins are the primary receptor for assembling focal adhesions whereas heparan sulfate proteoglycans are also required (Woods et al., 1986). Syndecan-4 is required for integrin mediated fibronectin induced focal adhesion formation (Saoncella et al., 1999) by binding its GAG chains to the heparin-binding domain of fibronectin (Lyon et al., 2000; Woods et al., 2000). Overexpression of syndecan-4 induced more cell spreading and focal adhesion formation, whereas the overexpression of a truncated syndecan-4 without the V region of the cytoplasmic domain decreased cell spreading and focal adhesion formation (Longley et al., 1999). This indicates an important role for the V region of the syndecan-4 cytoplasmic domain in focal adhesion formation.

The tyrosine residue in the amino acid sequence KKPIYKK in the V region of the syndecan-4 cytoplasmic domain binds to phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (Oh et al., 1998; Horowitz et al., 1999; Couchman et al., 2002) and then interacts with and strongly activates PKC$_\alpha$ (Oh et al., 1997b, 1998; Horowitz and Simons, 1998a; Couchman et al., 2002) to regulate adhesion formation and actin organization (Sun and Rotenberg, 1999). The phosphorylation of the serine residue in the C1 region of the syndecan-4 cytoplasmic domain leads to the decreased affinity of the PIP$_2$ binding to the V region of syndecan-4, decreases PKC$_\alpha$ activity (Couchman et al., 2002; Murakami et al., 2002), and then regulates focal adhesion formation.
In the current study, the role of the turkey syndecan-4 cytoplasmic domain, the serine residue 178 (Ser\textsuperscript{178}) in the C1 region of turkey syndecan-4 and the tyrosine residue 187 (Tyr\textsuperscript{187}) in the sequence KKPIYKK in the V region of the turkey syndecan-4, in combination with the N-glycosylated and GAG chains, in turkey satellite cell apoptosis and focal adhesion formation were explored. Syndecan-4 clones without the cytoplasmic domain with or without GAG chains and N-glycosylated chains, Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants with or without GAG chains and N-glycosylated chains were transfected into turkey myogenic satellite cells, and cell apoptosis and focal adhesion formation were measured. The results from the current study provide new information about the function of the syndecan-4 cytoplasmic domain, in combination with N-glycosylated chains and GAG chains, during turkey satellite cell apoptosis and focal adhesion formation.

7.2 Materials and Methods

7.2.1 Clone Generation

Turkey cDNA generated from embryonic day 18 pectoralis major muscle was used as a template to generate S4C. Syndecan-4 without any N-glycosylated chains (S4-N0), syndecan-4 without any GAG chains (S4-S0), and syndecan-4 without any GAG and N-glycosylated chains (S4-S0N0) cloned into the pCMS-EGFP vector (Song et al., 2011) were used as templates to generate syndecan-4 cytoplasmic domain mutants with or without N-glycosylated chains and GAG chains (S4C-N0, S4C-S0, and S4C-S0N0). The list of the syndecan-4 mutants were: syndecan-4 without the cytoplasmic domain (S4C); syndecan-4 without the cytoplasmic domain and the N-glycosylated chains (S4C-
N0); syndecan-4 without the cytoplasmic domain and the GAG chains (S4C-S0); syndecan-4 without the cytoplasmic domain and the GAG and N-glycosylated chains (S4C-S0N0) syndecan-4 Ser^{178} mutant with Ser mutated to Ala (S4SA); syndecan-4 Ser^{178} mutant without N-glycosylated chains (S4SA-N0); syndecan-4 Ser^{178} mutant without GAG chains (S4SA-S0); syndecan-4 Ser^{178} mutant without N-glycosylated and GAG chains (S4SA-S0N0); syndecan-4 Tyr^{187} mutant with Tyr mutated to Phe (S4YF); syndecan-4 Tyr^{187} mutant without N-glycosylated chains (S4YF-N0); syndecan-4 Tyr^{187} mutant without GAG chains (S4YF-S0); and syndecan-4 Tyr^{187} mutant without N-glycosylated and GAG chains (S4YF-S0N0). The primers used were: forward primer 5’-ATA CTC GAG _ATG_ CCG CTG CTC CGC GCC GCC TTC_{24} -3’, and reverse primer 5’- CGC TCT AGA TTA _ATA_ GAC TAA GAG GAG GAT CAG G_{486} -3’. Amplification consisted of an initial denaturation at 94 °C for 15 min, and 35 cycles of 30 s at 94 °C, 20 s at 55 °C, 30 s at 72 °C followed by 5 min at 72 °C. The PCR product was inserted into the pCMS-EGFP vector. The ligation reaction was performed by using T4 ligase (Promega) according to the manufacturer’s protocol.

The Ser^{178} and Tyr^{187} mutants were generated using the Quick Change Multi Site-Directed Mutagenesis kit (Stratagene Corporation) according to the manufacturer’s protocol. The templates used were S4, S4-N0, S4-S0, and S4-S0N0 (Velleman et al., 2006; Song et al., 2011). The primers used for Ser^{178} mutants were 5’-GAA GAA AAA GGA TGA AGG C GC CTA CGA CCT TGG GAA GAA A-3’ and for Tyr^{187} mutants were 5’-CTT GGG AAG AAA CCA ATC TTC AAG AAA GCC CCT ACA AAT-3’. The Ser residue was mutated to Ala, and the Tyr residue was mutated to phenylalanine (highlighted in the primer sequences). All of the mutations were confirmed by DNA
sequencing in the Molecular and Cellular Imaging Center (Ohio Agricultural and Development Center, The Ohio State University).

The S4 clone was subcloned into a pcDNA3.1/V5 His TOPO vector (Invitrogen) previously by Song et al. (2011). In addition, the S4C, S4C-N0, S4C-S0, S4C-S0N0, S4SA, S4SA-N0, S4SA-S0, S4SA-S0N0, S4YF, S4YF-N0, S4YF-S0, and S4YF-S0N0 cloned into the pCMS-EGFP vector were used as templates to subclone into the pcDNA3.1/V5 His TOPO vector. The primers for S4C mutants subcloning were: forward primer 5’- ATA 1ATG CCG CTG CTC CGC G16 -3’, and reverse primer 5’- 507ATA GAC TAA GAG GAG GAT CAG GAA G3’. The forward primer for S4SA and S4YF mutants subcloning is the same as S4C subcloning primer, and the reverse primer was: 5’-591AGC GTA GAA CTC ATT TGT AGG GGC 568-3’. The PCR condition was denaturation at 95 °C for 15 min, and 35 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 75 °C followed by 15 min at 75 °C. The ligation reaction contained 3 µl of PCR product from the above reaction, 1 µl of pcDNA3.1/V5-His-TOPO Vector (Invitrogen) and 1 µl of salt solution (1.2 M NaCl and 0.06 M MgCl2). Clones were confirmed by DNA sequencing at the Molecular and Cellular Imaging Center.

7.2.2 Satellite Cell Culture and Transfection

Satellite cells isolated from the pectoralis major muscle of 7-week-old male randombred control 2 line turkeys were used in the current study. Satellite cells were plated in gelatin-coated cell culture plates (Greiner Bio-One) in plating medium [Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) containing 10% chicken serum (Invitrogen), 5% horse serum (Invitrogen), 1% antibiotic/antimycotic (Invitrogen),
and 0.1% gentamicin (Invitrogen)] and incubated in a 37.5 °C 5%CO₂/95% air incubator. After 24 h attachment, the cells were transfected with pCMS-EGFP empty vector, S4, S4SA, and S4YF mutants using the Optifect transfection system (Invitrogen) with 0.5 μg of plasmid DNA according to the manufacturer’s protocol or cotransfected with S4C mutants and a small interfering RNA (siRNA) targeting the syndecan-4 cytoplasmic domain using Lipofectamine 2000 transfection reagent (Invitrogen) with 0.5 μg plasmid DNA and 20 pmol siRNA according to the manufacturer’s protocol. The siRNA used was, sense: 5’- 544GGG AAG AAA CCA AUC UAC AAG AAA G568 -3’, and antisense: 5’- CUU UCU UGU AGA UUG GUU UCU UCC C -3’. After 6 h of transfection, the medium was changed to feeding medium [McCoy’s 5A medium (Sigma-Aldrich) containing 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic, and 0.1% gentamicin]. Feeding medium was changed daily until 72 h post-transfection. Differentiation was induced when cells reach 60% confluency by changing the feeding medium to fusion medium [DMEM containing 3% horse serum, 1% antibiotic/antimycotic, 0.01 mg/mL porcine gelatin (Sigma-Aldrich), 0.1% gentamicin, and 1.0 mg/mL bovine serum albumin (Sigma-Aldrich)]. The fusion medium was changed every 24 h until 48 h of differentiation.

