THE EXPRESSION OF CELL SURFACE
HEPARAN SULFATE PROTEOGLYCANS AND THEIR ROLES
IN TURKEY SKELETAL MUSCLE FORMATION

DISSERTATION

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By

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Skeletal muscle myogenesis is a series of highly organized processes including cell migration, adhesion, proliferation, and differentiation that are precisely regulated by the extrinsic environment of muscle cells. Fibroblast growth factor 2 (FGF2) is one of the key growth factors involved in the regulation of skeletal muscle myogenesis. Since FGF2 is a potent stimulator of skeletal muscle cell proliferation but an intense inhibitor of cell differentiation, changes in FGF2 signaling to muscle cells will influence cell behavior and result in differences in cell proliferation and differentiation. As the cell surface heparan sulfate proteoglycans (HSPG), syndecans and glypicans, are the low-affinity receptors of FGF2 and function to regulate the binding of FGF2 to the high-affinity fibroblast growth factor receptors (FGFR) and affect the activity of FGF2, differences in the expression of these molecules may cause alterations in cell responsiveness to FGF2 stimulation, which can lead to changes in skeletal muscle development and growth. However, the precise functional differences of syndecans and glypicans in FGF2 signaling are unknown to date. Our hypothesis is that syndecans and glypicans may play different roles in regulating the FGF2-FGFR interaction, and the relative expression of these molecules is critical for determining the cell status in proliferation and differentiation. To address how changes in the expression of cell surface HSPG syndecan-1 and glypican-1 are related to muscle growth properties, and
how the differences in the expression of syndecan-1 and glypican are related to FGF2 and FGFR 1 expression, a turkey line (F) selected for increased body weight at 16-wk posthatch and its control line (RBC2) which was unselected for any trait were used as the animal model. The *pectoralis major* muscle tissue isolated from individuals of the F and RBC2 line at different embryonic and posthatch developmental ages as well as myogenic satellite cells isolated from these two lines of turkeys were used to investigate the expression of syndecan-1, glypican, FGF2, and FGFR 1 during *in vivo* and *in vitro* myogenesis. In addition to line and developmental differences, the sex differences in syndecan-1, glypican, FGF2, and FGFR 1 expression were also measured. The results showed that in the F line satellite cells the increase in syndecan-1 expression was correlated with the increase in FGF2 expression and cell proliferation as compared to the RBC2 line cells. The expression of syndecan-1 and glypican expression was enhanced in the F line *p. major* muscle compared to the RBC2 line samples during both embryonic and posthatch development. The results from this study provide novel information about the expression of syndecan-1 and glypican as it relates to FGF2 signaling and skeletal muscle formation.
To

my husband, Xiangfei Lu

my son, Eric Lu

and

my parents
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TABLE OF CONTENTS

Abstract ............................................................................................................................................. ii

Dedication......................................................................................................................................... iv

Acknowledgements ......................................................................................................................... v

Vita ..................................................................................................................................................... vi

List of Tables ................................................................................................................................... xii

List of Figures................................................................................................................................... xiii

Chapters:

1. Literature review ............................................................................................................................... 1

   1.1 Introduction .................................................................................................................................. 1

   1.2 Skeletal muscle development and growth .................................................................................... 1

       1.2.1 Skeletal muscle myogenesis .............................................................................................. 2

       1.2.2 Molecular control of skeletal muscle development ......................................................... 6

   1.3 Extracellular matrix (ECM) ......................................................................................................... 8

       1.3.1 Collagen .............................................................................................................................. 9

       1.3.2 Elastic fibers ....................................................................................................................... 11

       1.3.3 Glycoproteins .................................................................................................................... 11

       1.3.4 Proteoglycans ................................................................................................................... 12

   1.4 Proteoglycans expressed in skeletal muscle ................................................................................. 14

       1.4.1 Versican ............................................................................................................................ 14

       1.4.2 Decorin ............................................................................................................................. 15

       1.4.3 Syndecan .......................................................................................................................... 17

       1.4.4 Glypican .......................................................................................................................... 18

       1.4.5 Perlecan .......................................................................................................................... 19

       1.4.6 Dynamic expression of proteoglycans in skeletal muscle .............................................. 20

   1.5 Purpose of the current study ....................................................................................................... 21
2. Turkey lines used in this study ....................................................................................46
   2.1 Introduction .............................................................................................................47
   2.2 Materials and methods ..........................................................................................49
      2.2.1 Animals ..............................................................................................................49
      2.2.2 Sex identification ..............................................................................................49
      2.2.3 Sample collection and measurement .....................................................................51
      2.2.4 Statistical analysis .............................................................................................51

2.3 Results ..................................................................................................................... 51
   2.3.1 Sex identification of turkey embryos .................................................................51
   2.3.2 Embryonic \textit{p. major} muscle weight ..........................................................52
   2.3.3 Posthatch \textit{p. major} muscle weight ............................................................53

2.4 Discussion..................................................................................................................53

3. Heterogeneity of growth and differentiation characteristics in male and female
   satellite cells isolated from turkey lines with different growth rates .............................62
   3.1 Introduction .............................................................................................................63
   3.2 Materials and methods ..........................................................................................64
      3.2.1 Isolation of turkey myogenic satellite cells ......................................................64
      3.2.2 Proliferation assay .............................................................................................65
      3.2.3 Differentiation assay ..........................................................................................66
      3.2.4 Myotube morphological analysis .......................................................................66
      3.2.5 Statistical analysis .............................................................................................67

3.3 Results and discussion .............................................................................................67
   3.3.1 Satellite cell proliferation ....................................................................................67
   3.3.2 Satellite cell differentiation ................................................................................68
   3.3.3 Morphological characteristics during differentiation ........................................68

4. Developmental expression of skeletal muscle heparin sulfate proteoglycans in turkeys
   with different growth rates..........................................................................................79
   4.1 Introduction .............................................................................................................80
   4.2 Materials and methods ..........................................................................................83
      4.2.1 Animal model ......................................................................................................83
      4.2.2 Sample collection and sex identification ............................................................83
      4.2.3 Cellular acetate chromatography .......................................................................84
      4.2.4 Cell culture .........................................................................................................85
      4.2.5 ELISA analysis ....................................................................................................86
      4.2.6 Statistical analysis .............................................................................................88

4.3 Results ..................................................................................................................... 88
   4.3.1 Heparan sulfate glycosaminoglycan analysis .....................................................88
   4.3.2 Heparan sulfate proteoglycan concentration .....................................................89
   4.3.3 Satellite cell heparin sulfate proteoglycan concentration ..................................90
5. Developmental regulated expression of syndecan-1 and glypican in *pectoralis major* muscle in turkeys with different growth rates .................................................................106

5.1 Introduction ........................................................................................................107

5.2 Materials and methods ......................................................................................110

5.2.1 Animal model ...............................................................................................110

5.2.2 Sample collection and sex identification ......................................................110

5.2.3 Cell culture ..................................................................................................111

5.2.4 Extraction of total RNA ...............................................................................111

5.2.5 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) .................................................................111

5.2.6 *In Situ* hybridization ...............................................................................113

5.2.7 Statistical analysis .......................................................................................116

5.3 Results ...............................................................................................................116

5.3.1 Syndecan-1 expression in turkey *p. major* muscle .....................................116

5.3.2 Syndecan-1 expression on turkey satellite cells ........................................117

5.3.3 Glypican expression in turkey *p. major* muscle .......................................118

5.3.4 Glypican expression in turkey satellite cells .............................................119

5.3.5 *In situ* hybridization of syndecan-1 and glypican expression in turkey *p. major* muscle ...........................................................119

5.4 Discussion .........................................................................................................120

6. Expression of fibroblast growth factor 2 and its receptor during skeletal muscle development from turkeys with different growth rates ........................................144

6.1 Introduction .......................................................................................................145

6.2 Materials and methods ......................................................................................148

6.2.1 Animal model ...............................................................................................148

6.2.2 Sample collection ........................................................................................148

6.2.3 Cell culture ..................................................................................................148

6.2.4 Extraction of total RNA ...............................................................................149

6.2.5 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) .................................................................149

6.2.6 FGF2 responsiveness assay ........................................................................151

6.2.7 Statistical analysis .......................................................................................152

6.3 Results ...............................................................................................................152

6.3.1 FGF2 expression in turkey *p. major* muscle .............................................152

6.3.2 FGF2 expression in turkey satellite cells ....................................................153

6.3.3 FGFR1 expression in turkey *p. major* muscle ........................................154

6.3.4 FGFR1 expression in turkey satellite cells ................................................154

6.3.5 Satellite cell responsiveness to FGF2 treatments ....................................155
6.4 Discussion ..............................................................................................................155

7. Summary ....................................................................................................................169

List of references ..........................................................................................................178
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Percent nuclei in myotubes during fusion</td>
<td>78</td>
</tr>
<tr>
<td>4.1 Means of reflective density (n = 3) of heparan sulfate glycosaminoglycan at 14, 16, 18, 20, 22, and 24 embryonic day (ED) development stages</td>
<td>105</td>
</tr>
<tr>
<td>5.1 Percent muscle fiber expression syndecan-1 mRNA in <em>pectoralis major</em> muscle at different embryonic development days (ED) and posthatch stages detected by <em>in situ</em> hybridization</td>
<td>142</td>
</tr>
<tr>
<td>5.1 Percent muscle fiber expression glypican mRNA in <em>pectoralis major</em> muscle at different embryonic development days (ED) and posthatch stages detected by <em>in situ</em> hybridization</td>
<td>143</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 A schematic illustration of the stages in the development of skeletal muscle cell</td>
<td>40</td>
</tr>
<tr>
<td>1.2 Diagram of transverse tubule system and sarcoplasmic reticulum system in a longitudinal view of skeletal muscle</td>
<td>41</td>
</tr>
<tr>
<td>1.3 Diagram of skeletal muscle showing general muscle structure and the epimysium, perimysium, and endomysium connective tissue layers</td>
<td>42</td>
</tr>
<tr>
<td>1.4 Location of satellite cells residing between the plasma membrane and basal lamina of muscle fibers</td>
<td>43</td>
</tr>
<tr>
<td>1.5 Repeating units of glycosaminoglycan structures</td>
<td>44</td>
</tr>
<tr>
<td>1.6 Schematic representation of proteoglycan structures</td>
<td>45</td>
</tr>
<tr>
<td>2.1 Polymerase chain reaction products for sex identification separated on a 1% agarose gel</td>
<td>57</td>
</tr>
<tr>
<td>2.2 <em>Pectoralis major</em> muscle weights from male and female F and RBC2 line embryos at 14 to 24 days of embryonic development</td>
<td>58</td>
</tr>
<tr>
<td>2.3 <em>Pectoralis major</em> muscle weights from F and RBC2 line embryos at 14 to 24 days of embryonic development</td>
<td>59</td>
</tr>
<tr>
<td>2.4 <em>Pectoralis major</em> muscle weights from male and female F and RBC2 line turkeys at 1 to 16wk of age</td>
<td>60</td>
</tr>
</tbody>
</table>
2.5 Pectoralis major muscle weights from F and RBC2 line turkeys at 1 to 16wk of age ..................................................................................................................................................61

3.1 Proliferation rate of (A) F-line male and female satellite cells; and (B) RBC2-line male and female satellite cells ....................................................................................................................72

3.2 Proliferation rate of (A) F-line male satellite cells compared to RBC2-line male satellite cells; and (B) F-line female satellite cells compared to RBC2-line female satellite cells ..................................................................................................................................73

3.3 Differentiation of F and RBC2-line male and female satellite cells. (A) F-line male and female satellite cells; and (B) RBC2-line male and female satellite cells ..........74

3.4 Differentiation of (A) F-line male satellite cells compared to RBC2 line male satellite cells; and (B) F-line female satellite cells compared to RBC2-line female satellite cells ..................................................................................................................................75

3.5 Myotube length during differentiation ........................................................................76

3.6 Myotube length of (A) F-line male satellite cells compared to RBC2-line male satellite cells; and (B) F-line female satellite cells compared to RBC2-line female satellite cells ..................................................................................................................................77

4.1 HSPG expression in p. major muscle from male and female F and RBC2 line embryos at 14 to 24 days of embryonic development .........................................................99

4.2 HSPG expression in p. major muscle from F and RBC2 line male and female embryos at 14 to 24 days of embryonic development .........................................................100

4.3 HSPG core protein expression in p. major muscle from male and female F and RBC2 line turkeys from 1 to 16 wk of age ..................................................................................101

4.4 HSPG expression in p. major muscle from male and female F and RBC2 line turkeys from 1 to 16 wk of age ..................................................................................102
4.5 HSPG expression during proliferation (P) and differentiation (D) of male and female F and RBC2 line satellite cells .................................................................103

4.6 HSPG expression during proliferation (P) and differentiation (D) of male and female F and RBC2 line satellite cells ........................................................................104

5.1 Reverse transcription polymerase chain reaction analysis of syndecan-1 gene expression in F and RBC2 line pectoralis major muscle at different embryonic developmental days (ED) .................................................................130

5.2 Reverse transcription polymerase chain reaction analysis of syndecan-1 gene expression in F and RBC2 line pectoralis major muscle at different posthatch stages ..........................................................................................131

5.3 Reverse transcription polymerase chain reaction analysis of syndecan-1 gene expression in F and RBC2 line satellite cells during proliferation (P) and differentiation (D) ......................................................................................132

5.4 Reverse transcription polymerase chain reaction analysis of glypican gene expression in F and RBC2 line pectoralis major muscle at different embryonic developmental days (ED) ..............................................................133