7.2.3 Caspase Assay

Satellite cells were cultured and transfected in 24-well cell culture plates. At 24 h following the transfection, and 48 h of differentiation, cell culture medium were removed and washed twice with PBS. CaspACE-fluoroisothiocyanate-valylalanyl-aspartyl [O-methyl]-fluoromethylketone in situ marker (1:500; Promega) was added into the cell
cultures and incubated in dark for 40 min at 37 °C in a 95% air/5% CO₂ cell culture incubator. After incubation, the cell cultures were washed twice with PBS and then fixed with 3% paraformaldehyde for 30 min at room temperature in dark. After fixation, 1µg/ml 4, 6-diamidino-2-phenylindole was added into the cell culture and incubated for 5 min. The cell cultures were then viewed and recorded with an Olympus XI 70 microscope and an Optronics digital camera (Optronics).

7.2.4 Guava Nexin Assay

Satellite cells were cultured and transfected in 24-well cell culture plates. Culture medium was changed daily, collected for each sample and stored at 37 °C in a 95% air/5% CO₂ cell culture incubator for further analysis. At 24 and 48 h following the transfection, and 48 h of differentiation, cells were scraped off the plates and added to each tube containing collected culture medium. The cells were collected by centrifuging at 350 x g for 10 min at 4 °C. After removing the supernatant, the cells were washed twice by resuspending the pellet in 1 ml cold 1X Nexin buffer (Millipore). After the second wash, cell concentration was adjusted to 2 X 10⁶ cells/ml. Forty µl of each sample was added to 5 µl of Annexin V conjugated with Phycoerythrin (MBL International) to detect early apoptotic cells, and 5 µl of 7-Amino-actinomycin D (BD Biosciences) to detect late apoptotic cells. After 20 min incubation on ice, 450 µl of cold 1X Nexin buffer was added to each sample and analyzed on a Guava EasyCyte system (Millipore) following the manufacturer’s Guava Nexin assay protocol.

7.2.5 Western Blot
Satellite cells were cultured and transfected with the syndecan-4 mutants. At 48 h following the transfection, cell cultures were removed from the incubator. Total cell protein was extracted using RIPA buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 10 mM NaF, and 2 mM Na$_3$VO$_4$, and freshly prepared protease inhibitor cocktail (Roche Applied Science)). After incubating with 200 µl of RIPA buffer on ice for 5 min, cells were scraped off the plates, collected and homogenized on ice for 30 min. Following centrifugation at 16,000 x g at 4 °C for 15 min, proteins in the supernatant were collected.

Protein concentration was determined by the Bradford assay (Bradford, 1976). Equal amounts of proteins were mixed with reducing sample buffer (0.125 M Tris–HCl, pH6.8, 4.1% SDS, 20% glycerol, 2% β-mercaptoethanol, and 0.001% bromphenol blue), boiled for 5 min and then separated on an 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) by the method of Laemmli (1970). Proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Millipore). After being blocked for 1 h with 5% nonfat milk dissolved in Tris-buffered saline containing 20 mM Tris–HCl, pH7.4, 150 mM NaCl, and 0.05% Tween 20 (TBS-T), the PVDF membrane was incubated with the indicated primary antibodies in blocking buffer for 2 h at room temperature or overnight at 4 °C. Antibodies used were rabbit anti-human FAK (pY$^{397}$) (Invitrogen, 1:1,000) and rabbit anti-human FAK (Santa Cruz; 1:3,000). The secondary antibody used was donkey anti-rabbit conjugated with IgG alkaline phosphatase (Santa Cruz, 1:10,000). After incubating with chemiluminescent alkaline phosphatase substrate (Millipore) for 5 min at room temperature, immunoreactive bands were visualized with a
Bio Rad ChemiDoc XRS imaging system (Bio Rad). The relative quantity and size of the bands were determined with Quantity One 4.6.6 Software (Bio Rad).

**7.2.6 Confocal Immunofluorescence Microscopy**

Cells were seeded on gelatin coated polystyrene slides (Electron Microscopy Sciences) and transfected. At 48 h post transfection, cells were rinsed with PBS for 3 X and fixed with 3% paraformaldehyde for 30 min at room temperature. After 30 min of being blocked with 1% BSA in TBS-T, cells were incubated with the indicated primary antibody overnight at 4 °C or 2 h at room temperature. Cells were then rinsed 3 X with 1% BSA in TBS-T and incubated with the appropriate secondary antibody for 1 h. Primary antibodies used were sheep anti-V5 tag (Abcam, 1:12,000), mouse anti-human PKCα (BD Biosciences, 1:5,000), mouse monoclonal vinculin (Abcam, 1:300), mouse monoclonal β-actin (Sigma, 1:300), and mouse anti-chicken β1-integrin (CSAT, 1:300) antibodies. The secondary antibodies used were Alexa Fluor 488 donkey anti-sheep (Invitrogen, 1:1,000), and Alexa Fluor 568 rabbit anti-mouse (Invitrogen, 1:1,000). Samples were then mounted with gel/mount media (Biomeda) and covered with microscope cover glasses No. 1.5 (VWR). The cell cultures were then viewed and recorded by a Leica TCS SP confocal scanning microscope system (Leica).

**7.2.7 Statistical Analysis**

All experiments were repeated independently at least three times. For the Guava Nexin assay there were three replicates for each treatment, and the data from each repeat were used to calculate a mean and the standard error of the mean (SEM). Data are graphed as the mean ± SEM. The SAS PROC GLM (SAS Institute Inc.) was used for
statistical analyses. Differences among means were detected using the Fisher’s least significance method. Differences among means in each experiment were evaluated using an ANOVA and detected using Fisher's least-significant-difference. Two-tailed $P$ values of $P < 0.05$ were considered statistically significant.

7.3 Results

7.3.1 Syndecan-4 cytoplasmic domain regulation of focal adhesion formation

The activity of FAK was measured by the ratio of phosphorylated FAK to total FAK at 48 h following the transfection. Syndecan-4 cytoplasmic domain mutants had different effects on FAK activity (Figure 7.1). Compared to the pCMS-EGFP empty vector, the transfection of wild type syndecan-4 (S4) increased FAK activity. Compared to S4, all of the Ser$^{178}$ mutants (S4SA, S4SA-N0, S4SA-S0, and S4SA-S0N0) and Tyr$^{187}$ mutants including S4YF, S4YF-N0, and S4YF-S0 had decreased FAK activity (Figure 7.1a). However, S4YF-S0N0 increased FAK activity compared to S4. The deletion of the entire syndecan-4 cytoplasmic domain in combination with the GAG and N-glycosylated chains (S4C, S4C-N0, S4C-S0, and S4C-S0N0) also decreased FAK activity compared to S4 (Figure 7.1b).