5.5 Reverse transcription polymerase chain reaction analysis of glypican gene expression in F and RBC2 line pectoralis major muscle at different posthatch stages ...........134

5.6 Reverse transcription polymerase chain reaction analysis of glypican gene expression in F and RBC2 line satellite cells during proliferation (P) and differentiation (D) .................................................................................135

5.7 In situ hybridization for syndecan-1 and glypican in pectoralis major muscle tissue from F and RBC2 line turkeys at embryonic day 14 .................................................136

5.8 In situ hybridization for syndecan-1 and glypican in pectoralis major muscle tissue from F and RBC2 line turkeys at embryonic day 18 .................................................138
5.9 In situ hybridization for syndecan-1 and glypican in pectoralis major muscle tissue from F and RBC2 line turkeys at embryonic day 24 .........................................................140

6.1 Reverse transcription polymerase chain reaction analysis of FGF2 gene expression in RBC2 and F line p. major muscle at different embryonic developmental days (ED) ........................................................................................................................................163

6.2 Reverse transcription polymerase chain reaction analysis of FGF2 gene expression in RBC2 and F line satellite cells during proliferation (P) and differentiation (D) ........................................................................................................................................164

6.3 Reverse transcription polymerase chain reaction analysis of FGFR 1 gene expression in RBC2 and F line p. major muscle at different embryonic developmental days (ED) ........................................................................................................................................165

6.4 Reverse transcription polymerase chain reaction analysis of FGFR 1 gene expression in RBC2 and F line p. major muscle at different posthatch stages ..........................166

6.5 Reverse transcription polymerase chain reaction analysis of FGFR 1 gene expression in RBC2 and F line satellite cells during proliferation (P) and differentiation (D) ........................................................................................................................................167

6.6 F and RBC2 satellite cell responsiveness to increasing concentrations of FGF2 .....168

7.1 Model of FGF2 and cell surface HSPG syndecan-1 and glypican regulation of skeletal muscle proliferation and differentiation .................................................................177
CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Skeletal muscle is one major source of food from agricultural animals. In order to enhance meat productivity and improve meat quality, knowledge about how skeletal muscle develops and how the development processes are regulated needs to be established.

Skeletal muscle myogenesis is a series of highly organized processes. The central events of these processes are the proliferation, fusion, and differentiation of muscle cells, the myoblasts. Both internal transcription factors and external regulatory factors such as growth factors and extracellular matrix are involved in the control of these events. This literature review summarizes the origination of muscle cells, and the process of myogenesis, followed by a discussion of regulators of skeletal muscle myogenesis with a focus on the extracellular matrix.

1.2 Skeletal muscle development and growth

Skeletal muscle myogenesis is a series of complex events including the proliferation of myoblasts, muscle cell migration and alignment, and fusion of myoblasts into muscle fibers. Myogenesis is under precise control of myogenic transcription factors, whose expressions and activities are regulated by the extrinsic environment of muscle cells.
1.2.1 Skeletal muscle myogenesis

Skeletal muscle cells originate from the embryonic mesoderm (Gilbert, 2000). After proliferation, the precursor muscle cells align and fuse to form myotubes, which develop into muscle fibers. Although the number of muscle fibers is fixed before or shortly after birth, a group of quiescent myogenic cells, the satellite cells, contribute to postnatal muscle growth and muscle regeneration after injury (Seale and Rudnicki, 2000).

A) Embryological origin of skeletal muscle

The mesoderm in the neurula-stage embryo can be divided into five regions (Gilbert, 2000). The central chordamesoderm forms the notochord that induces the formation of neural tube and anterior-posterior axis of the animal body. A pair of paraxial mesoderm located on either side of the chordamesoderm, which are segmented and give rise to somites. Lateral to the paraxial mesoderm are a pair of intermediate mesoderm that give rise to the urinary system and genital ducts. Following the intermediate mesoderm are a pair of lateral plate mesoderm, which develops into heart, blood vessels, blood cells, and all mesodermal components of limbs except skeletal muscle.

The somite originates from the paraxial mesoderm and contains two different regions (Gilbert, 2000). The ventral region of the somite forms sclerotome, the precursor of cartilage and bone (Huang et al., 2000). The epithelial portion of the somite contains two layers. The dorsal layer is known as dermatome, which generates fibroblasts that develop into dermis. The inner layer is myotome that forms the myoblasts, which will develop into skeletal muscle (Stockdale et al., 2000).
B) Muscle cell proliferation and differentiation

Cells that are committed to form myoblasts migrate from the myotome region of the somite and accumulate at the sites where skeletal muscle will form. These cells are known as pre-myoblasts (Pearson and Young, 1989). They are bipolar or spindle shaped mononuclear cells that do not express muscle-specific gene products. However, these pre-myoblasts are capable of cell division. At a certain point of embryogenesis, the pre-myoblasts develop into committed myoblasts. Myoblasts, like the pre-myoblasts, are spindle-shaped mononuclear cells. Unlike the pre-myoblasts, the myoblasts are capable of synthesizing muscle-specific proteins. Under appropriate conditions, the myoblasts withdraw from the cell cycle, migrate and align with adjacent myoblasts, and fuse to form long myotubes (Figure 1.1).

Myotubes are elongated cylindrical multinucleated cells that result from the fusion of mononuclear myoblasts (Boudjelida and Muntz, 1987). Initially the nuclei are located at the central region of the myotube but gradually moved to the periphery of the cell (Alberts et al., 1989). Myotubes are terminally differentiated muscle cells that are capable of producing muscle specific proteins such as actin and myosin. In myotubes, the muscle specific proteins are arranged into organized bundles known as myofibrils (Pearson and Young, 1989). Each myofibril consists of two types of filament, thick and thin filaments. The thick filament is composed of myosin whereas the thin filament is mainly made up of actin, with a small portion of tropoactin and tropomyosin. These two filaments are spatially organized into the repeating contractile units, known as sarcomeres. Myotubes keep on developing to form myofibers by expressing a complex of muscle specific proteins.

In myofibers, more myofibrils are synthesized and further organized than that in the myotubes (Pearson and Young, 1989). The myofibers have a striated appearance due to the sarcomeres contained within the myofibrils (Figure 1.2). The myofibrils occupy
most of the intracellular space and push the nuclei to a peripheral position right underneath the plasma membrane. The myofibrils are surrounded by a transverse tubule system (T system) that is perpendicular to the long axis of the cell, and by a sarcoplasmic reticulum system that is parallel to the long axis (Huxley and Taylor, 1958). These two tubular systems are involved in regulating intracellular Ca\(^{2+}\) concentration that controls myofiber contraction and relaxation.

Individual myofibers are surrounded by a fine connective tissue sheath, the endomysium, that overlays the plasma membrane and the basal lamina of the cell (Pearson and Young, 1989). Myofibers are arranged longitudinally to form bundles that are enveloped in thin connective tissue sheets, the primary perimysium. Small myofiber bundles assemble into larger secondary and tertiary myofiber bundles surrounded by secondary and tertiary perimysium, respectively. The entire muscle is enclosed by a thick layer of connective tissue named epimysium (Figure 1.3).

C) Satellite cells

Although the number of skeletal muscle fibers is fixed before or shortly after hatch, the size of the myofibers and the nuclear numbers within the cells increase significantly with animal growth (Smith, 1963). A population of quiescent myogenic stem cells, the satellite cells, is responsible for postnatal skeletal muscle growth and muscle regeneration (Seale and Rudnicki, 2000). Satellite cells were identified by Alexander Mauro (1961) as cells that are located outside the plasma membrane but within the basal lamina of muscle fibers (Figure 1.4). De Angelis et al. (1999) reported that precursors of satellite cells are readily isolated from mouse embryonic dorsal aorta, which is derived from the paraxial mesoderm. This finding suggests a possible origin of the satellite cells.
Moss and LeBlond (1971) reported that satellite cells proliferate and fuse to adjacent muscle fibers in postnatal muscle, which results in an increase of nuclei number and a relatively constant nuclear to cytoplasmic ratio in growing muscle fibers. Accumulation of nuclei is a prerequisite of muscle growth. Satellite cells are usually quiescent and function as reserved myogenic cells in adult animals. However, in certain situations such as myofiber injury, exercise, and denervation, satellite cells may be activated to proliferate and fuse with existing myofibers, or form new myofibers to replace the injured ones (Carlson and Faulkner, 1983; McGeachie, 1985; Darr and Schultz, 1987).

It is largely unknown how satellite cells are activated and how their activities are controlled. However, in vitro and in vivo studies indicate that satellite cell proliferation and differentiation are regulated by many growth factors (McFarland, 1999). Hepatocyte growth factor has been shown to stimulate quiescent satellite cells to enter the cell cycle (Allen et al., 1995; Tatsumi et al., 1998, Miller et al., 2000). Fibroblast growth factors (FGF) and platelet-derived growth factor stimulate satellite cell proliferation but inhibit cell differentiation (Allen et al., 1984; Yablonka-Reuveni et al., 1990; McFarland et al., 1993; Sheehan and Allen, 1999). Insulin-like growth factors and epidermal growth factors enhance both satellite cell proliferation and differentiation (Ham et al., 1988; Greene and Allen, 1991; McFarland et al., 1993) whereas transforming growth factor-β (TGF-β) inhibits the proliferation and differentiation of satellite cells (Greene and Allen, 1991; Yun et al., 1997). Targeted inactivation of a TGF-β family member, growth/differentiation factor 8 that is expressed specifically in developing and adult skeletal muscle, results in increased muscle mass (McPherron et al., 1997).
1.2.2 Molecular controls of skeletal muscle development

Skeletal muscle myogenesis represents a defined series of processes that are precisely regulated. Several different families of transcription factors are involved. Transcription factors are regulatory proteins that control the transcription of given genes. The signals to activate these transcription factors are from the extrinsic environment of the cells.

One transcription factor family involved in the control of skeletal muscle myogenesis is the paired-box (Pax) domain transcription factors. The Pax 3 and Pax 7 from this family have critical roles during early stages of myogenesis. The Pax 3 is expressed in the myogenic cells in the myotome and is required for myogenic cell specification (Williams and Ordahl, 1994). The Pax 3-deficient mice can not form skeletal muscle due to a lack of migration of myogenic cells from the somites (Daston et al., 1996). The Pax 3 controls the expression of a cell surface receptor, c-met, which is required for myogenic precursor cell migration (Bladt et al., 1995). The Pax 7 is required for satellite cell specification. The Pax 7-deficient mice are significantly smaller then the wild type ones and have no satellite cell-derived myoblasts (Seale et al., 2000).

Another group of transcription factors that play a central role in the activation of skeletal muscle cell differentiation is the myogenic regulatory factors (MRF). This family contains four members, MyoD, myogenin, Myf-5, and MRF4, and belongs to a basic helix-loop-helix (bHLH) superfamily that share structural homology in a basic domain followed by a helix-loop-helix (HLH) domain. These transcription factors form heterodimers with E-proteins through their HLH domain. The heterodimers are capable of binding through the basic domain to a conserved DNA sequence termed E-box (CANNTG) that is present in the enhancer region of many muscle-specific genes (Perry and Rudnicki, 2000).
The Myf-5 is the first member of the MRF activated during myogenesis. Inactivation of Myf-5 results in a delay of myotome formation and an inhibition of precursor muscle cells migration (Tajbakhsh et al., 1996). The MyoD expression is under the control of both Myf-5 and Pax 3 (Tajbakhsh et al., 1997). The Myf-5 and MyoD are functionally redundant to some degree and are required for myogenic determination (Rudnicki et al., 1992; Braun et al., 1992; Rudnicki et al., 1993). Satellite cells from the MyoD -/- mice fail to express up-regulated MRF4 mRNA during later cell differentiation stages (Cornelison et al., 2000), and the transition from proliferation to differentiation is delayed (Yablonka-Reuveni et al., 1999). Myogenin appears at the onset of myoblast differentiation and is necessary to initiate myotube formation (Venuti et al., 1995). Targeted mutations in the myogenin gene results in severe muscle deficiency and neonatal death in mice (Hasty et al., 1993). The MRF4 is expressed at the terminal stages of myogenesis. Inactivation of MRF4 gene results in apparently normal muscle (Braun and Arnold, 1995) but increasing myogenin expression (Zhang et al., 1995). Thus, MRF4 has some overlapping functions with myogenin (Zhu and Miller, 1997) and MyoD (Rawis et al., 1998).

Members of the myogenic enhancer factor 2 (MEF2) family of transcription factors also play important roles in muscle cell differentiation (Naya and Olson, 1999). The MEF2 family consists of four members: MEF2A, MEF2B, MEF2C, and MEF2D. The MEF2 transcription factors bind to a conserved A/T rich DNA sequence (CTA[A/T]₄TAG/A) existing in the enhancer-promoter regions of many muscle-specific genes. Like MRF, different MEF2 transcription factors are expressed at different stages of myogenesis. MEF2D is present in proliferating myoblasts and throughout cell differentiation stages (Breitbart et al., 1993). The MEF2A is expressed at the onset of differentiation (Gossett et al., 1989), whereas MEF2C appears at a late differentiation stage (McDermott et al., 1993).
The expression of MRF and MEF2 transcription factors is under the control of growth factors. For example, FGF2 inhibits myogenin gene expression in muscle cells during differentiation (Brunetti and Goldfine, 1990). The MyoD expression is down-regulated by myostatin, a growth factor belonging to TGF-β superfamily (Langley et al., 2002). The activity of MEF2 transcription factors is inhibited by TGF-β (De Angelis et al., 1998). The influence of FGF2 and TGF-β on myogenic transcription factor expression suggests a possible mechanism by which these growth factors block muscle cell differentiation. By affecting MRF and MEF2 transcription factor expression and in turn regulating muscle-specific gene activity, the growth factors may function by regulating cell proliferation and differentiation.

1.3 Extracellular matrix (ECM)

Myogenic transcription factors regulate the expression of muscle-specific genes. However, the signals to activate these transcription factors come from the external environment of muscle cells. Many external regulatory factors such as nerves, extracellular matrix, growth factors, and hormones are involved in the regulation of myogenesis, and their functions are not fully understood. The ECM is one of the external factors that may determine the fate of myogenic cells.

The ECM is a complex network of polysaccharides and proteins that are secreted by cells and fill the extracellular space (Scott, 1995). Once the ECM was thought of as an inactive structural scaffold with supporting protein fibers (collagens and elastic fibers) and an amorphous ground substance (proteoglycans and glycoproteins). It is now known as a precisely organized dynamic structure that is tissue specific and plays an active role in tissue formation and maintenance (Scott, 1995).

Myofibers are surrounded by three layers of connective tissue, the endomysium around single muscle fibers, the perimysium surrounding muscle bundles, and the
epimysium that encloses the whole muscle (Pearson and Young, 1989). Except for these three layers, each muscle cell has a layer of basal lamina that covers the plasma membrane. These structures all belong to the ECM of the skeletal muscle. The ECM forms a complex architecture around the muscle cells that is capable of interacting with extracellular molecules such as growth factors as well as with cell surface receptors such as integrin. Through these interactions, the ECM regulates muscle cell behaviors such as adhesion, migration, proliferation, and differentiation (Geiger et al., 2001). The ECM is a requisite for completion of skeletal muscle differentiation (Melo et al., 1996; Osses and Brandan, 2002).

1.3.1 Collagen

Collagen is the most abundant protein in animal body (Pearson and Young, 1989). It is an insoluble fibrous component of the ECM. The most important function of collagen is to provide tensile strength to maintain and support tissues. Nineteen different collagens, Type I through XIX collagens, have been identified to date. The Type I and Type III collagen are the primary ones in skeletal muscle. Type V and VI collagen associate with the Type I and III collagen. These collagens are located in the endomysium, perimysium, and epimysium of the muscle tissue, whereas Type IV collagen exists in the basal lamina (Nishimura et al., 1997). All collagens are composed of three $\alpha$-chains that twist around one other to form a right-hand triple-helix structure linked together by the interchain disulfide bonds (Brodsky and Ramshaw, 1997). The triple-helical regions on $\alpha$-chains are often interrupted by non-triple-helical regions, which contain diverse functional domains (Brown and Timpl, 1995).

Based on the differences in $\alpha$-chain composition, size, and function, collagens can be classified into three groups: fibrillar collagens (Type I, II, III, V, and X), non-fibrillar collagens (Type IV, VI, VII, VIII, and XI), and fibril-associated collagens with interrupted
triple helices (FACIT) (Type IX, XII, XIV, XVI, and XIX) (Brown and Timpl, 1995). Fibrillar collagens are the most abundant collagens. Collagens in this group share the structural characteristic of a large triple-helical region about 1,000 amino acid long with perfect Gly-X-Y repeats, where X and Y can be any amino acid but frequently X is proline and Y is hydroxyproline (Brodsky and Ramshaw, 1997). Fibrillar collagens provide tensile strength to tissues. The non-fibrillar collagens contain a large number of non-triple-helical regions. These collagens form a variety of structures such as microfibrillar meshwork, short filament, and sheet-like network. They function as structural components, interact with other ECM molecules, and are involved in regulating cell adhesion. The roles of some non-fibrillar collagens are still unclear. The FACIT collagens contain short triple-helical regions that are separated by non-triple-helical regions. This group of collagens associates with fibrillar collagens and may modify the interactions between fibrillar collagens and other ECM molecules (Olsen et al., 1995). The function of Type XIII, XV, XVII, and XVIII collagens are unknown.

Collagen is synthesized in cells as a procollagen peptide chain that contains collagen domains flanked by N-terminal and C-terminal propeptide domains (Pearson and Young, 1989). Once transported into the extracellular space, the N-terminal and C-terminal propeptides are cleaved by procollagen peptidase. Collagen molecules align as a quarter-stagger array in the extracellular space, initiate cross-linking among the microfibrils, and form collagen fibers. There are two types of cross-link, enzymatic cross-link and non-enzymatic cross-link, formed among collagen microfibrils (Reiser at al., 1992). The enzymatic cross-link is also known as the lysine-derived cross-link. Specific lysine and hydroxylysine are oxidatively deaminated by lysyl oxidase, forming lysine or hydroxylysine aldehydes. This type of cross-link is usually formed in the non-triple-helical regions of collagen. The non-enzymatic cross-link, or glucose-derived cross-link, is formed by the non-enzymatically condensation of specific lysine and
hydroxylysine residues to form hydroxylysylpyridinoline (Reiser et al., 1992). Cross-linking among collagen microfibrils stabilizes the collagen fiber structure. There is an increase of hydroxylysylpyridinoline cross-link with animal aging. This change may result in meat toughness, reduce tissue elasticity, and affect meat quality (McCormick, 1999)

1.3.2 Elastic fibers

Elastic fibers are another important insoluble fibrous component of the ECM. Elastic fibers provide elasticity and resilience to tissues, allowing tissues to rapidly restore their original states after deformation (Bailey and Light, 1989).

Elastin is the major component comprising 90% of the elastic fibers (Pasquali-Ronchetti and Baccarni-Contri, 1999). It forms the core of the elastic fiber with the microfibrils located around the periphery. Only a small proportion of microfibrils exists in matured elastic fibers, and is believed to play no role in mechanical properties of the elastic fibers.

Elastic fibers have been found in the epimysium and perimysium in skeletal muscle (Bailey and Light, 1989). Elastic fibers may increase the elasticity and residual tensile strength of the muscle and play a role in muscle contraction. They may also function in maintaining muscle integrity by holding the myofibrils together (Bailey and Light, 1989).

1.3.3 Glycoproteins

Glycoproteins constitute a small proportion of the ECM. Unlike collagen and elastic fibers, glycoproteins are soluble components of the ECM. The common characteristic of glycoproteins is that they all contain carbohydrate (glycan) components covalently linked to proteins (Alberts et al., 1989). The carbohydrate components can be
monosaccharides, disaccharides, oligosaccharides, or their derivatives. By interacting with cell surface receptors and other ECM molecules, glycoproteins have diverse biological functions such as maintaining cell shapes, mediating cell behaviors, and stabilizing ECM structure.

1.3.4 Proteoglycans

Proteoglycans (PG) are a soluble component of the ECM involved in the regulation of ECM assembly, growth factor signaling, and cell migration, adhesion, proliferation, and differentiation. Proteoglycans are macromolecules that consist of a core protein to which one or more unbranched glycosaminoglycan (GAG) side chains are covalently attached (Hardingham and Fosang, 1992). The core proteins are encoded by specific genes and the GAG chains are added to the core proteins by post-translation modifications.

The core proteins of PG are diverse in size and structure (Scott, 1988). The small core proteins are globular with only one or two attached GAG chains. However, large and very large core proteins are composed of multi-domains with up to 100 GAG chains.

Glycosaminoglycans are glycosamine containing glycans (polysaccharides). A high content of sulfate groups gives the GAG chains a strong negative charge, allowing them to interact with a large number of molecules, including water, by ionic interactions.

All GAG chains are polymers of disaccharide repeats that contain a hexosamine (glucosamine) and non-nitrogenous sugar (Hardingham and Fosang, 1992). Based on different repeating disaccharide units, the GAG chains can be divided into four types: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), and keratan sulfate (KS). The PG can then be classified into chondroitin sulfate PG (CSPG), dermatan sulfate PG (DSPG), heparan sulfate PG (HSPG), and keratan sulfate PG (KSPG), respectively.
Chondroitin sulfate contains the repeating units of a D-galactosamine (D-GalNAc) and a D-glucuronic acid (D-GlcUA) (Bhavanandan and Davidson, 1992) (Figure 1.5). Based on the position of sulfate groups on D-GalNAc (C-4 or C-6), the CS can be further classified as chondroitin-4-sulfate (chondroitin sulfate A) or chondroitin-6-sulfate (chondroitin sulfate C). Versican is the primary CSPG expressed in skeletal muscle (Carrino and Caplan, 1989).

Dermatan sulfate contains the repeating units of D-GalNAc and L-iduronic acid (L-IdUA) or D-GlcUA (Bhavanandan and Davidson, 1992) (Figure 1.5). Dermatan sulfate is also termed as chondroitin sulfate B. The small leucine-rich proteoglycans (SLRP) are most well known DSPG. Decorin, biglycan, and fibromodulin are the representatives of this family. The SLRP are widely distributed in tissue and are the key regulators of ECM assembly and cell growth (Iozzo, 1997).

Heparan sulfate is composed of repeating D-Glucosamine (D-GlcNAc) and L-iduronic acid (L-IdUA) or D-GlcUA (Bhavanandan and Davidson, 1992) (Figure 1.5). The HSPG are expressed mainly on the cell surface and in the basement membrane. The HS GAG chains are capable of binding to diverse ligands including growth factors, ECM molecules, cell surface receptors, and cell adhesion molecules. The HSPG are involved in cell behaviors such as recognition, migration, adhesion, proliferation, and differentiation that are critical for skeletal muscle development (McKeehan et al., 1998). Cell surface HSPG bind to FGF2, a strong stimulator of skeletal muscle cell proliferation and a potent inhibitor of cell differentiation, and protect FGF2 from proteolysis degradation. The HSPG act as the low affinity receptor of FGF2 that enhances the binding of FGF2 to its high-affinity tyrosine kinase receptors (Rapraeger et al., 1991, Yayon et al., 1991).

Keratan sulfate is composed of repeating D-GlcNAc and D-galactose (D-Gal) disaccharides units (Hardingham and Fosang, 1992) (Figure 1.5). In some PG such as
aggrecan, KS chains are co-located with CS chains (Greiling, 1994). No KSPG have been reported in skeletal muscle tissue to date.

1.4 Proteoglycans expressed in skeletal muscle

In skeletal muscle, CSPG, DSPG, and HSPG have been identified. The expression of these PG displays a dynamic pattern during muscle development, indicating that different PG may have unique functions, and the regulation of their expression is critical for muscle development and growth.

1.4.1 Versican

Versican, or PG-M, is a large aggregating CSPG (Schwartz et al., 1999) and the predominant CSPG found in skeletal muscle tissue (Carrino and Caplan, 1989). The versican core protein contains three domains (Zimmermann and Ruoslahti, 1989) (Figure 1.6). The N-terminal G1 domain is composed of an IgG-like motif and two tandem repeats. The C-terminal G3 domain consists of several motifs, including two epidermal growth factor (EGF)-like motifs, a carbohydrate recognition domain, and a complement-binding protein (CBP) motif. The central domain located between the G1 and G3 domains is the CS binding domain. The length of this domain varies in different versican isoforms. Four versican isoforms (V₀, V₁, V₂, and V₃) have been reported (LeBaron, 1996) with molecular mass of 370, 260, 180, and 74 KD, respectively. They are transcribed from the same gene but are derived from different alternative splicing of mRNA.

Versican associates with hyaluronan in the ECM through its G1 domain (LeBaron et al., 1992). The G1 domain reduces cell adhesion but enhances cell proliferation in vitro (Ang et al., 1999; Yang et al., 1999). The G3 domain enhances cultured fibroblast proliferation through the EGF-like motifs (Zhang et al., 1998). The G3 domain promotes
CS chains attachment and versican secretion via its CBP motif, whereas the G1 domain inhibits versican expression (Yang et al., 2000). In addition, the binding of the G3 domain to cell surface β1-integrin activates focal adhesion kinase activity, mediates cell adhesion, and protects cells from apoptosis (Wu et al., 2002). However, the function of versican in skeletal muscle still remains unclear. Inhibition of versican synthesis does not affect muscle cell differentiation (Carrino and Caplan, 1994), suggesting that versican may not involved in regulating muscle cell differentiation. Versican is the major PG synthesized in embryos at early embryonic stages (Carrino and Caplan, 1984), and its expression decreases with muscle development. When muscle is injured, versican levels are up-regulated in regenerating muscle tissue (Carrino et al., 1988). These facts indicate that versican may be involved in early myogenesis. Carrino and Caplan (1989) postulated that versican regulates the spacing of muscle fibers at early stages of myogenesis due to its ionic interaction with water.

1.4.2 Decorin

Decorin is a CS or DS PG belonging to the SLRP family (Iozzo, 1997). Decorin is expressed in skeletal muscle from different species (Young et al., 1988; Andrade and Brandan, 1991; Eggen et al., 1994; Velleman et al., 1996, 1997).

Decorin has a globular core protein of 45 KD and a total molecular weight of 100 KD (Rosenberg et al., 1985) (Figure 1.6). Decorin core protein contains three domains. The N-terminal domain has four cysteine residues that form disulfide bonds. The CS or DS GAG chain is attached to this domain. The central region of the core protein is a leucine-rich domain consisting of 10 to 14 repeat sequences. The C-terminal domain contains two cysteine residues that form a loop structure stabilized by a disulfide bond (Neame et al., 1989). In mammals, decorin contains one CS or DS GAG chain.
However, decorin with two DS chains has been reported in the chicken (Blaschke et al., 1996).