At 48 h following the transfection, confocal microscopy showed that cells transfected with S4, and all of the syndecan-4 cytoplasmic domain mutants, and Ser$^{178}$ and Tyr$^{187}$ mutants had higher cell membrane localization of $\beta 1$-integrin compared to the cells without transfection (Figure 7.2). The cell membrane localization of $\beta$-actin was increased by the transfection of S4, S4SA-N0, and S4YF-S0 (Figure 7.3a, g, and l) but
not other syndecan-4 cytoplasmic domain mutants, and Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants. Furthermore, S4 and syndecan-4 cytoplasmic domain mutants, and Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants did not influence the cell membrane localization of vinculin compared to the cells without transfection (Figure 7.4).

7.3.2 Apoptotic effect of syndecan-4 cytoplasmic domain

At 24 h following the transfection and 48 h of differentiation, satellite cells transfected with S4, syndecan-4 cytoplasmic domain mutants, and Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants had no detectable apoptotic cells when hybridized with the CaspACE marker specific for apoptotic cells (Figure 7.5, 6; not all mutants are shown). Cell cytometry also indicated that the cytoplasmic domain of syndecan-4 has limited function on cell apoptosis. At 48 h of differentiation the transfection of S4C had a higher level of late (Annexin +/7-AAD+) apoptotic cells (Table 1) and at 48 h following the transfection the transfection of S4SA had a higher level of late apoptotic cells (Table 2). Other than that, at 24 and 48 h following the transfection, and 48 h of differentiation, transfection of all of the syndecan-4 cytoplasmic domain mutants, Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants had no significant difference in the number of early (Annexin V +/7AAD-) and late apoptotic cells compared to the S4 transfection (Table 1-3). These data suggest that the cytoplasmic domain of syndecan-4 generally has a limited effect on turkey satellite cell apoptosis.

7.4 Discussion

The heparan sulfate proteoglycan, syndecan-4, is an essential component in focal adhesion complexes (Saoncella et al., 1999). It functions with the integrins to promote
the formation of focal adhesions and actin stress fiber assembly (Saoncella et al., 1999). Lyon et al. (2000) and Woods et al. (2000) reported that syndecan-4 GAG chains are needed in integrin-mediated focal adhesion formation by binding GAG chains to the heparin-binding domain of fibronectin. This is supported by the study of LeBaron et al. (1988) that GAG-deficient syndecan-4 reduces focal adhesion formation and actin stress fiber formation. However, Echtermeyer et al. (1999) demonstrated that syndecan-4 core protein is sufficient to increase focal adhesion formation and decreases cell migration. Furthermore, a study by Longley et al. (1999) found that the V region of the syndecan-4 cytoplasmic domain is critical in focal adhesion formation and cell migration. In the current study, the role of the syndecan-4 cytoplasmic domain in combination with the GAG and N-glycosylated chains in turkey skeletal muscle satellite cell focal adhesion formation was explored.

Syndecan-4 can either support β1-integrin mediated focal adhesion formation (Saoncella et al., 1999) directly by activating PKC α (Oh et al., 1997a, b; Lim et al., 2003; Keum et al., 2004) or function through enhancing FAK activity (Guan et al., 1991; Kornberg et al., 1992). The serine residue in the C1 region of the syndecan-4 cytoplasmic domain and the tyrosine residue in the amino acid sequence KKPIYKK in the V region of the syndecan-4 cytoplasmic domain have been reported to be critical in regulating PKC α activity (Oh et al., 1997a, b, 1998; Horowitz and Simons, 1998a, b; Couchman et al., 2002). Thus, the Ser and Tyr residues may function in the formation of focal adhesions. In the present study, the overexpression of the syndecan-4 cytoplasmic domain mutants, and the Ser^{178} and Tyr^{187} mutants increased β1 integrin cell membrane localization, but did not increase the formation of the vinculin-containing focal
adhesions. Only S4, S4SA-N0, and S4YF-S0 increased β-actin cell membrane localization, whereas the other mutants had no effect. These data indicate that the syndecan-4 cytoplasmic domain is essential for stress fiber formation, and that Ser\textsuperscript{178} and Tyr\textsuperscript{187} are critical in this process. How syndecan-4 and integrin interact with each other is still not clear. Ligand affinity can change the function of β1-integrin. The activation of β1-integrin may involve multiple intermediate conformational changes to an adhesion-competent conformation (Mould and Humphries, 2004).

Beta 1 integrin focal adhesion signals activate FAK. The phosphorylation of FAK in response to β1 integrin leads to the activation of cell signaling pathways which are important for cell survival (Ginacotti and Ruoslahti, 1999). Wilcox-Adelman et al. (2002) reported that syndecan-4 can regulate the activation of FAK. The overexpression of S4 increased the FAK activity whereas syndecan-4 cytoplasmic domain mutants and the Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants decreased FAK activity compared to the S4. These data indicated that the cytoplasmic domain of syndecan-4 is critical in regulating FAK activity, and Ser\textsuperscript{178} and Tyr\textsuperscript{187} are critical in regulation of FAK activity. Overexpression of S4YF, S4YF-N0, and S4YF-S0 decreased FAK activity, whereas S4YF-S0N0 increased FAK activity, indicating that there are complex interactions between the syndecan-4 cytoplasmic domain, the GAG chains, and the N-glycosylated chains. The deletion of the N-glycosylated chains may change the 3-dimensional structure of the syndecan-4 core protein, and the deletion of the GAG chains may influenced the interaction of syndecan-4 with extracellular matrix molecules which together modulated the function of syndecan-4 in regulation of FAK activity. The current study also indicated that the syndecan-4 cytoplasmic domain, and the Ser\textsuperscript{178} and Tyr\textsuperscript{187} in the
syndecan-4 cytoplasmic domain, in combination with the GAG chains and N-glycosylated chains, had no effect on turkey satellite cell apoptosis.
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References