Decorin is capable of binding to fibrillar collagens through its central domain leucine-rich repeats 4 to 5 (Svensson et al., 1995). By this binding, decorin acts as a spacer during lateral assembly of collagen fibers (Weber et al., 1996). Targeted inactivation of the decorin gene in mice resulted in fragile skin and abnormal collagen fiber morphology with coarse and irregular collagen fibers. Individual collagen fibers showed abrupt increases and decreases in mass along the axes, indicating that lateral fusion of collagen fibrils becomes uncontrollable in decorin knock-out mice (Danielson et al., 1997). These results indicate that decorin may inhibit the lateral fusion of adjacent collagen fibrils and regulate collagen fibrillogenesis in vivo.

Decorin plays a role in controlling cell behaviors such as proliferation, migration, and differentiation. It inhibits cell proliferation in Chinese hamster ovary cells (Yamaguchi and Ruoslahti, 1988) and human colon cancer cells (Santra et al., 1995) by activating the EGF receptor-MAP kinase signaling pathway (Moscatello et al., 1998) and increases intracellular Ca2+ level in cells (Patel et al., 1998). Decorin-induced cell growth suppression is associated with the up-regulation of p21 mRNA and protein, an inhibitor of cyclin-dependent kinase (Luca et al., 1996; Santra et al., 1997). Decorin also binds to fibronectin, an ECM glycoprotein, via its core protein and plays a role in cell adhesion by affecting the interaction between fibronectin and cell surface integrin receptors (Schmidt et al., 1987, 1991). The GAG chain is critical for decorin to inhibit cell migration (Merle et al., 1999). The expression of decorin is up-regulated by TGF-β (Bassols and Massage, 1998). Transfection studies indicated that inhibition of decorin expression stimulates myogenin expression which is suppressed by TGF-β, and enhances cell differentiation (Riquelme et al., 2001). These results suggest that decorin inhibits
muscle cell terminal differentiation by activating a TGF-β-dependent signaling pathway to influence MRF expression.

1.4.3 Syndecan

Syndecan family includes four vertebrate members, syndecan-1 through 4 (Tumova et al., 2000). All syndecan core proteins have a diverse N-terminal ectodomain that contains several conserved amino acid sequences for GAG chain binding (Figure 1.6). Besides the ectodomain, syndecans contain a highly conserved transmembrane domain, and a short C-terminal cytoplasmic domain that contains two conserved motifs (C1 and C2) interrupted by a variable region (V). The V region is diverse among syndecans. The majority of GAG chains binding to the syndecan core proteins are HS chains, though CS chains are found in syndecan-1 and -4. The GAG chain mediates the binding of syndecans with a large number of molecules include growth factors, enzymes, ECM molecules, cell adhesion molecules, and lipid-binding proteins (Carey, 1997).

Syndecans play important roles in many cell events. Over-expression of syndecan-1 enhances cell spreading and alters cell morphology, accompanied with re-organization of the cytoskeletal structures (Carey et al., 1994). Increased syndecan-1 expression in transfected myoblasts strongly inhibits myogenin expression and myoblast differentiation (Larraín et al., 1998). Syndecan-2, along with activated β1 integrin, specifically controls the assemble of fibronectin and laminin in the ECM (Klass et al., 2000). Inhibition of syndecan-3 expression in myoblasts enhances myogenin expression and accelerates cell terminal differentiation (Fuentealba et al., 1999). Syndecan-4 mediates focal adhesion formation and regulates protein kinase C activity (Oh et al., 1997).

Expression of syndecans is affected by growth factors. An enhancer on the syndecan-1 gene is activated only by FGF (Jaakkola et al., 1997). Syndecan-1 expression
increases in response to FGF2 and PDGF but decreases in response to TGF-β. In contrast, syndecan-2 expression is up-regulated by TGF-β (Worapamorn et al., 2001).

The binding of syndecans to FGF2 has been well studied. Rapraeger et al. (1991) reported that in the absence of cell surface HSPG, FGF2 interacts poorly with its high-affinity receptor and does not activate the downstream signal transduction pathway. The syndecans form a ternary complex with FGF2 and FGF receptors, protects FGF2 from proteolysis degradation and acts as a regulator of FGF2 signaling (Park et al., 2000). The cytoplasmic domains of syndecans are also important in FGF2-dependent signal transduction. Increased syndecan cytoplasmic domain expression leads to more in cell growth and migration in response to FGF2 (Volk et al., 1999). Since FGF2 stimulates muscle cell proliferation and inhibits cell differentiation, differences in syndecan expression may cause differences in cell responsiveness to FGF2, which in turn will likely result in changes in myogenesis.

1.4.4 Glypican

Glypicans are another family of cell surface HSPG. Unlike the syndecans that have a transmembrane domain, glypicans are covalently linked to the cell surface via a glycosylphosphatidylinositol (GPI) anchor (David, 1993). Six glypicans, glypican-1 through 6, have been identified (David et al., 1990; Stipp et al., 1994; Filmus et al., 1995; Watanabe et al., 1995; Saunder et al., 1996; Veugelers et al., 1997; Paine-Saunders et al., 1999; Veugelers et al., 1999). However, only glypican-1 (glypican) has been reported in skeletal muscle (Campos et al., 1993).

The core proteins of all glypicans are similar in size (~ 60 KD) and contain Ser-Gly repeats. The heparan sulfate binding sites is contained in their C-terminal domain. A unique feature of glypican core proteins is that they all contain a highly conserved pattern of 14 cysteine residues which are involved in the formation of disulfide bonds that
maintain the core proteins in a compact tertiary structure (Herndon and Lander, 1990). Like in syndecans, the majority of the GAG chains binding to glypican core proteins are HS chains. The HS chains show a great deal of diversity on their fine structures such as the N-deacetylation of glucosamine units and the position of sulfate groups (De Cal and David, 2001; Nakato and Kimata, 2002). The diversity in the HS chain fine structure results in different affinities for FGF1 and FGF2 (Kreuger et al., 2001), suggesting a possible regulation mechanism of glypicans through the specificity of HS chain in FGF-dependent signaling.

Decreased expression of glypican-1 is associated with a significant decrease of growth-stimulatory effects of FGF2 and HGF on cells (Matsuda et al., 2001). Mutations in the glypican-3 gene (GPC3) cause abnormal embryonic and postnatal overgrowth in humans (Pilia et al., 1999). Since IGF-II receptor-deficient mice display the same developmental overgrowth as the GPS3 -/- mice, it is possible that glypican-3 negatively regulates IGF-II signaling (Wang et al., 1994; Lau et al., 1994). Like syndecans, the glypicans have been reported to interact with FGF-2 and stimulate FGF receptor-1 occupancy and signaling through their HS chains (Steinfeld et al., 1996). However, little is known about the specific functions of different glypicans, or their functional differences from syndecans during skeletal muscle development and growth.

### 1.4.5 Perlecan

Perlecan is a large HSPG and one of the major components of basal lamina (Iozzo et al., 1994). Perlecan interacts with laminin-1, a glycoprotein in the basement lamina, with its HS GAG chains. It also interacts with entactin-1/nidogen-1 and type IV collagen through the core protein. By these interactions, perlecan helps to establish the network structure of the basement lamina which is essential in maintaining the structural integrity of skeletal muscle tissue (Battaglia et al., 1992; Reinhardt et al., 1993).
Perlecan has a large core protein with a molecular weight of 400 to 467 KD in different species (Noonan et al., 1991; Murdoch et al., 1992). The core protein of perlecan contains five domains (Figure 1.6). The N-terminal Domain I contains three consecutive Ser-Gly-X-Gly sequences for the attachment of HS GAG chains. Domain II is homologous with the low-density lipoprotein receptor. Domain III is homologous with the short arm of laminin A and B chains. Domain IV is a immunoglobulin-like domain. The C-terminal Domain V is homologous with the C-terminus of laminin A chain and with epidermal growth factor. The complex of perlecan core protein structure indicates the multiple and diverse functions of the perlecan, and the possible interactions between perlecan and other molecules.

Perlecan interacts with FGF2 and promotes FGF2 receptor binding (Aviezer et al., 1994). Inhibition of perlecan expression significantly decreases FGF2-high affinity binding and blocks autocrine and paracrine activities of FGF2 (Aviezer et al., 1997). Since FGF2 enhances skeletal muscle cell proliferation and inhibits cell differentiation, the regulation of FGF2 binding by perlecan may affect muscle cell development. Mongia et al. (2001) reported that perlecan binds to a fibroblast growth factor-binding protein (FGF-BP) via its second EGF repeat of domain III that is close to the binding site for FGF. Thus, interaction with FGF-BP may replace the binding of FGF2 and release the FGF2 into the microenvironment of the cells.

1.4.6 Dynamic expression of proteoglycans in skeletal muscle

At early stages of chicken embryonic development, the large CSPG versican is the predominant PG expressed in skeletal muscle. With muscle development, the versican is gradually replaced by small CSPG, DSPG, and HSPG (Carrino and Caplan, 1984). A similar transition pattern of PG expression has been reported in mouse and turkey, respectively (Young et al., 1990; Velleman et al., 1999). In cultured muscle cells,
individual PG also displays a dynamic expression pattern during cell proliferation and differentiation. Syndecan-1, -3, and -4 expression are down-regulated during muscle cell differentiation, whereas syndecan-2 remains unchanged (Larraín et al., 1997b; Fuentealba et al., 1999). Glypican gene expression is up-regulated significantly during cell terminal differentiation (Brandan et al., 1996) whereas perlecan expression decreased (Larraín et al., 1997a). Considering all the HSPG interact with FGF2, a stimulator of muscle cell proliferation and a potent inhibitor of cell differentiation, it remains an enigma as to what the functional difference between syndecans and glypicans since their expression patterns during myogenesis are different. Brandan and Larraín (1998) suggested that syndecans may function as a presenter of FGF2 during cell proliferation whereas glypicans may function as a sequester of FGF2 to attenuate the inhibitory effect of FGF2 during cell differentiation. However, there is no data about different HSPG expression in skeletal muscle tissue as it relates to muscle development and growth. The impact of syndecans and glypican expression on muscle tissue growth remains unclear. It is unknown if \textit{in vivo} syndecan expression is higher at early embryonic stages corresponding to myoblast proliferation, and glypican expression increases later with cell differentiation as has been shown \textit{in vitro}. It is also unknown if syndecan and glypican expression is different in animals with different growth rates. If changes in HSPG expression do exist, they may affect muscle cell responsiveness to FGF2 signaling, and have influences on cell proliferation and differentiation \textit{in vivo}, which would be reflected in differences in muscle growth rates.

\section*{1.5 Purpose of the current study}

Cell surface HSPG are co-receptors for FGF2 and have been shown to modulate the FGF2 signaling (Yayon et al., 1991). Since FGF2 stimulates skeletal muscle cell proliferation and inhibits cell differentiation, differences in cell surface HSPG expression
may directly affect cellular responses to FGF2, resulting in differences in muscle growth rates due to the changes in cell proliferation and differentiation. However, no data has been reported relating HSPG expression in skeletal muscle to muscle growth rate.

In order to address how cell surface HSPG expression is related to skeletal muscle development and growth, a turkey line (F) selected only for increased body weight at 16-wk posthatch (Nestor, 1984) and its unselected randombred control line (RBC2) were used in this study. The F line turkeys have a heavier body weight and *pectoralis major* muscle weight compared to the RBC2 line turkeys (Nestor et al., 1987; Lilburn and Nestor, 1991). Therefore, the F line represents a faster muscle growth model and the RBC2 line serves as the genetic control. The expression of cell surface HSPG syndecan-1 and glypican, and the expression of FGF2 and FGF receptors in the F and RBC2 line turkey *p. major* muscle tissue as well as in the satellite cells derived from these two lines were examined in the present study.

In addition to investigating the influence of growth difference on cell surface HSPG expression, the influence of sex differences on HSPG expression and FGF2 signaling were also examined. Differences in body weight and muscle mass of male and female chicken and turkey embryos have been reported (Burke and Sharp, 1989; Burke, 1994). The mechanism underlying these differences is yet unclear. Gonadal steroid hormone may be indirectly involved in the control of muscle growth through regulating the expression of growth factors such as TGF-β and FGF2 that influence muscle cell proliferation and differentiation. The results from the present study will provide novel information about HSPG expression, especially syndecan-1 and glypican expression, in regulating muscle development and growth. These data will improve the understanding of the roles that syndecan-1 and glypican play in FGF2 signaling during *in vivo* myogenesis and muscle growth.
References:


Figure 1.1 A schematic illustration of the stages in the development of skeletal muscle cell. (Adapted from Muntz, 1990)
Figure 1.2 Diagram of transverse tubule system and sarcoplasmic reticulum system in a longitudinal view of skeletal muscle. (Adapted from Pearson and Young, 1989)
Figure 1.3 Diagram of skeletal muscle showing general muscle structure and the epimysium, perimysium, and endomysium connective tissue layers. (Adapted from Bailey and Light, 1989)
Figure 1.4 Location of satellite cells residing between the plasma membrane and basal lamina of muscle fibers. (Adapted from McFarland, 1999)
Figure 1.5 Repeating units of glycosaminoglycan structures. (Adapted from Bhavanandan and Davidson, 1992)
Figure 1.6 Schematic representation of proteoglycan structures. (Adapted from Ruoslahti et al., 1992)
CHAPTER 2

TURKEY LINES USED IN THIS STUDY

Abstract

A turkey line, F, selected for increased 16-wk body weight and its genetic control line, RBC2, which is unselected and maintained with no change over generations were used to investigate the differential expression of proteoglycans (PG) involved in the regulation of skeletal muscle development and growth. *Pectoralis major* muscle was isolated from F and RBC2 line embryos and posthatch turkeys at different developmental stages and weighed. The F line of both sexes had significantly heavier muscle weights during embryonic and posthatch stages compared to the RBC2 line. In the F line male embryos had heavier *p. major* muscle than females beginning at embryonic day 16, but no differences were found between sexes in the RBC2 line until embryonic day 20. The *p. major* muscle weights of the females in both lines increased at a faster rate than the males during posthatch development and there was no sex difference by 16 wk. The results indicated that the muscle growth potentials in the F and RBC2 line turkeys of both sexes were different and these two lines of turkeys can be used as a model to investigate the influence of muscle growth and sex on PG expression.
2.1 Introduction

Muscle cell proliferation, migration, adhesion, fusion to form multinucleated myotubes that further differentiate into mature muscle fibers are central events in skeletal muscle myogenesis (Swartz et al., 1994). The extracellular matrix of the muscle cells is largely involved in the control of these processes (Dodson et al., 1996), but the mechanism is not completely understood. In order to understand how proteoglycans (PG) in the extracellular matrix are involved in the regulation of skeletal muscle development and growth, an animal model with the same genetic background but different muscle growth properties is required for the comparison of differences in PG expression in regard to muscle growth.

Nestor (1984) developed a fast growing line of turkeys (F) through long-term selection only for increased body weight at 16-wk posthatch. This line of turkeys is derived from a randombred control line, RBC2. The RBC2 line has not been selected for any trait and is maintained in a condition that little genetic change is expected or observed (Nestor, 1977a, b). Thus, the RBC2 line turkeys can be used as an unselected control for the F line. A previous study by Nestor et al. (1987) demonstrated that the F line turkeys had significantly increased amounts of thigh, drumstick, and breast muscle compared to the RBC2 turkeys at 8, 16, and 20-wk posthatch. Another study by Lilburn and Nestor (1991) showed that the pectoralis major muscle weight in the F line was significantly higher with an average of 0.882 g at hatch compared to 0.652 g in the RBC2 line, and the differences continued at 4, 8, 12, and 16-wk posthatch. Since the F line is derived from the RBC2 line, genetic background variations between the F and RBC2 lines that may cause differences in muscle growth can be excluded. Differences in
skeletal muscle growth can be attributed to changes in expression of genes involved in the control of muscle development.

Embryonic muscle development, hyperplasia, is mainly due to the proliferation and differentiation of myoblasts to form myotubes and muscle fibers. The number of muscle fibers is fixed before hatch and does not increase during posthatch stages (Smith, 1963). The increase in skeletal muscle weight during posthatch growth is the result of the activation of quiescent myogenic satellite cells (Moss and LeBlond, 1971). Satellite cells are located between the plasma membrane and basal lamina (Mauro, 1961). The activated satellite cells proliferate and fuse with the existing muscle fibers and increase the DNA content in muscle fibers. Higher DNA content results in increased protein synthesis potential in the muscle fibers that leads to muscle growth through hypertrophy (Yablonka-Reuveni, 1995).

Summers and Medrano (1997) reported that the fusion of myoblasts was delayed in high-growth mice, which may cause an increase in the myoblast population and result in more hyperplasia. In Japanese quail selected for rapid growth, larger *p. major* muscle during posthatch development was primarily related increased total nuclei number in muscle fibers (Fowler et al., 1980). It is possible that the faster muscle growth in the F line turkeys is the result of both hyperplasia and hypertrophy. However, data about the F line muscle growth is from studies on posthatch turkeys and no data about embryonic muscle development has been published. In order to understand how PG are involved in the regulation of myogenesis, it is necessary to relate PG expression to both embryonic muscle development and posthatch muscle growth. In this study, the *p. major* muscle
from the F and RBC2 line embryos and growing turkeys at different ages were isolated and the muscle was weighed.

In addition to the differences in muscle growth in different turkey lines, differences in body weight and muscle mass of male and female chicken and turkey embryos have been reported (Burke and Sharp, 1989; Burke, 1994). In turkeys, this difference starts as early as embryonic day 12 day (Burke, 1994). Currently, it is unknown what mechanism causes this sex difference in muscle growth. In order to relate PG expression to male and female muscle growth, turkey \textit{p. major} muscle of both sexes were isolated at different embryonic and posthatch ages and the muscle was weighed.

2.2 Materials and methods

2.2.1 Animals

The F and RBC2 line turkeys (Nestor, 1984) used in this study were from flocks maintained at the Ohio Agricultural Research and Development Center.

2.2.2 Sex identification

Genomic DNA used as the template for polymerase chain reaction (PCR) to identify the sex of turkey embryos was isolated from blood samples at embryonic day 14, 16, 18, 20, 22, and 24. Fifty microliters of blood was collected from each embryo by using a heparinized micro-hematocrit capillary tube (Fisher Scientific, Pittsburg, PA). The blood was diluted in 1 ml of phosphate buffered saline (PBS, 170 mM NaCl, 3 mM KCl, 10 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$, pH 7.4). Six hundred microliters of diluted blood was then mixed with 800 µl of lysis buffer (10 mM Tris, pH 7.5, 5 mM MgCl$_2$, 320
mM sucrose, and 1% Triton X-100), vortexed, and centrifuged at 10,000 × g for 3 min at 4 °C. After removing the supernatant, the pellet was resuspended in 1 ml of TEN buffer (10 mM Tris, pH 8.0, 2 mM EDTA, and 400 mM NaCl) containing 14 µl of proteinase K (10 mg/ml) and 50 µl of 10% SDS, and incubated at 37 °C for 16 h with gentle agitation. The sample was extracted twice with equal volume of phenol/chloroform/isoamyl (25:24:1, pH 6.7) (Fisher Scientific, Pittsburg, PA) and centrifuged at 4 °C for 10 min. The supernatant was then precipitated by two volumes of 95% ethanol and 1/10 volume of 3 M NaAc (pH 5.2) for 2 h at -20 °C. The sample was centrifuged at 4 °C for 15 min and the supernatant was removed. The DNA pellet was washed two times in 1 ml of 70% ethanol and two times in 1 ml of 95% ethanol, and air-dried for 20 min at room temperature. The DNA pellet was then resuspended in 100 µl of TE (10 mM Tris, 0.2 mM EDTA, pH 8.0).

Sex identification of the turkey embryos was performed using a PCR method adapted from D’Costa and Petitte (1998). The primer pair amplified a 177 bp PSTI sequence in the female W chromosome with the forward primer: 5’-CAGGAAATGCCAGTTTTATCG-3’ and the reverse primer: 5’-ATGTTTTGGGGGCAAAAAATCC-3’. A second primer pair amplified a 250 bp turkey adenosine triphosphate (ATP) synthase DNA fragment with the forward primer: 5’-TTGGAGGAGGCTCTCTGACTGCTTTGCCCG-3’ and the reverse primer: 5’-CCAACGTGGATGGCTGGACGGATACCTTTG-3’. Both the primer sets were commercially synthesized by Operon (Alameda, CA) and used in a multiplex PCR reaction according to D’Costa and Petitte (1998). After heating the PCR components at 95 °C for 5 min, the PCR was performed as 2 cycles of 95 °C for 5 min, 52 °C for 1 min,
and 72 °C for 3 min, and then followed by 30 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 3 min, and a final extension at 72 °C for 10 min. The PCR products were separated on a 1% agarose gel containing ethidium bromide and visualized under an UV light. The females were identified by the presence of a 177 bp \( PstI \) W chromosome repeat. The sex of the posthatch birds was identified by dissection.

2.2.3 Sample collection and measurement

For each set of samples, the \( p. major \) muscle tissue was isolated with all visible skin and fat removed from 5 males and 5 females from the F and RBC2 lines at 14, 16, 18, 20, 22, and 24 day of embryonic development, and at 1, 8, 12, and 16 wk posthatch. The muscle tissue was weighed. Three sets of samples were collected. The average weights of \( p. major \) muscle according to sex and line at each developmental age were calculated.

2.2.4 Statistical analysis

Statistical analysis was performed on the data by a student’s \( t \)-test using the general linear model procedure of SAS (1985). The effect of sex and line was measured. Differences were considered significant at \( P < 0.05 \).

2.3 Results

2.3.1 Sex identification of turkey embryos

The sex of avian embryo was determined by the presence of the \( W \) sex chromosome. Unlike mammals, the male bird has homogametic sex chromosomes (ZZ)
and the female bird has heterogametic sex chromosomes (ZW). Thus, the W chromosome is female specific. The W chromosome contains a repetitive \textit{Pst}I DNA fragment that is 0.4 kb long and contains about 10,000 repeat sequences. A 177 bp DNA sequence of this \textit{Pst}I unit was amplified in all female samples and was undetectable in all male samples (Figure 2.1). The ATP synthase gene is located on autosome and exists in both male and female birds. This gene was amplified as a control for the PCR reaction. A 250-bp turkey ATP synthase DNA fragment was amplified in the same PCR reactions with the \textit{Pst}I sequence, and was found in both the male and female samples (Figure 2.1). To confirm the PCR product, the amplified 177 bp \textit{Pst}I fragment was sequenced and had 100\% homology to turkey \textit{Pst}I sequence (Genebank #X17583). To further confirm the accuracy of the PCR method, blood samples were taken from 8, 11, and 12 embryos at 16, 18, and 20 day of embryonic development, respectively. The sex of those embryos was identified by the PCR method, and the embryos incubated until hatch. At 2 days of age, the sex of birds was confirmed by dissecting. There was a 100\% match of the sex based on the PCR method and the visual determination.

2.3.2 Embryonic \textit{p. major} muscle weight

The F line male embryos had significantly heavier \textit{p. major} muscle weights than those of the RBC2 line males at all ages during embryonic development (Figure 2.2). The muscle weights of the F line females embryos were also higher then those of the RBC2 line females except at day 14 and 22 (Figure 2.2). Comparison of the \textit{p. major} muscle weights within the F and RBC2 lines showed no differences between male and female embryos in the RBC2 line until day 20 (Figure 2.3). However, the F line male
embryos had heavier *p. major* muscle than the F line females as early as day 16 (Figure 2.3).

### 2.3.3 Posthatch *p. major* muscle weight

The *p. major* muscle weights in the posthatch birds differed significantly between the F and RBC2 lines (*P* < 0.05). At all developmental stages, the F line males had heavier muscle weight than the RBC2 line males (Figure 2.4). The F line females also had heavier muscle weight compared to the RBC2 line females (Figure 2.4). However, no significant differences were found between the RBC2 line males and females during the posthatch stages except at 12 wk (Figure 2.5). In the F line, no significant difference was found between the males and females at most of the posthatch stages except at 8 wk (Figure 2.5).

### 2.4 Discussion

The results from this study indicated that the F line turkeys had heavier *p. major* muscle weights compared to the RBC2 line birds at both embryonic and posthatch stages. This is in agreement with the study by Nestor et al. (1987) showing that the F line turkeys had heavier body weights as well as heavier muscle weights than the RBC2 line turkeys at 8, 16, and 20 wk posthatch. Another study by Lilburn and Nestor (1991) also indicated that the F line turkeys had heavier *p. major* muscle weights than the RBC2 line birds from hatch to 16-wk posthatch. In addition, data from the present study demonstrated that the differences in muscle weight between the F and RBC2 lines started from early
embryonic developmental stages and these differences continued during posthatch growth.

Muscle myogenesis during embryonic development is attributed to both proliferation and differentiation of myoblasts that form multinucleated myotubes, which eventually develop into mature muscle fibers (Muntz, 1990). However, posthatch muscle growth is mainly dependent upon the activation and fusion of quiescent satellite cells with the existing muscle fibers (Moss and LeBlond, 1971). Since the F line turkeys are selected only for increased body weight, the selection may cause changes in the expression of genes, including extracellular matrix PG genes, that regulates myogenesis and result in the differences in muscle development and growth. The F and RBC2 line turkeys provide an excellent model to investigate the relation between PG expression changes and muscle growth potentials.

Differences in body weight and muscle mass related to sex have been previously reported in chicken and turkey embryos (Burke and Sharp, 1989; Burke, 1994). Data from the current study confirmed these reports in that the male embryos had heavier skeletal muscle weights than the females at embryonic stages in both the F and RBC2 lines. Interestingly, muscle of females grow faster than those of males during posthatch growth in both lines, indicating that there may be sex-related regulation differences in skeletal muscle development and growth. The F and RBC2 line of turkeys provide a model to study the influence of sex on PG expression in regard to skeletal muscle myogenesis.
References


Figure 2.1 Polymerase chain reaction products for sex identification separated on a 1% agarose gel. The template genomic DNA for PCR analysis was isolated from whole blood samples collected from 16-day old F line embryos. A 250 bp fragment of turkey ATP synthase gene was amplified in both male and female samples. A 177 bp female-specific W chromosome PstI sequence was used to identify female embryos. Lane 1) 50 bp DNA ladder; Lane 2) genomic DNA; Lane 3 and 7) female samples; Lane 4 to 6) male samples.
Figure 2.2 *Pectoralis major* muscle weights from male and female F and RBC2 line embryos at 14 to 24 days of embryonic development. A) male embryos from the RBC2 and F lines; B) female embryos from the RBC2 and F lines. Bars represent the standard error of the mean. Asterisks indicate a significant difference (*P* < 0.05).
Figure 2.3 *Pectoralis major* muscle weights from F and RBC2 line embryos at 14 to 24 days of embryonic development. A) male and female embryos from the RBC2 line; B) male and female embryos from the F lines. Bars represent the standard error of the mean. Asterisks indicate a significant difference ($P < 0.05$).
Figure 2.4 *Pectoralis major* muscle weights from male and female F and RBC2 line turkeys at 1 to 16wk of age. A) male turkeys from the RBC2 and F lines; B) female turkeys from the RBC2 and F lines. Bars represent the standard error of the mean. Asterisks indicate a significant difference ($P < 0.05$).
Figure 2.5 *Pectoralis major* muscle weights from F and RBC2 line turkeys at 1 to 16wk of age. A) male and female turkeys from the RBC2 line; B) male and female turkeys from the F lines. Bars represent the standard error of the mean. Asterisks indicate a significant difference (*P* < 0.05).
CHAPTER 3

HETEROGENEITY IN GROWTH AND DIFFERENTIATION

CHARACTERISTICS IN MALE AND FEMALE SATELLITE CELLS

ISOLATED FROM TURKEY LINES WITH DIFFERENT GROWTH RATES*

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Reformatted for dissertation.