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<th>48 h following the transfection</th>
<th>48 h of differentiation</th>
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Table 7.1  Cell apoptosis (mean ± SEM) measured by Guava Nexin assay. Cell apoptosis was measured at 24 and 48 h following the transfection and 48 h of differentiation after transfection with the empty pCMS-EGFP vector (pCMS), wild type syndecan-4 (S4), syndecan-4 mutant without any N-linked glycosylated chains attached to the core protein (S4-N0), syndecan-4 mutants with only one N-linked glycosylated chains attached to the core protein at Asn^{124} or Asn^{139} (S4-N1 or S4-N2), respectively. The percentage of early and late apoptotic cells in the total cells was measured. Numbers within columns without common letters are significantly different (P < 0.05).
Table 7.2  Cell apoptosis (mean ± SEM) measured by Guava Nexin assay. Cell apoptosis was measured at 24 and 48 h following the transfection and 48 h of differentiation after transfection with the empty pCMS-EGFP vector (pCMS), wild type syndecan-4 (S4), syndecan-4 mutant without any glycosaminoglycan (GAG) chains attached to the core protein (S4-S0), syndecan-4 mutant without any N-linked glycosylated chains and GAG chains attached to the core protein (S4-S0N0), syndecan-4 mutants with only one N-linked glycosylated chains attached to the core protein at Asn\textsuperscript{124} or Asn\textsuperscript{139} without any GAG chains attached (S4-S0N1 or S4-S0N2), respectively. The percentage of early and late apoptotic cells in the total cells was measured. Numbers within columns without common letters are significantly different ($P < 0.05$).
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Table 7.3  Cell apoptosis (mean ± SEM) measured by Guava Nexin assay. Cell apoptosis was measured at 24 and 48 h following the transfection and 48 h of differentiation after transfection with the empty pCMS-EGFP vector (pCMS), wild type syndecan-4 (S4), syndecan-4 mutant without any glycosaminoglycan (GAG) chains attached to the core protein (S4-S0), syndecan-4 mutant without any N-linked glycosylated chains and GAG chains attached to the core protein (S4-S0N0), syndecan-4 mutants with only one N-linked glycosylated chains attached to the core protein at Asn\(^{124}\) or Asn\(^{139}\) without any GAG chains attached (S4-S0N1 or S4-S0N2), respectively. The percentage of early and late apoptotic cells in the total cells was measured. Numbers within columns without common letters are significantly different (\(P < 0.05\)).
Figure 7.1 Western blot analysis of focal adhesion kinase (FAK) activity in syndecan-4, the cytoplasmic domain deletion mutations, and the Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants transfected into randombred control 2 line turkey satellite cells at 48 h following the transfection. a) pCMS-EGFP empty vector (pCMS); wild type syndecan-4 (S4); syndecan-4 Ser\textsuperscript{178} mutant with Ser mutated to Ala (S4SA); syndecan-4 Ser\textsuperscript{178} mutant without N-linked glycosylated (N-glycosylated) chains (S4SA-N0); syndecan-4 Ser\textsuperscript{178} mutant without glycosaminoglycan (GAG) chains (S4SA-S0); syndecan-4 Ser\textsuperscript{178} mutant without N-glycosylated and GAG chains (S4SA-S0N0); syndecan-4 Tyr\textsuperscript{187} mutant with Tyr mutated to Phe (S4YF); syndecan-4 Tyr\textsuperscript{187} mutant without N-glycosylated chains (S4YF-N0); syndecan-4 Tyr\textsuperscript{187} mutant without GAG chains (S4YF-S0); and syndecan-4 Tyr\textsuperscript{187} mutant without N-glycosylated and GAG chains (S4YF-S0N0); and b) syndecan-4 without the cytoplasmic domain (S4C); syndecan-4 without the cytoplasmic domain and the N-glycosylated chains (S4C-N0); syndecan-4 without the cytoplasmic domain and the GAG chains (S4C-S0); and syndecan-4 without the cytoplasmic domain and the GAG and N-glycosylated chains (S4C-S0N0). The FAK activity was calculated by the ratio of phosphorylated FAK (pFAK) to FAK.
Figure 7.2  β1-integrin cell membrane localization in syndecan-4 and its side chain mutants (in pcDNA3.1/V5-His-TOPO vector) transfected into randombred control 2 line turkey satellite cells at 48 h post transfection. Cells were transfected with a) wild type syndecan-4 (S4); b) syndecan-4 without the cytoplasmic domain (S4C); c) syndecan-4 without the cytoplasmic domain and the N-linked glycosylated (N-glycosylated) chains (S4C-N0); d) syndecan-4 without the cytoplasmic domain and the glycosaminoglycan (GAG) chains (S4C-S0); e) syndecan-4 without the cytoplasmic domain and the GAG and N-glycosylated chains (S4C-S0N0); f) syndecan-4 Ser\textsuperscript{178} mutant with Ser mutated to Ala (S4SA); g) syndecan-4 Ser\textsuperscript{178} mutant without N-glycosylated chains (S4SA-N0); h) syndecan-4 Ser\textsuperscript{178} mutant without GAG chains (S4SA-S0); i) syndecan-4 Ser\textsuperscript{178} mutant without N-glycosylated and GAG chains (S4SA-S0N0); j) syndecan-4 Tyr\textsuperscript{187} mutant with Tyr mutated to Phe (S4YF); k) syndecan-4 Tyr\textsuperscript{187} mutant without N-glycosylated chains (S4YF-N0); l) syndecan-4 Tyr\textsuperscript{187} mutant without GAG chains (S4YF-S0); and m) syndecan-4 Tyr\textsuperscript{187} mutant without N-glycosylated and GAG chains (S4YF-S0N0). Sheep anti-V5 antibody was used to detect the syndecan-4 core protein (green fluorescence); mouse anti-chicken β1-integrin antibody was used to detect β1-integrin (red fluorescence); and bright field (BF) images for each construct are shown. Scale bars in the BF images represent the size in μm for each construct.
Figure 7.3  β-actin cell membrane localization in syndecan-4 and its side chain mutants (in pcDNA3.1/V5-His-TOPO vector) transfected into randombred control 2 line turkey satellite cells at 48 h post transfection. Cells were transfected with a) wild type syndecan-4 (S4); b) syndecan-4 without the cytoplasmic domain (S4C); c) syndecan-4 without the cytoplasmic domain and the N-glycosylated chains (S4C-N0); d) syndecan-4 without the cytoplasmic domain and the GAG chains (S4C-S0); e) syndecan-4 without the cytoplasmic domain and the GAG and N-glycosylated chains (S4C-S0N0); f) syndecan-4 Ser^{178} mutant with Ser mutated to Ala (S4SA); g) syndecan-4 Ser^{178} mutant without N-glycosylated chains (S4SA-N0); h) syndecan-4 Ser^{178} mutant without GAG chains (S4SA-S0); i) syndecan-4 Ser^{178} mutant without N-glycosylated and GAG chains (S4SA-S0N0); j) syndecan-4 Tyr^{187} mutant with Tyr mutated to Phe (S4YF); k) syndecan-4 Tyr^{187} mutant without N-glycosylated chains (S4YF-N0); l) syndecan-4 Tyr^{187} mutant without GAG chains (S4YF-S0); and m) syndecan-4 Tyr^{187} mutant without N-glycosylated and GAG chains (S4YF-S0N0). Sheep anti-V5 antibody was used to detect the syndecan-4 core protein (green fluorescence); mouse monoclonal β-actin antibody was used to detect β-actin (red fluorescence); and bright field (BF) images for each construct are shown. Scale bars in the BF images represent the size in μm for each construct.
Figure 7.4 Vinculin cell membrane localization in syndecan-4 and its side chain mutants (in pcDNA3.1/V5-His-TOPO vector) transfected into randombred control 2 line turkey satellite cells at 48 h post transfection. Cells were transfected with a) wild type syndecan-4 (S4); b) syndecan-4 without the cytoplasmic domain (S4C); c) syndecan-4 without the cytoplasmic domain and the N-glycosylated chains (S4C-N0); d) syndecan-4 without the cytoplasmic domain and the GAG chains (S4C-S0); e) syndecan-4 without the cytoplasmic domain and the GAG and N-glycosylated chains (S4C-S0N0); f) syndecan-4 Ser$^{178}$ mutant with Ser mutated to Ala (S4SA); g) syndecan-4 Ser$^{178}$ mutant without N-glycosylated chains (S4SA-N0); h) syndecan-4 Ser$^{178}$ mutant without GAG chains (S4SA-S0); i) syndecan-4 Ser$^{178}$ mutant without N-glycosylated and GAG chains (S4SA-S0N0); j) syndecan-4 Tyr$^{187}$ mutant with Tyr mutated to Phe (S4YF); k) syndecan-4 Tyr$^{187}$ mutant without N-glycosylated chains (S4YF-N0); l) syndecan-4 Tyr$^{187}$ mutant without GAG chains (S4YF-S0); and m) syndecan-4 Tyr$^{187}$ mutant without N-glycosylated and GAG chains (S4YF-S0N0). Sheep anti-V5 antibody was used to detect the syndecan-4 core protein (green fluorescence); mouse monoclonal vinculin antibody was used to detect vinculin (red fluorescence); and bright field (BF) images for each construct are shown. Scale bars in the BF images represent the size in μm for each construct.
Figure 7.5 Randombred control 2 line turkey satellite cell apoptosis at 24 h following the transfection with syndecan-4 and its mutants in pcDNA3.1/V5-His-TOPO vector. NC, negative control without transfection; S4, wild type syndecan-4; S4C, syndecan-4 without the cytoplasmic domain; S4SA, syndecan-4 Ser$^{178}$ mutant with Ser mutated to Ala; S4YF, syndecan-4 Tyr$^{187}$ mutant with Tyr mutated to Phe. BF, bright field images; DAPI, cells were stained with 4',6-diamidino-2-phenylindole; CaspACE, cells were stained with CaspACE-fluoroisothiocyanate-valylalanyl-aspartyl [O-methyl]-fluoromethylketone in situ marker. The scale bar represents 50 µM.
Figure 7.6  Randombred control 2 line turkey satellite cell apoptosis at 48 h of differentiation that transfected with syndecan-4 and its mutants in pcDNA3.1/V5-His-TOPO vector. NC, negative control without transfection; S4, wild type syndecan-4; S4C, syndecan-4 without the cytoplasmic domain; S4SA, syndecan-4 Ser\textsuperscript{178} mutant with Ser mutated to Ala; S4YF, syndecan-4 Tyr\textsuperscript{187} mutant with Tyr mutated to Phe. BF, bright field images; DAPI, cells were stained with 4',6-diamidino-2-phenylindole; CaspACE, cells were stained with CaspACE-fluoroisothiocyanate-valylalanyl-aspartyl [O-methyl]-fluoromethylketone in situ marker. The scale bar represents 50 µM.
CHAPTER 8: DISCUSSION AND CONCLUSION