Abstract

The effects of growth- and gender-related differences on satellite cell proliferation and differentiation were investigated using satellite cells isolated from the *pectoralis major* muscle of a turkey line selected for increased 16-week body weight (F-line) and its unselected randombred control (RBC2-line). Proliferation rates within the F- and RBC2-lines did not differ between sexes. The F-line male and female satellite cells when compared to the RBC2-line male and female satellite cells proliferated at a faster rate. Differentiation rates were increased for the F-line male cells compared to both the F-line female and RBC2-line male satellite cells. No difference in differentiation rate was noted within the RBC2-line satellite cells. For satellite cells from females, the RBC2-line differentiated faster than the F-line. Morphological data on myotube length and the number of nuclei per myotube supported the differentiation data in that F-line male
satellite cells had the longest myotubes with the most nuclei, there was no significant
difference between myotubes within the RBC2-line, and female-derived myotubes from
the RBC2-line were longer than those of the F-line by 96 h of fusion. These data are
suggestive of both growth- and gender- related differences in satellite cell proliferation
and differentiation.

3.1 Introduction

Embryonic development of skeletal muscle occurs as a result of proliferation and
differentiation of myoblasts that fuse to form multinucleated myotubes. These myotubes
ultimately differentiate into mature muscle fibers producing muscle specific contractile
proteins. However, prior to hatching and during postnatal development, continued
skeletal muscle growth is largely dependent upon the proliferation and differentiation of
myogenic satellite cells. Satellite cells are located between the basement membrane and
the plasmalemma of skeletal muscle fibers (Mauro, 1961) and donate their nuclei to
adjacent muscle fibers (Stockdale and Holtzer, 1961; Moss and LeBlond, 1971; Allen et
al., 1979) leading to muscle growth. To date, little research has been done comparing
myogenic satellite cell proliferation and differentiation in animals with different postnatal
growth rates. Nestor (Nestor, 1977) developed a line of turkeys (F) selected for increased
16-week body weight from a randombred control population (RBC2). During the first
nine generations of selection, 16-week body weight of the F-line increased 2.36 kg in
males and 1.73 kg in females. The realized heritability for 16-week body weight in the F-
line was 0.29±0.02 for males and 0.24±0.01 for females with an average of 0.26±0.02
(Nestor, 1984). The F-line pectoral muscle, based on the percent of total body weight,
begins to show a significant increase from that of the RBC2-line at 16-week posthatch. Therefore, comparing pectoral muscle satellite cell proliferation and differentiation properties in the F-line to its genetic control, the RBC2-line, will provide new information regarding the role of satellite cell proliferation and differentiation in postnatal muscle growth properties.

### 3.2 Materials and methods

#### 3.2.1 Isolation of turkey myogenic satellite cells

*Pectoralis major* muscles were removed from 7-week-old males and females from the two turkey lines. Visible connective tissue and blood vessels were removed, the muscle was ground using sterile conditions, and then treated with pronase (0.8 mg/ml) for 1 h at 37°C to liberate satellite cells from the muscle fibers. Following the incubation, the samples were centrifuged at 1500 × g for 6 min at room temperature and the pellet was retained. The pellet was resuspended in phosphate buffered saline (PBS) and centrifuged for 10 min at 400 × g. Following centrifugation, the supernatant was decanted and retained. The collected supernatants were centrifuged at 1800 × g for 10 min and the pellet was retained. The pellets were resuspended in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies Inc., Grand Island, NY) containing 10% horse serum (HS) (Life Technologies Inc., Grand Island, NY). The cell suspension was filtered with a 500 mm Nitex cloth. Aliquots of 15 ml of the pooled material were plated into 15 cm diameter cell culture dishes with an additional 15 ml of DMEM containing 10% HS added to each dish. These preplates were placed into a 95% air/5% CO2 incubator at 38.5°C for 2 h. The contents of each preplate was decanted into a 50 ml centrifuge tube
and centrifuged at 1500 \( \times \) g for 10 min. The supernatant was discarded and the cell pellets were resuspended in DMEM containing 20% HS. The samples were filtered through a 28 mm Nitex cloth and frozen in medium containing 9.1% dimethyl sulfoxide in liquid nitrogen.

### 3.2.2 Proliferation assay

Turkey satellite cells from males and females of the F- and RBC2-lines were grown in 16 mm gelatin coated plates (Corning Costar, Corning, NY) at a density of 15 000 cells per well. The cells were initially plated for 24 h in DMEM containing 10% HS and changed to McCoy’s 5A medium (Life Technologies Inc., Grand Island, NY) containing 10% chicken serum (CS) (Sigma Chemical Co., St. Louis, MO), 5% HS, 1% antibiotic:antimycotic (Life Technologies Inc., Grand Island, NY), and 0.1% gentamicin (Life Technologies Inc., Grand Island, NY) (feeding medium). The feeding medium was changed every 24 h for 120 h. There were five well replicates for each satellite cell line at each sampling time. At 24 h intervals, the feeding medium was removed from one plate and rinsed with sterile PBS, air dried, and stored at -70°C until use. Proliferation was assessed by the DNA content of the wells. DNA was analyzed by using Hoechst 33258 fluorochrome (Sigma, Chemical Co., St. Louis, MO) by the method of McFarland et al. (1995) adapted from the fluorometric procedure described by Rago et al. (1990) using double-stranded calf thymus DNA as the standard.
3.2.3 Differentiation assay

Turkey male and female satellite cells from the F- and RBC2-lines were cultured in 6.4 mm diameter gelatin coated plates at a density of 1,700 cells per well. The cells were initially plated for 24 h in DMEM and changed to McCoy’s 5A feeding medium until the cells reached 60% confluency. Differentiation was induced by using medium containing DMEM, 3% HS, 0.01 mg/ml porcine gelatin (Sigma, St. Louis, MO), 1.0 mg/ml bovine serum albumin (BSA). Differentiation was determined by measuring muscle specific creatine kinase protein levels by the procedure of Florini (Florini, 1989).

3.2.4 Myotube morphological analysis

Turkey male and female satellite cells from the F- and RBC2-lines were grown in 16 mm gelatin coated plates at a density of 15,000 cells per well. The cells were initially plated for 24 h in DMEM containing 10% CS and 5% HS and changed to McCoy’s 5A feeding medium until 60% confluency. Differentiation was induced by using medium containing DMEM, 3% HS, 0.01 mg/ml porcine gelatin, and 1.0 mg/ml BSA. At 24 h intervals during the differentiation process, the fusion medium was removed from one plate and the wells rinsed 2X with cold PBS. The cells were then fixed with 1 ml/well of 3% paraformaldehyde for 30 min at room temperature. After fixation, the cells were rinsed 2X with PBS. The myotube nuclei were stained with 1 mg/ml 4,6-diamidino-2-phenylindole (DAPI) for 2 h at 4°C. The sample wells were rinsed 2X with PBS and each well was covered with a 60:40 combination of glycerol: PBS. The stained myotubes were observed with an Olympus IX 70 microscope (Melville, KY) and the number of nuclei were determined in \( n = 25 \) myotubes for each sample. Using an Olympus IX 70
microscope attached to a Vay Tec image deconvolution system (Fairfield, IA) equipped with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD), the length of \( n = 20 \) myotubes was determined at each sampling time for each myoblast cell line.

3.2.5 Statistical analysis

Statistical analysis of the proliferation, differentiation, and morphology data were performed at each sampling time by a student’s \( t \)-test using the general linear model procedure of SAS (1985). The effect of line and sex was measured. Stated differences were significant at the 0.05 level of probability.

3.3 Results and discussion

3.3.1 Satellite cell proliferation

The F- and RBC2- male and female satellite cells were cultured and assayed for DNA accretion as an indication of proliferation. Comparison of the F-line male and female satellite cells showed no significant difference within the line. Similarly, the RBC2 male and female satellite cells did not proliferate at different rates, except at 120 h of culture, at which time the male cells proliferated at a faster rate (Figure 3.1). However, comparison of the F and RBC2 male satellite cells demonstrated that the growth-selected F-line cells proliferated at a faster rate than the RBC2-line. This trend was also observed in the F- and RBC2- line female satellite cells (Figure 3.2).
3.3.2 Satellite cell differentiation

Comparison of male and female F-line differentiation showed by 48 h that the fusion rate for the male satellite cells was faster than the female satellite cells (Figure 3.3). This increased rate of differentiation continued for the duration of the 96 h. In contrast, no difference in differentiation rate was observed between males and females of the RBC2-line (Fig. 3.3). The F-line male satellite cells compared to the RBC2-line male satellite cells differentiated at a faster rate beginning at 72 h of fusion whereas the female RBC2-line satellites differentiated faster than the female F-line satellite cells (Figure 3.4). The decline in creatine kinase levels at 96 h in all cultures is likely due to detachment and loss of myotubes from the substratum. It is not unexpected to detect differentiation differences based on gender as sex-linked differences in the actual weight of the breast muscle in the F- and RBC2-lines have been reported (Nestor et al., 1988).

3.3.3 Morphological characteristics during differentiation

The length of the myotubes and number of nuclei per myotube were measured throughout differentiation. The F-line male satellite cells produced longer myotubes than the female F-line satellite cells and no significant difference was observed within the RBC2-line (Figure 3.5). Comparison of male myotube length indicated that the F-line satellite cells developed longer myotubes whereas the RBC2-line female satellite cell myotube length was increased over that observed for the F-line female satellite cells (Figure 3.6). With differentiation, the number of nuclei per myotube increased in all satellite cell lines examined in this study (Table 3.1). By 96 h of differentiation, the F-line male myotubes had the highest percent of nuclei with 44% of the myotubes with seven or
more nuclei. Both the RBC2-line male and female myotubes by 96 h of differentiation had 28% of their myotubes with seven or more nuclei. Only 16% of the F-line female myotubes had seven or more nuclei by 96 h of differentiation. The results from this study demonstrate both line and sex differences in turkey satellite cell proliferation and differentiation. The results of this investigation are not unique in reporting differences in satellite cell proliferation and differentiation. McFarland et al. (1993) demonstrated heterogeneity in the differentiation characteristics of satellite cells isolated from an unselected line of turkeys (Merriam’s) compared to commercial varieties selected for growth rate. However, it is still an enigma as to why satellite cells isolated from animals with different growth rates exhibit different proliferation and differentiation characteristics. In consideration of possible mechanisms influencing satellite cell growth and differentiation, the extracellular environment needs to be considered. The proteoglycan component of the extracellular matrix plays a key role in regulating the cellular response to growth factors. For example, cell surface heparan sulfate proteoglycans are one group of proteoglycans that may be extremely important for skeletal muscle growth due to their regulation of basic fibroblast growth factor binding to its high affinity cell receptor (Yayon et al., 1991; Steinfeld et al., 1995). Basic fibroblast growth factor is a potent stimulator of skeletal muscle cell proliferation and an inhibitor of differentiation (Clegg et al., 1987). Future research will address the influence of the extracellular environment including the role of heparan sulfate proteoglycans on both proliferation and differentiation in male and female F- and RBC2-line satellite cells.
References


Figure 3.1 Proliferation rates of (A) F-line male and female satellite cells; and (B) RBC2-line male and female satellite cells. Asterisks indicate when proliferation levels were significantly different between cell lines ($P<0.05$).
Figure 3.2 Proliferation rates of (A) F-line male satellite cells compared to RBC2-line male satellite cells; and (B) F-line female satellite cells compared to RBC2-line female satellite cells. Asterisks indicate when proliferation levels were significantly different between cell lines ($P<0.05$).
Figure 3.3 Differentiation of F and RBC2-line male and female satellite cells. (A) F-line male and female satellite cells; and (B) RBC2-line male and female satellite cells. Asterisks indicate when differentiation was significantly different between cell lines ($P<0.05$).