Introduction

Skeletal muscle growth and development are highly organized processes that are regulated by cell to cell interactions and cell to matrix interactions. The extracellular matrix (ECM) is composed of fibrous proteins like collagens and non-fibrous proteins including proteoglycans. Heparan sulfate proteoglycans play pivotal roles in regulating muscle growth and development. The objective of the current study was to elucidate how the heparan sulfate proteoglycans, glypican-1 and syndecan-4, modulate muscle growth and development and the pathways that might be involved. It was hypothesized that glypican-1 may regulate muscle growth, development and responsiveness to fibroblast growth factor 2 (FGF2) through its heparan sulfate and N-linked glycosylated (N-glycosylated) chains; and syndecan-4 may function in muscle development by modulating focal adhesion formation through its central core protein cytoplasmic domain and the attached heparan sulfate and N-glycosylated chains. The FGF2 signal transduction and the protein kinase C alpha (PKCα) pathway may be involved in this process. According to this hypothesis, studies were designed to address how glypican-1 N-glycosylated chains influence satellite cell proliferation, differentiation, and cellular responsiveness to FGF2, and how the syndecan-4 cytoplasmic domain, heparan sulfate chains, and N-glycosylated chains regulate satellite cell proliferation, differentiation,
cellular responsiveness to FGF2, the activity of PKCα, focal adhesion formation, and cell apoptosis.

The role of glypican-1 in skeletal muscle growth and development

The heparan sulfate proteoglycan, glypican-1 has both membrane-associated and shed forms. The membrane-associated form is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor and has been hypothesized to increase FGF2 binding to its signaling receptor (Steinfeld et al., 1996; Brandan and Larraín, 1998). The shed form of glypican-1 may be incorporated into the extracellular matrix during muscle differentiation and sequester FGF2 away from its receptor, permitting differentiation (Brandan and Larraín, 1998). Both forms of turkey glypican-1 are composed of a Cys-rich core protein with 3 covalently attached heparan sulfate chains and 3 N-glycosylated chains. Zhang et al. (2007) reported that glypican-1 heparan sulfate chains are required for its function to regulate turkey myogenic satellite cell differentiation and the cellular responsiveness to FGF2 during proliferation. However, the role of glypican-1 N-glycosylated chains has received no research attention with regard to these functions. The N-glycosylated chains have been reported to function in many biological processes. For example, they are involved in proper folding of proteins (Parodi, 2000; Helenius and Aebi, 2001) and localization of membrane proteins to the cell surface (Martinez-Maza et al., 2001; Yan et al., 2002). With the heparan sulfate chains attached, the glypican-1 N-glycosylated chain mutants did not affect turkey satellite cell proliferation, however, when both the heparan sulfate chains and N-glycosylated chains were deleted, turkey
myogenic satellite cell proliferation was increased (Chapter 2). These data suggest that both of the glypican-1 heparan sulfate chains and N-glycosylated chains are required for glypican-1 regulation of turkey satellite cell proliferation.

Zhang et al. (2007) reported that glypican-1 heparan sulfate chains play an important role in cellular responsiveness to FGF2. This finding is supported by the data in chapter 2 that not only the heparan sulfate chains but also N-glycosylated chains are important in regulating satellite cell responsiveness to FGF2. Furthermore, there are interactions between heparan sulfate chains and N-glycosylated chains in this process.

One possibility to explain these results is that the glypican-1 heparan sulfate chains and N-glycosylated chains interact with each other and influence the shedding of glypican-1. Compared with the cells transfected with wild-type glypican-1, the deletion of the heparan sulfate chains may decrease the binding and presentation of FGF2 to its tyrosine kinase receptors, thus the cells will have decreased responsiveness to FGF2. With the heparan sulfate chains attached to the core protein and N-glycosylated chains deleted, the core protein 3-dimensional structure may be changed, but glypican-1 can still be shed normally. In this situation, the glypican-1 core protein directly presents FGF2 to its receptor. When both the glypican-1 heparan sulfate chains and N-glycosylated chains were deleted, the 3-dimensional structure of the core protein may have been modified and inhibited the shedding of glypican-1, thus more glypican-1 core protein would remain on the cell surface. The increased glypican-1 core protein can directly bind to FGF2 and present it to its tyrosine kinase receptor (Kirkpatrick et al., 2006). As a result, the satellite cells will be more responsive to FGF2 compared with the cells transfected with
wild-type glypican-1. Another possibility is that the deletion of N-glycosylated chains changes the 3-dimensional structure of the glypican-1 core protein and changes the availability of its heparan sulfate chains and its core protein to FGF2.

At the time of differentiation, the deletion of only heparan sulfate or N-glycosylated chains decreased cell differentiation whereas the deletion of both heparan sulfate and N-glycosylated chains increased cell differentiation (Zhang et al., 2007; Chapter 2). The deletion of glypican-1 heparan sulfate chains likely reduced the amount of FGF2 sequestered by glypican-1 resulting in a decrease in differentiation compared with the cells transfected with wild-type glypican-1. With the heparan sulfate chains, the deletion of N-glycosylated chains may not influence the shedding of glypican-1; thus, there was no change in cell differentiation. Glypican-1 can be released or shed from the cell membrane through the action of phospholipases (Mythreye and Blobe, 2009). When glypicans are shed into the ECM, they can function as an inhibitor of their ligands. The shedding of glypican-1 N-glycosylated mutants without heparan sulfate chains into the ECM will decrease the binding of FGF2 to its tyrosine kinase receptor and result in an increase in cell differentiation.

In addition to FGF2 signaling, glypicans can regulate other signaling pathways, such as Wnts, hedgehogs, and bone morphogenetic proteins (Jackson et al., 1997; Lin and Perrimon, 1999; Paine-Saunders et al., 2000; Topczewski et al., 2001; Lum et al., 2003; Ohkawara et al., 2003; Song et al., 2005). Thus, glypican-1 may regulate cell signaling in a complex manner including FGF2 signal transduction.
The role of syndecan-4 in skeletal muscle growth and development

Syndecan-4 is a heparan sulfate proteoglycan that plays an essential role in muscle maintenance and regeneration (Cornelison et al., 2001, 2004; Tanaka et al., 2009). Syndecan-4 has been reported to be a marker for regenerating satellite cells (Tanaka et al., 2009). This is supported by the observation that syndecan-4 knockout mice lose their ability to regenerate damaged muscle (Cornelison et al., 2004). Syndecan-4 negative satellite cells have deficient muscle cell proliferation and differentiation (Cornelison et al., 2004). In contrast, the overexpression of syndecan-4 decreases turkey satellite cell proliferation and differentiation (Velleman et al., 2007; Zhang et al., 2008; Chapter 3). These conflicting results can be explained by the formation of focal adhesions, which is one of the important roles of syndecan-4 (see below).

Syndecan-4 has 3 heparan sulfate chains attached to the extracellular domain of the core protein. The heparan sulfate chains play important roles in interacting with extracellular matrix molecules, growth factors, chemokines, and cytokines (Esko and Selleck 2002). For example, heparan sulfate chains are required for stable binding of FGF2 to its high affinity receptor (Yayon et al., 1991; Aviezer et al., 1994). However, in turkey satellite cells, syndecan-4 heparan sulfate chains are not required for cellular responsiveness to FGF2 during proliferation (Zhang et al., 2008). The N-glycosylated chains alone and in combination with the heparan sulfate chains also have no effect on cellular responsiveness to FGF2 (Chapter 3). The deletion of only syndecan-4 heparan sulfate chains or N-glycosylated chains has no effect on turkey satellite cell proliferation and differentiation, but the deletion of both the heparan sulfate and N-glycosylated chains increased cell proliferation (Zhang et al., 2008; Chapter 3). These data indicated that
there are interactions between syndecan-4 heparan sulfate and N-linked glycosylated chains in regulating of turkey satellite cell proliferation, and both the heparan sulfate and N-glycosylated chains are not required for syndecan-4 modulated turkey myogenic satellite cell differentiation.

Volk et al. (1999) showed that the cytoplasmic domain of syndecan-4 modulates cell proliferation and migration in a human endothelial cell line. Mutations in the cytoplasmic domain of syndecan-4 lead to reduced cell migration and proliferation and the capacity to form tubes in endothelial cells (Horowitz et al., 2002). In turkey satellite cells, overexpression of the syndecan-4 mutants without the cytoplasmic domain caused increased proliferation and FGF2 responsiveness during proliferation, but did not influence cell differentiation (Chapter 4). Further studies on the function of the two amino acids (Ser\textsuperscript{178} residue in the C1 region and Tyr\textsuperscript{187} residue in the sequence of KKPIYKK in the V region of the syndecan-4 cytoplasmic domain) on turkey satellite cell proliferation and differentiation revealed that the Ser\textsuperscript{178} and Tyr\textsuperscript{187} residues in the cytoplasmic domain of syndecan-4 are critical in modulating turkey satellite cell proliferation, and Ser\textsuperscript{178} residue is responsible for syndecan-4 cytoplasmic domain regulated cellular responsivness to FGF2 (Chapter 5). Furthermore, in contrast to the conclusion in Chapter 4 that the syndecan-4 cytoplasmic domain had no effect on turkey satellite cell differentiation, Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants increased cell differentiation compared to the wild type syndecan-4. Ser\textsuperscript{178} and Tyr\textsuperscript{187} are critical for regulating turkey satellite cell differentiation. However, the mechanism of how Ser\textsuperscript{178} and Tyr\textsuperscript{187} modulate cell differentiation needs to be further investigated. The function of syndecan-4 heparan
sulfate chains, N-glycosylated chains, and the cytoplasmic domain in turkey satellite cell proliferation, differentiation, and responsiveness to FGF2 is summarized in Table 8.1.

**Signaling pathways affected by syndecan-4**

Cell to cell and cell to matrix interactions are critical to many processes including but not limited to cell growth, development, migration, and apoptosis. Cell membrane receptors play important roles in transducing signals from the matrix or other cells into the cell. Integrins are the primary cell membrane receptors and syndecan-4 is also important.

How syndecan-4 is involved in signal transduction remains a matter of active question. Syndecan-4 was hypothesized to function as a co-receptor for heparin-binding growth factors because the heparan sulfate chains attached to the extracellular domain of the core protein can bind to many growth factors including FGF2. Syndecan-4 functions as a co-receptor for FGF2 by binding FGF2 to its heparan sulfate chains, presenting FGF2 to its high affinity tyrosine kinase receptor and then initiating downstream signaling pathways (Volk et al., 1999). Heparinase-treated smooth muscle cells have decreased responsiveness to FGF2 (Chu et al., 2004). However, in turkey satellite cells, syndecan-4 heparan sulfate chains are not required for syndecan-4 regulated cellular responsiveness to FGF2 (Zhang et al., 2008), which suggested that the function of heparan sulfate chains in regulating FGF2 signaling are cell-type dependent.

In addition to the heparan sulfate-dependent signaling, the syndecan-4 core protein is also involved in signal transduction through protein to protein interactions.
Signals transduced through the heparan sulfate chains and the extracellular domain of the core protein can initiate signaling events via the cytoplasmic domain of syndecan-4. The chimera experiments by Volk et al. (1999) and Horowitz et al. (2002) suggested that the cytoplasmic domain of syndecan-4 core protein is also important in mediating FGF2 signaling. The syndecan-4 and glypican-1 chimera experiments in endothelial cells demonstrated that the cytoplasmic domain of syndecan-4 is critical in cellular responsiveness to FGF2 (Volk et al., 1999). The deletion of the cytoplasmic domain of syndecan-4 and the Ser\(^{178}\) and Tyr\(^{187}\) mutant experiments also supported the conclusion that the cytoplasmic domain of the syndecan-4 is critical in regulating cellular responsiveness to FGF2 (Chapter 4 and 5). The mechanism whereby the deletion of the syndecan-4 cytoplasmic domain increases cellular responsiveness to FGF2 may involve the Ser residue in the C1 region of the syndecan-4 cytoplasmic domain (Horowitz and Simons 1998). Horowitz and Simons (1998) reported that the Ser residue in the C1 region of syndecan-4 cytoplasmic domain can be phosphorylated by growth inhibition and dephosphorylated by the addition of FGF2. The mutation or deletion of the Ser residue in the syndecan-4 cytoplasmic domain may increase syndecan-4 binding of FGF2 and presentation of FGF2 to its high affinity tyrosine kinase receptor, thus increasing the cellular responsiveness to FGF2.

Zhang et al. (2008) and the results presented in Chapter 3 demonstrated that syndecan-4 heparan sulfate chains and N-glycosylated chains are not involved in FGF2 signaling, however, the deletion of heparan sulfate chains and N-glycosylated chains together with the cytoplasmic domain of syndecan-4 suggested that there are complex interactions between syndecan-4 heparan sulfate chains, N-glycosylated chains, and the
cytoplasmic domain. Based on the fact that N-linked glycosylated chains are involved in proper folding of proteins (Parodi 2000; Helenius and Aebi, 2001), and localization of membrane proteins to the cell surface (Martínez-Maza et al., 2001; Yan et al., 2002), the deletion of N-glycosylated chains from syndecan-4 without cytoplasmic domain mutant may change the three dimensional structure of the core protein leading to decreased binding of FGF2 to the heparan sulfate chains and the core protein causing decreased cellular responsiveness to FGF2. The deletion of heparan sulfate chains from syndecan-4 without cytoplasmic domain mutant may increase core protein binding of FGF2 by exposing more space of the core protein to FGF2. The deletion of both heparan sulfate chains and N-glycosylated chains from syndecan-4 without cytoplasmic domain mutant may change core protein three dimensional structure; however, without heparan sulfate chains attached to the core protein, there may be more available space for the core protein to bind to FGF2 or direct interactions between the heparan sulfate and N-glycosylated chains.

Another syndecan-4 signaling function is the activation of PKCα in the presence of phosphatidylinositol (4,5) bisphosphate (PIP₂) to activate the RhoA family of G proteins (Dovas et al., 2006). Syndecan-4 increased PKCα activity (cell membrane localization) in turkey satellite cells during proliferation (Chapter 6 and 7). The cytoplasmic domain of syndecan-4 is specific for interacting with PIP₂ and then forming dimers or oligomers (Oh et al., 1997; Lee et al., 1998) which have an unusual twisted clamp motif formed at either end of the V region (Lee et al., 1998; Shin et al., 2001). The binding of PIP₂ at the amino sequence KKPIYKK (Oh et al., 1998; Horowitz et al., 1999; Couchman et al., 2002) triggers the activation of PKCα (Oh et al., 1997; Horowitz and
Simons, 1998a; Oh et al., 1998; Couchman et al., 2002). Lim et al. (2003) reported that the overexpression of syndecan-4 increased the amount of PKCα localized in focal adhesions. The activity of PKCα can also be regulated by the Ser residue in the C1 region of the syndecan-4 cytoplasmic domain. The dephosphorylation of Ser will increase the affinity of the PIP2 binding to the V region of syndecan-4, and increase PKCα activity. This is supported by the experiments that the mutation of Ser to Ala results in increased PKCα activity, whereas mutation of tyrosine in the sequence of KKPIYKK leads to decreased PKCα activity (Chapter 5).