![Graph A](image1)

**A)**

![Graph B](image2)

**B)**
Figure 3.4 Differentiation of (A) F-line male satellite cells compared to RBC2 line male satellite cells; and (B) F-line female satellite cells compared to RBC2-line female satellite cells. Asterisks indicate when differentiation was significantly different between cell lines ($P<0.05$).
Figure 3.5 Myotube lengths during differentiation. (A) F-line male and female satellite cells; and (B) RBC2-line male and female satellite cells. Asterisks indicate a significant difference in myotube length ($P<0.05$).
Figure 3.6 Myotube lengths of (A) F-line male satellite cells compared to RBC2-line male satellite cells; and (B) F-line female satellite cells compared to RBC2-line female satellite cells. Asterisks indicate a significant difference in myotube length ($P<0.05$).
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Table 3.1 Percent nuclei in myotubes during fusion.
CHAPTER 4

DEVELOPMENTAL EXPRESSION OF SKELETAL MUSCLE HEPARAN SULFATE PROTEOGLYCANS IN TURKEYS WITH DIFFERENT GROWTH RATES*


Abstract

Heparan sulfate proteoglycans (HSPG) are a group of extracellular matrix molecules that link skeletal muscle cells to their extrinsic environment. To investigate if HSPG expression is affected by muscle growth and gender, a turkey line (F) selected for increased 16-wk body weight and its unselected randombred control line, RBC2, were used in the present study. Heparan sulfate (HS) and HSPG levels were measured in embryonic and posthatch pectoralis major muscle. Heparan sulfate levels plateaued at embryonic day (ED) 16 in both lines. A significant decrease of HS occurred at ED 18 in F line males and females, and at ED 20 and 22 in the RBC2 line males and females, respectively. Embryonic HSPG levels peaked at ED 18, and were significantly higher from ED 14 through 18 in F line males and females compared with those of the RBC2 line. Male p. major muscle had more HSPG at early embryonic stages than female muscle in both lines. During 1 to 16-wk posthatch, F line male and female p. major
muscle contained more HSPG than the RBC2 line samples, and HSPG levels in F line males were higher than those of the females. Myogenic satellite cells derived from F and RBC2 line male and female *p. major* muscle were cultured to measure HSPG expression during proliferation and differentiation. No significant difference in HSPG level was found between the RBC2 and F line cells. However, in both lines, male-derived satellite cells had more HSPG than the female cells during proliferation and differentiation. These data show that HS and HSPG expression are affected by muscle growth properties and sex.

4.1 Introduction

Skeletal muscle myogenesis is a complex process that involves muscle cell proliferation, migration, adhesion, fusion to form multinucleated myotubes, and further differentiation to become mature muscle fibers (Swartz et al., 1994). This process is under precise control through the interactions of muscle cells with their extrinsic environment. One major extrinsic factor involved in the regulation of skeletal muscle development is the extracellular matrix (ECM). The ECM is an organized structure located outside cells that is composed of proteins and polysaccharides locally produced by the cells (Scott, 1995). The ECM is required for skeletal muscle myogenesis (Melo et al., 1996). Through interactions with growth factors such as transforming growth factor β (TGF-β) and fibroblast growth factor 2 (FGF2), and with cell surface receptors, the ECM is involved in the regulation of cell gene expression, proliferation, migration, adhesion, and differentiation, which are all essential for muscle development and growth (Yanagishita, 1993).
Proteoglycans (PG), one major component of the ECM, contain a central core protein to which one or more glycosaminoglycan (GAG) chains are attached. Based on GAG composition, PG are classified into chondroitin sulfate (CS) PG, dermatan sulfate (DS) PG, heparan sulfate (HS) PG, and keratan sulfate (KS) PG (Hardingham and Fosang, 1992). Proteoglycan expression changes during muscle development from a matrix containing large CS-rich PG to a mixture of CS, DS, and HS PG (Young et al., 1990; Fernandez et al., 1991; Velleman et al., 1999). This conserved expression pattern suggests that different PG may have distinct functions at different stages of myogenesis. However, the precise roles of these PG during skeletal muscle development and growth are largely unknown.

In skeletal muscle, HSPG are found mainly on cell plasma membrane and in basement membrane. Heparan sulfate proteoglycans interact with many molecules including FGF2 (Bernfield et al., 1999; Tumova et al., 2000). The FGF2 is an intense stimulator of skeletal muscle cell proliferation but a potent inhibitor of differentiation (Dollenmeier et al., 1981). Heparan sulfate proteoglycans act as co-receptors of FGF2 and regulate the binding of FGF2 to its high-affinity signaling receptors (Raprager et al., 1991; Yayon et al., 1991). The expression of HSPG increases with muscle development (Velleman et al., 1999), and HSPG are dynamically expressed during myoblast differentiation (Brandan et al., 1996; Larraín et al., 1997; Brandan and Larraín, 1998; Fuentealba et al., 1999). Changes in HSPG expression during skeletal muscle cell differentiation may play a significant role in regulating cellular response to FGF2 and may result in changes in muscle cell differentiation rates (Larraín et al., 1998; Fuentealba et al., 1999).
Information about the expression and role of HSPG in myogenesis has been mainly obtained from established muscle cell lines in vitro. Little is known about the in vivo expression of HSPG during skeletal muscle development and growth. In order to address how the expression of HSPG is influenced by muscle growth, a line (F) of turkeys selected for increased 16-wk body weight (Nestor, 1984) and a randombred control line (RBC2) from which the F line was derived were used in this study. The F line turkeys have a faster growth rate and heavier p. major muscle weights than the RBC2 line birds (Nestor et al., 1987; Lilburn and Nestor, 1991). Because the F line was developed from the RBC2 line by selection only for increased body weight and the RBC2 line is not selected for any trait, changes in gene expression during muscle development and growth can be attributed to changes in growth. Velleman et al. (2000) showed that the F and RBC2 line myogenic satellite cells have different proliferation and differentiation characteristics with the F line cells proliferating and differentiating at a faster rate.

Both male chickens and turkeys have heavier body weight and more muscle mass compared with corresponding females beginning at early embryonic stages (Burke and Sharp, 1989; Burke, 1994). Similar gender-influenced patterns of muscle growth have been observed in humans (Wang et al., 1999; Janssen et al., 2000). The physiological mechanisms of these sex differences in muscle growth have not been identified. There is no evidence that gonadal steroid hormones are directly involved in the regulation of muscle development. However, gonadal hormones affect the expression of certain growth factors (Murphy et al., 1988; Murphy and Potzlaw, 1989; Bacher et al., 1993) that influence muscle cell proliferation and differentiation. Although growth factors have
been found to regulate HSPG expression (Romarís et al., 1995; Jaakkola et al., 1997), there is no reported data about sex influence on PG expression.

Satellite cells are myogenic cells that are responsible for postnatal muscle growth and muscle regeneration (Mauro, 1961; Moss and LeBlond, 1971). In order to address the question of how HSPG are expressed during myogenic cell proliferation and differentiation, satellite cells derived from \textit{p. major} muscles obtained from males and females of the F and RBC2 line were used in the present study. Results from the current study will provide new information about the influence of muscle growth and sex on HSPG expression during \textit{in vivo} muscle development, and \textit{in vitro} satellite cell proliferation and differentiation.

4.2 Materials and methods

4.2.1 Animal model

The F and RBC2 line turkeys (Nestor, 1984) used in the present study were from flocks maintained at the Ohio Agricultural Research and Development Center. The F line is a turkey line selected for increased body weight at 16-wk posthatch and was developed from the randombred control RBC2 line. The RBC2 line has not been selected for any trait and is maintained in such a manner (Nestor, 1977a, b) that little genetic change is expected or observed.

4.2.2 Sample collection and sex identification

For each set of samples, the \textit{p. major} muscle tissue was isolated with all visible skin and fat removed from 5 F and 5 RBC2 male and female embryos at 14, 16, 18, 20,
22, and 24 day of embryonic development, and from 5 male and 5 female F and RBC2 turkeys at 1, 8, 12, and 16 wk posthatch. The muscle tissue was quick frozen in liquid nitrogen and stored at -70 °C. Three sets of samples were collected.

Sex identification of the turkey embryos was performed using a polymerase chain reaction (PCR) method adapted from D’Costa and Petitte (1998). All primers used were according to D’Costa and Petitte (1998) and commercially synthesized by Operon (Alameda, CA). After heating the PCR components at 95 °C for 5 min, the PCR cycle conditions were 2 cycles of 95 °C for 5 min, 52 °C for 1 min, and 72 °C for 3 min. This was followed by 30 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 3 min, and a final extension at 72 °C for 10 min. The sex of the posthatch birds was identified by dissection.

4.2.3 Cellulose acetate chromatography

Equal amounts of muscle from 5 embryos or birds at each developmental age were homogenized in a Tris-EDTA buffer (TE; 10 mM Tris, pH 8.0, 0.2 mM EDTA) at a concentration of 1 g tissue/2 ml TE. Tissue debris was removed by centrifugation at 10,000 ×g for 5 min. Protein concentration of the supernatant was measured by the Bradford (1976) method. Thirty microgram of each sample was digested with an equal volume of protease type XIV (20 µg/µl) (Sigma Chemical Co., St. Louis, MO) at 55 °C for 16 h. The digested samples and 3 µl of a standard containing 0.05 µg/µl of CS A (Sigma Chemical Co., St. Louis, MO), CS C (Sigma Chemical Co., St. Louis, MO), DS (Sigma Chemical Co., St. Louis, MO), and HS (Sigma Chemical Co., St. Louis, MO) were loaded onto cellulose acetate paper (Pall Corporation, Ann Arbor, MI) pre-wetted in
running buffer (200 mM Ca-acetate, pH 7.2). The chromatography paper was electrophoresed in a Gelman Sciences (Ann Arbor, MI) chromatography apparatus at 6 mAmps per piece of paper for 6 h in running buffer. The cellulose acetate paper was then stained in 0.05% Alcian Blue (Eastman Kodak Co., Rochester, NY) containing 3% acetic acid and 50 mM MgCl₂ (pH 3.9) for 15 minutes with gentle agitation. The paper was destained in 1% acetic acid containing 50 mM MgCl₂ (pH 3.9) until the background was white. The bands were scanned and analyzed with a PDI Quantity One scanner (PDI, Huntington Station, NY), and the reflective densities were measured. Each sample was chromatographed three times and an average reflective density was calculated. The assay was repeated three times with different sets of sample.

4.2.4 Cell culture

Satellite cells were isolated from the p. major muscle of 7-wk-old male and female F and RBC2 line turkeys (Velleman et al., 2000). The satellite cells were grown on 35-mm gelatin-coated plates (Corning Costar, Corning, NY) at a density of 35,000 cells per well. The cells were plated in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies Inc., Grand Island, NY) containing 10% chicken serum (Sigma Chemical Co., St. Louis, MO), 5% horse serum (Life Technologies Inc., Grand Island, NY), 1% antibiotic/antimycotic (Life Technologies Inc., Grand Island, NY) and 0.1% gentamicin (Life Technologies Inc., Grand Island, NY) for 24 h, and then switched to McCoy’s 5A medium (Life Technologies Inc., Grand Island, NY) containing 10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic, and 0.1% gentamicin. When the satellite cells reached 60% confluency, differentiation was induced by culturing the
cells in DMEM containing 3% horse serum, 0.01 mg/ml gelatin, and 1 mg/ml bovine serum albumin until 96 h. At 24-h intervals during cell proliferation and 12-h intervals during differentiation, the medium was removed from one plate and the wells were rinsed twice with sterile PBS (170 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.08). The plates were stored at -70 °C until analysis.

4.2.5 ELISA analysis

Muscle from 5 male and 5 female F and RBC2 line embryos or birds at each developmental age were weighed and equal amounts of muscle from the samples were homogenized in the TE buffer at a concentration of 1 g tissue/2 ml TE. Tissue debris was removed by centrifugation at 10,000 ×g for 5 min. Protein concentration of the supernatant was measured by the Bradford (1976) method. The supernatant was then used for ELISA analysis.

Satellite cells were scraped with a rubber policeman and disrupted with a dounce homogenizer in a HEPES buffer (20 mM HEPES, pH 4.2, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and 1 µg/ml leupeptin). Samples were dialyzed overnight against Nanopure H₂O (Barnstead Thermolyne, Cheswick, PA) at 4 °C overnight. Protein concentrations were measured by the Bradford (1976) method.

Samples from tissue or satellite cells were lyophilized, and then resuspended in a carbonate-bicarbonate buffer (50 mM Na₂CO₃, 50 mM NaHCO₃, pH 9.8) at a concentration of 10 µg in 100 µl of buffer. Ten micrograms of antigen were plated per well on a pro-bind assay plate (Falcon, Lincoln Park, NJ). The plate was incubated at 37 °C for 16 h. Controls without primary antibody were run with each assay. After
incubation, the plate was washed twice with PBS-Tween 20 (PBS-T; PBS and 0.05\% Tween-20) and twice with Nanopure H$_2$O. One hundred microliter of primary HSPG monoclonal antibody (Development Studies Hybridoma Bank, Iowa City, IA) at a dilution of 1:500 in PBS-T was added to each well, and the plate was incubated at 37 °C for 2 h. The plate was then washed twice with PBS-T and twice with Nanopure H$_2$O. To each well, 100 µl of alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma, St. Louis, MO) at a dilution of 1:30,000 in PBS-T was added and incubated at 37 °C for 2 h. The plate was washed three times with PBS-T and twice with Nanopure H$_2$O, and 100 µl of the substrate $p$-nitrophenyl phosphate (Sigma, St. Louis, MO) was added to each well and incubated in the dark at room temperature for 30 min. The optical density (OD) was read at 405 nm with a Revelation microplate reader (Dynex Technologies, Inc., Chantilly, VA). Each sample was run in triplicate per assay and an average OD calculated. The ELISA assay was repeated three times with different sets of muscle or cultured cells.

The OD of HSPG in the muscle was adjusted to the concentration of protein in the entire $p$. major muscle. Total protein concentration in 1 g of tissue was calculated and multiplied by the weight of the $p$. major muscle. The ELISA assay was performed with 10 µg of antigen, the total amount of protein in the muscle tissue was divided by 10 µg to obtain an adjustment factor. The OD was multiplied by the adjustment factor to derive the adjusted OD.
4.2.6 Statistical analysis

A randomized complete block model using the general linear model procedure of SAS Institute (1985) was used to evaluate the effects of turkey line, sex, and development stage on HS GAG expression as measured by cellulose acetate chromatography. Differences were considered significant at $P < 0.05$.