In addition to the cytoplasmic domain of syndecan-4, Chapter 6 demonstrated that both heparan sulfate chains and N-glycosylated chains are critical for syndecan-4 regulation of PKCα activity. This function is probably due to the property of heparan sulfate chains binding to other molecules and N-glycosylated chains control the conformation of the syndecan-4 core protein.

The colocalization of syndecan-4 and PKCα in focal adhesions (Oh et al., 1997b) suggests a role in cell migration, stress fiber formation, and cell adhesion. Focal adhesions form where the cells attach to the substrate. Focal adhesions contain transmembrane receptors including integrins and syndecan-4, cytoplasmic structural proteins such as talin, vinculin, α-actinin, paxillin, and tensin, and the signaling proteins including protein kinase C, focal adhesion kinase (FAK), and Src (Hynes 1992; Schwartz et al., 1995; Clark and Brugge, 1995; Burridge and Chrzanowska-Wodnicka, 1996). They act not only as anchorage points for the cells, but also function in signal transduction pathways. Integrins are involved in cell proliferation, adhesion, and apoptosis in muscle cells (Liu et al., 2008). Saoncella et al. (1999) reported that
syndecan-4 is required for integrin mediated fibronectin-induced focal adhesion formation. Syndecan-4 plays an important role in promoting focal adhesion formation (Echtermeyer et al., 1999), regulates cell migration (Echtermeyer et al., 2001), and protects cells from apoptosis (Jeong et al., 2001). The overexpression of syndecan-4 in Chinese hamster ovary K1 cells resulted in increased focal adhesion formation and decreased cell migration (Longley et al., 1999). It is possible that when syndecan-4 is overexpressed, more focal adhesions form and cell migration is decreased. Because migration is important for satellite cells to interact with each other to receive signals to promote proliferation, the increased number of focal adhesions may inhibit cell proliferation which would explain why the overexpression of syndecan-4 in turkey satellite cells decreased cell proliferation (Chapter 3).

The cytoplasmic domain of syndecan-4 plays important roles in focal adhesion formation and signal transduction (Echtermeyer et al., 1999; Saoncella et al., 1999; Woods et al., 2000; Woods and Couchman, 2001; Chapter 7). Oligomerization of syndecan-4 is important in the regulation of focal adhesion formation (Choi et al., 2005). Syndecan-4 has the tendency to form SDS-resistant dimers or oligomers (Rapraeger, 2000). Except for the transmembrane domain, syndecan-4 cytoplasmic domain and extracellular domain participate in syndecan-4 dimer formation (Carey 1997; Oh et al., 1997). Syndecan-4 is required for integrin-mediated, fibronectin-induced focal adhesion formation (Saoncella et al., 1999) by binding its heparan sulfate chains to the heparin-binding domain of fibronectin (Lyon et al., 2000; Woods et al., 2000). Overexpression of syndecan-4 induced more cell spreading and focal adhesion formation whereas the overexpression of a truncated syndecan-4 without the V region of the cytoplasmic
domain decreased cell spreading and focal adhesion formation (Longley et al., 1999). This indicates the important role of the V region of the syndecan-4 cytoplasmic domain in focal adhesion formation.

Echtermeyer et al. (1999) reported that the syndecan-4 core protein is sufficient to assemble focal adhesions and actin stress fibers. However, Lyon et al. (2000) and Woods et al. (2000) reported that syndecan-4 heparan sulfate chains are needed in integrin-mediated focal adhesion formation by binding heparan sulfate chains to the heparin-binding domain of fibronectin. This is supported by the study of LeBaron et al. (1988) that heparan sulfate-deficient syndecan-4 reduces focal adhesion formation and actin stress fiber formation. Syndecan-4 heparan sulfate chains and N-glycosylated chains may also be involved in focal adhesion formation through dimer formation. Sareneva et al. (1994) reported that the N-glycosylated chains are required for proteins to form dimers. Syndecan-4 N-glycosylated chains may influence syndecan-4 dimer formation. Syndecan-4 heparan sulfate chains have been reported to play an important role in the initial binding of syndecan-4 to focal adhesions (Woods and Couchman, 2001). It is possible that when both heparan sulfate chains and N-glycosylated chains are deleted, syndecan-4 regulated focal adhesion formation is decreased; thus, the cells may have increased migration and a higher rate of proliferation. When only the heparan sulfate chains or N-glycosylated chains are deleted, syndecan-4 still has the ability to increase focal adhesion formation. As a result, the cells will have decreased migration and proliferation (Chapter 3).

With regard to the mechanism, syndecan-4 can either support β1-integrin mediated focal adhesion formation (Saoncella et al., 1999) through enhancing FAK
activity (Guan et al., 1991; Kornberg et al., 1992) or function directly by activating PKC α (Oh et al., 1997; Lim et al., 2003; Keum et al., 2004). The interaction between β1-integrin and fibronectin induces phosphorylation of FAK, and then changes the number and size of the focal adhesions (Pirone et al., 2006; Israeli et al., 2010). The size and number of focal adhesions will influence cell motility and shape (Goffin et al., 2006). Focal adhesions can inhibit cell rounding and shedding from the substrate, decreasing cell apoptosis (Jeong et al., 2001) and modulating cell proliferation (Wozniak et al., 2004). The appropriate amount of focal adhesions may promote cell migration and then increase cell proliferation. However, too many focal adhesions may inhibit cell migration and proliferation.

The overexpression of syndecan-4 increased the cell membrane localization of β1-integrin and FAK activity, but did not affect vinculin-containing focal adhesion formation (Chapter 6 and 7). Compared to syndecan-4, the syndecan-4 heparan sulfate and N-glycosylated chain mutants, the cytoplasmic domain mutants, and the Ser<sup>178</sup> and Tyr<sup>187</sup> mutants also increased cell membrane localization of β1-integrin, but decreased FAK activity. The cell membrane localization of vinculin was also not regulated by the overexpression of syndecan-4 heparan sulfate and N-glycosylated chain mutants, the cytoplasmic domain mutants, and the Ser<sup>178</sup> and Tyr<sup>187</sup> mutants (Chapter 6 and 7). Based on these data, it appears that syndecan-4 and its covalently attached side chains affect the recruitment of β1-integrin to the cell membrane, but did not influence vinculin-containing focal adhesion formation. How syndecan-4 and integrin interact with each other is still not clear. Ligand affinity can change the function of β1-integrin. Mould and Humphries (2004) suggested that integrin activation involves multiple intermediate conformation
changes to an adhesion-competent conformation. The deletion of syndecan-4 N-glycosylated chains may change the conformation of syndecan-4 and then influence the interaction of syndecan-4 with β1-integrin directly or indirectly. However, the increased localization of β1-integrin on the cell membrane did not affect vinculin-containing focal adhesions. Only wild type syndecan-4 increased β-actin cell membrane localization, whereas the heparan sulfate and N-glycosylated chains mutants, the cytoplasmic domain mutants, and the Ser\textsuperscript{178} and Tyr\textsuperscript{187} mutants did not, which suggested that syndecan-4 side chains, and the cytoplasmic domain are essential for stress fiber formation, and the Ser\textsuperscript{178} and Tyr\textsuperscript{187} are critical in this process. Jeong et al. (2001) showed that syndecan-4 plays an important role in preventing cells from undergoing apoptosis. However, syndecan-4 had no effect on turkey satellite cell apoptosis (Chapter 6 and 7).

The results from this research demonstrated the importance of the cytoplasmic domain, the glycosaminoglycan (GAG), and the N-glycosylated chains in turkey satellite cell proliferation, differentiation, FGF2 responsiveness, PKCα activity, and focal adhesion formation. The effect of each of the mutants in the regulation of skeletal muscle growth and development is summarized in Table 8.1.
Conclusions

Glypican-1 and syndecan-4 belong to two different families of heparan sulfate proteoglycans and they differently regulate skeletal muscle growth and development. Glypican-1 is expressed at high levels during turkey satellite cell differentiation whereas syndecan-4 is predominantly expressed during proliferation (Liu et al., 2006). Glypican-1 has both the shed form and the cell membrane associated form which is attached to the cell membrane through a glycosylphosphatidylinositol structure. In contrast, syndecan-4 attaches to the cell membrane through its transmembrane core protein domain. Both glypican-1 and syndecan-4 have GAG chains and N-glycosylated chains attached to the extracellular domain of the core protein. The GAG chains of glypican-1 and syndecan-4 can interact with growth factors like FGF2 and present FGF2 to its high affinity tyrosine kinase receptor to initiate cell signaling events that regulate cell proliferation and differentiation. The shed form of glypican-1 may sequester FGF2 away from its tyrosine kinase receptor and inhibit FGF2 signal transduction. The N-glycosylated chains attached to the extracellular domain of glypican-1 and syndecan-4 may function in cell membrane localization and proper folding of the core proteins.

Based on the results from the current study, the glypican-1 heparan sulfate chains and N-glycosylated chains are important in regulating turkey satellite cell proliferation, differentiation, and cellular responsiveness to FGF2. Furthermore, there are interactions between heparan sulfate chains and N-glycosylated chains that are involved in this process. In terms of syndecan-4, the data from this study is supportive of the regulation
of skeletal muscle growth and development through the GAG chains, N-glycosylated chains, and the cytoplasmic domain especially the serine residue in the C1 region and tyrosine residue in the V region of the cytoplasmic domain through both FGF2 signaling and the PKCα pathway, and the formation of focal adhesions. The deletion of only GAG or N-glycosylated chains had no effect on satellite cell proliferation, but the deletion of both of them increased cell proliferation which indicated the interaction between syndecan-4 GAG and N-glycosylated chains.

The fact that the deletion of the syndecan-4 cytoplasmic domain increased satellite cell proliferation and the alteration of the serine residue in the C1 region and tyrosine residue in the V region had no effect on satellite cell proliferation suggested that the cytoplasmic domain of syndecan-4 is essential for cell proliferation but the serine and tyrosine residues had no effect on cell proliferation. Syndecan-4 N-glycosylated chain mutants and cytoplasmic domain mutants did not change the differentiation process of satellite cells, however, the mutation of either the serine residue in the C1 region or the tyrosine residue in the V region promoted cell differentiation. This indicated the critical role of the serine residue in the C1 region and tyrosine residue in the V region in satellite cell differentiation.

This study demonstrated for the first time that FGF2 is involved in syndecan-4 regulation of turkey satellite cell proliferation and differentiation. The deletion of the cytoplasmic domain of syndecan-4, the deletion of the cytoplasmic domain and GAG chains, and the deletion of the cytoplasmic domain, GAG, and N-glycosylated chains increased cellular responsiveness to FGF2 during proliferation. Furthermore, the mutation of the serine residue in the C1 region, the mutation of the serine residue in the
C1 region and GAG chains, and the mutation of the serine residue in the C1 region, GAG, and N-glycosylated chains also promoted cellular responsiveness to FGF2 during proliferation, demonstrating the critical role of the serine residue in the C1 region in satellite cell differentiation. Protein kinase C alpha activity was measured by both western analysis and confocal microscopy. Wild type syndecan-4 increased the activity of PKCα, suggesting that PKCα signal transduction may be involved in syndecan-4 regulated skeletal muscle growth and development. Confocal microscopy was done comparing cell membrane localization of PKCα in the syndecan-4 mutant transfected cells and non-transfected cells. Compared to the non-transfected cells, all of the syndecan-4 GAG and N-glycosylated chain mutants, the cytoplasmic domain mutants, and the serine and tyrosine mutants increased the cell membrane localization of PKCα. In western analysis, compared to the wild type syndecan-4, all of the syndecan-4 GAG and N-glycosylated chain mutants, the cytoplasmic domain mutants, and the serine and tyrosine mutants had decreased PKCα activity (cell membrane localization) which suggested an important role of the GAG and N-glycosylated chains, the cytoplasmic domain, and the serine and tyrosine residues in regulation of PKCα activity.

The phosphorylation of FAK was increased by the transfection of wild type syndecan-4, and decreased by the syndecan-4 GAG and N-glycosylated chain mutants, the cytoplasmic domain mutants and the serine and tyrosine residue mutants compared to the wild type syndecan-4. The β1-integrin cell membrane localization was increased by the transfection of wild type syndecan-4 and syndecan-4 GAG and N-glycosylated chain mutants, the cytoplasmic domain mutants and the serine and tyrosine residue mutants, which suggests that syndecan-4 plays an important role in the cell membrane recruitment
of β1-integrin and the GAG and N-glycosylated chains, the cytoplasmic domain and the serine and tyrosine residues have limited functions in the cell membrane recruitment of β1-integrin. The cell membrane localization of vinculin was not regulated by wild type syndecan-4 and syndecan-4 mutants, indicating that syndecan-4 has limited if any function in vinculin-containing focal adhesion formation. Syndecan-4 also had no function in cell apoptosis. To summarize, syndecan-4 signaling interactions addressed in this research are illustrated in Figure 8.1. Syndecan-4 regulates cell proliferation and differentiation through modulating FGF2 signal transduction, focal adhesion formation, and a PKCα pathway.
References


Table 8.1 Function of syndecan-4 different domain mutants in skeletal muscle growth and development. Effect of syndecan-4 was compared to the empty pCMS-EGFP vector, and the effects of all of the other syndecan-4 mutants were compared to the wild type syndecan-4 (S4). Syndecan-4 mutants: syndecan-4 without any N-linked glycosylated (N-glycosylated) chains (S4-N0); syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{124}$ (S4-N1); syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{139}$ (S4-N2); syndecan-4 with both N-glycosylated chains attached to the core protein without any glycosaminoglycan (GAG) chains (S4-S0); syndecan-4 without any N-glycosylated chains and GAG chains attached to the core protein (S4-S0N0); syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{124}$ without any GAG chains (S4-S0N1); syndecan-4 with only one N-glycosylated chain attached to the core protein at Asn$^{139}$ without any GAG chains (S4-S0N2); syndecan-4 without cytoplasmic domain (S4C); syndecan-4 without cytoplasmic domain and N-glycosylated chains (S4C-N0); syndecan-4 without cytoplasmic domain and GAG chains (S4C-S0); syndecan-4 without cytoplasmic domain, N-glycosylated chains, and GAG chains (S4C-S0N0); syndecan-4 Ser$^{178}$ mutant (S4SA); syndecan-4 Ser$^{178}$ mutant without N-glycosylated chains (S4SA-N0); syndecan-4 Ser$^{178}$ mutant without GAG chains (S4SA-S0); syndecan-4 Ser$^{178}$ mutant without both N-glycosylated chains and GAG chains (S4SA-S0N0); syndecan-4 Tyr$^{187}$ mutant (S4YF); syndecan-4 Tyr$^{187}$ mutant without N-glycosylated chains (S4YF-N0); syndecan-4 Tyr$^{187}$ mutant without GAG chains (S4YF-S0); and syndecan-4 Tyr$^{187}$ mutant without both N-glycosylated chains and GAG chains (S4YF-S0N0). ↓ indicates there is a decreased effect, ↑ indicates there is an increased effect, and → indicates there is no effect. a, PKCα activity measured by western analysis; and b, PKCα activity measured by confocal microscopy and the results were compared to the cells without transfection.
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Table 8.1
Figure 8.1 The interactions addressed in this study of how syndecan-4 regulates skeletal muscle growth and development. Abbreviations: protein kinase C alpha (PKCα); focal adhesion kinase (FAK); and fibroblast growth factor 2 (FGF2).
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