For the ELISA assay of muscle tissue and satellite cells, a student’s two-tailed $t$-test using the general linear model procedure of SAS was used to determine the differences of HSPG expression between the F and RBC2 lines, or between male and female samples within the lines at each developmental stages (for muscle tissue) or cell culture time (for satellite cells). Differences were considered significant at $P < 0.05$.

4.3 Results

4.3.1 Heparan sulfate glycosaminoglycan analysis

Heparan sulfate levels during embryonic development are summarized in Table 4.1. At embryonic day (ED) 14, HS was high in all groups and plateaued at ED 16. Heparan sulfate contained in $p. major$ muscle gradually decreased with embryonic development. In both F line male and female samples, significant decreases in HS compared to those of ED 14 and 16 were observed at ED 18. Similar changes in HS occurred at ED 20 in RBC2 line males and at ED 22 in F line females. From 1 to 16 wk of posthatch development, HS was low in all groups, and no significant changes were observed. There were no line or sex differences in HS levels during posthatch development (data not shown).
4.3.2 Heparan sulfate proteoglycan concentration

In F and RBC2 line embryos of both sexes, HSPG concentration in *p. major* muscle continually increased from ED 14 through 18 (Figure 4.1A, 4.1B). Heparan sulfate proteoglycans then decreased at ED 20 and gradually increased again from ED 22 to day 24. At ED 14, 16, and 18, the F line male *p. major* muscle had significantly higher HSPG levels than the RBC2 line males (Figure 4.1). The F line females contained more HSPG than the RBC2 line females at ED 16 and 18 (Figure 4.1). In the F line male and female *p. major* muscle, HSPG were dramatically elevated from those of the RBC2 line samples at ED 18. Comparisons within the lines showed that HSPG concentration in the RBC2 line males were higher than the females at ED 14 and 16 (Figure 4.2). The F line males had more HSPG than the F line females from ED 14 through 20 (Figure 4.2).

Heparan sulfate proteoglycan concentration in posthatch F and RBC2 line *p. major* muscle showed that HSPG levels continually increased from 1 to 16 wk posthatch. The RBC2 line males had significantly lower HSPG than the F line males at all stages (Figure 4.3). A similar concentration pattern was observed in the F and RBC2 line female samples (Figure 4.3). Comparisons within the lines showed no significant difference in HSPG between RBC2 line males and females in posthatch muscle (Figure 4.4). However, the F line males had increased HSPG at all posthatch stages in comparison with those of the females (Figure 4.4).
4.3.3 Satellite cell heparan sulfate proteoglycan expression

During proliferation stages, HSPG were low in all groups and increased once differentiation was induced (Figure 4.5). The HSPG concentrations were high from 0 to 60 h of differentiation and then gradually decreased. There were no significant differences in HSPG between the RBC2 and F line male satellite cells during differentiation except at 96 h (Figure 4.5). As with the male cells, no significant differences in HSPG concentration between the RBC2 and F line female satellite cells during differentiation except at 12 and 96 h (Figure 4.5). However, higher amounts of HSPG were measured between 72 h of proliferation and 60 h of differentiation in male satellite cells compared with the females in both lines (Figure 4.6).

4.4 Discussion

During early embryonic muscle development, myoblasts are proliferating rapidly. In the growth selected F line, increased HSPG amounts were noted during early embryonic and posthatch stages compared with those of the RBC2 line. Since HSPG are co-receptors of FGF2, a stimulator of muscle cell proliferation and an inhibitor of differentiation, more HSPG in the F line p. major muscle may function by interacting and presenting more FGF2 to its high-affinity receptors. As a result, FGF2 signaling may be enhanced and myoblast proliferation stimulated. With increased proliferation, more myoblasts are generated which may result in muscle growth through hyperplasia. In support of this hypothesis, Summers and Medrano (1997) showed that high-growth mice have prolonged myoblast proliferation and delayed myotube formation during embryonic
development. Another period of rapid muscle growth, hypertrophy, caused by the fusion of activated satellite cells with existing muscle fibers (Moss and LeBlond, 1971) occurs during posthatch development. Higher HSPG amount in the F line at those stages may enhance satellite cell proliferation and result in muscle growth through hypertrophy.

The cell surface HSPG include syndecans and glypicans, which are differentially expressed during skeletal muscle cell differentiation. Syndecan-1 (Larraín et al., 1997), syndecan-3 (Fuentealba et al., 1999), and syndecan-4 (Brandan and Larraín, 1998) expression is down-regulated with cell differentiation, whereas glypican expression increases (Brandan et al., 1996). Both syndecan-1 and -3 enhance cell responsiveness to FGF2 and inhibit cell differentiation through an FGF2-dependent mechanism (Larraín et al., 1998; Fuentealba et al., 1999). Brandan and Larraín (1998) hypothesized that cell surface HSPG play different roles during muscle differentiation with syndecans presenting FGF2 to its high-affinity receptors and glypican sequestering FGF2 to permit differentiation to proceed. Furthermore, syndecans may also be involved in the regulation of myogenic regulatory factor expression that is necessary for muscle cell terminal differentiation. In a recent study by Olguin and Brandan (2001), syndecan-3 localization during limb muscle formation corresponded to myogenin expression. This co-localization of syndecan-3 and myogenin suggests a possible role of syndecan-3 in the regulation of myogenin expression.

The amount of HSPG in male derived satellite cells was higher compared with that of the female cells during differentiation. In a study by Velleman et al. (2000), the F line male satellite cells did not display a lower differentiation rate in vitro. These results
support the hypothesis that HSPG may be involved in interactions other than presenting FGF2 to its cellular receptors. If the HSPG functioned by only presenting FGF2 to its receptors, differentiation would be inhibited in the F line male satellite cells. In the present study, no difference in HSPG levels was found between F and RBC2 line satellite cells during \textit{in vitro} proliferation and differentiation though higher HSPG levels were observed \textit{in vivo} in the F line muscle tissue. The reason there is no difference in HSPG concentration \textit{in vitro} may be due to the separation of satellite cell proliferation and differentiation stages, during which the cells may express similar amounts of HSPG. In contrast, during \textit{in vivo} muscle development and growth, proliferation and differentiation are not distinct as some muscle cells are proliferating, whereas others are differentiating.

The \textit{p. major} muscle from the male embryos in both lines was heavier than those from the females (data not shown). Differences in body weight and muscle mass of male and female avian embryos have been previously reported (Burke and Sharp, 1989; Burke, 1994). It is unknown what mechanism causes the sex-related differences in muscle growth. Gonadal hormones may be indirectly involved in the regulation of skeletal muscle growth by affecting the expression of several growth factors including FGF2 (Murphy et al., 1988; Murphy and Potzlaw, 1989; Bacher et al., 1993). The FGF2 has been shown to enhance syndecan-1 expression (Jaakkola et al, 1997), but down-regulates glypican with the cooperation of TGF-β (Romarís et al., 1995). In the present study, both F and RBC2 line male embryos had higher HSPG levels during early embryonic muscle development that may result from changes in growth factor expression. The F line males had more HSPG at all posthatch stages investigated, but the \textit{p. major} muscle weights
were not heavier than those of the F line females except at 8-wk posthatch (data not shown). No difference in *p. major* muscle weights was observed between RBC2 line male and female birds (data not shown). These results suggest that the mechanism of sex differences on muscle growth is very complex and molecules other than HSPG may be involved.

In summary, sex and genetic selection for increased growth resulted in changes in HS and HSPG expression during skeletal muscle development and growth. In order to further understand the role of HSPG in skeletal muscle myogenesis, the expression of individual HSPG and their function in FGF2-dependent signaling needs to be elucidated.
References


Figure 4.1 HSPG expression in *p. major* muscle from male and female F and RBC2 line embryos at 14 to 24 days of embryonic development. A) male embryos; B) female embryos. HSPG concentrations were adjusted to *p. major* muscle weights. Bars represent the standard error of the mean. Asterisks indicate a significant difference ($P < 0.05$).
Figure 4.2 HSPG expression in *p. major* muscle from F and RBC2 line male and female embryos at 14 to 24 days of embryonic development. A) RBC2 line; B) F line. HSPG concentrations were adjusted to *p. major* muscle weights. Bars represent the standard error of the mean. Asterisks indicate a significant difference (*P < 0.05*).
Figure 4.3 HSPG expression in *p. major* muscle from male and female F and RBC2 line turkeys from 1 to 16 wk of age. A) male turkeys; B) female turkeys. HSPG concentrations were adjusted to *p. major* muscle weights. Bars represent the standard error of the mean. Asterisks indicate a significant difference (*P* < 0.05).
Figure 4.4 HSPG expression in *p. major* muscle from male and female F and RBC2 line turkeys from 1 to 16 wk of age. A) RBC2 line; B) F line. HSPG concentrations were adjusted to *p. major* muscle weight. Bars represent the standard error of the mean. Asterisks indicate a significant difference (*P* < 0.05).
Figure 4.5 HSPG expression during proliferation (P) and differentiation (D) of male and female F and RBC2 line satellite cells. A) satellite cells from male turkeys; B) satellite cells from female turkeys. Bars represent the standard error of the mean. Asterisks indicate a significant difference \((P < 0.05)\).
Figure 4.6 HSPG expression during proliferation (P) and differentiation (D) of male and female F and RBC2 line satellite cells. A) RBC2 line; B) F line. Bars represent the standard error of the mean. Asterisks indicate a significant difference ($P < 0.05$).
Table 4.1 Means of reflective density (n = 3) of heparan sulfate glycosaminoglycan at 14, 16, 18, 20, 22, and 24 embryonic day (ED) development stages.

<table>
<thead>
<tr>
<th></th>
<th>RBC2 Male</th>
<th>RBC2 Female</th>
<th>F Male</th>
<th>F Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 ED</td>
<td>0.097&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.080&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
<td>0.097&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.107&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>16 ED</td>
<td>0.100&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.090&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.100&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.100&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18 ED</td>
<td>0.073&lt;sup&gt;b,c,d,e&lt;/sup&gt;</td>
<td>0.067&lt;sup&gt;c,d,e&lt;/sup&gt;</td>
<td>0.060&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.063&lt;sup&gt;c,d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>20 ED</td>
<td>0.063&lt;sup&gt;c,d,e&lt;/sup&gt;</td>
<td>0.063&lt;sup&gt;c,d,e&lt;/sup&gt;</td>
<td>0.053&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.057&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>22 ED</td>
<td>0.060&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.050&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.050&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.053&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 ED</td>
<td>0.055&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.052&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.055&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>0.060&lt;sup&gt;d,e&lt;/sup&gt;</td>
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<sup>a-e</sup> Reflective densities with no common superscript are significant different (<i>P</i> < 0.05).

<sup>1</sup>The data were analyzed using a randomized complete block model.
CHAPTER 5

DEVELOPMENTAL REGULATED EXPRESSION OF SYNDECAN-1 AND GLYPCAN IN PECTORALIS MAJOR MUSCLE IN TURKEYS WITH DIFFERENT GROWTH RATES

Abstract

Heparan sulfate proteoglycans, syndecan-1 and glypican, are low-affinity receptors for fibroblast growth factor 2 (FGF2). Since FGF2 stimulates skeletal muscle cell proliferation but inhibits differentiation, differences in syndecan-1 and glypican expression may change the intensity of FGF2 signaling and affect muscle development and growth. In the current study, the pectoralis major muscle from 14 to 24-day-old embryos and from 1 to 16-wk-old birds from a turkey line (F) selected for increased 16-wk body weight and its genetic control line (RBC2) were used to address how syndecan-1 and glypican expression is related to muscle growth properties. The expression of syndecan-1 and glypican was measured by semi-quantitative reverse transcription polymerase chain reaction. For males, the F line embryos expressed more syndecan-1 (days 14 and 16) and glypican (days 14 and 18) than the RBC2 line. Similar line differences for males were observed for syndecan-1 and glypican expression at 8, 12, and 16-wk posthatch. The male embryos from both lines expressed more syndecan-1 at days 18 through 22 and more glypican at days 20 and 22 than the corresponding females. The temporal and spatial distribution of syndecan-1 was detected by in situ hybridization in
the *p. major* muscle at all embryonic stages studied but not in the posthatch tissue, whereas the glypican was detected from embryonic day 18 through 16-wk posthatch. The data from the current study provided novel information about the expression of syndecan-1 and glypican during muscle growth and development.

### 5.1 Introduction

Skeletal muscle tissue is composed of muscle cells embedded in an intricate network of extracellular matrix (ECM) that fills the intercellular space. The ECM comprises a variety of locally secreted polysaccharides and proteins that assemble into an organized meshwork (Scott, 1995). The major components of the ECM are collagens, proteoglycans (PG), and glycoproteins. These molecules form a tissue specific dynamic structure that changes in composition with development. The ECM is involved in the regulation of cellular behaviors such as migration, adhesion, proliferation, and differentiation that are essential for skeletal muscle development and growth (Geiger et al., 2001). The ECM is required for normal skeletal muscle myogenesis since interfering with the synthesis of ECM molecules and the assembly of ECM architecture results in an inhibition of skeletal muscle cell differentiation (Melo et al., 1996; Osses and Brandan, 2002).

Proteoglycans are one major component of the ECM. They represent a diverse family of glycosylated proteins that contain a core protein covalently attached by one or more glycosaminoglycan (GAG) chains (Hardingham and Fosang, 1992). Proteoglycans have important functions with respect to cell adhesion, migration, and growth factor signaling. During skeletal muscle development, PG expression changes from a
chondroitin sulfate-rich matrix to a mixture of chondroitin sulfate, dermatan sulfate, and heparan sulfate PG (Young et al., 1990; Fernandez et al., 1991; Velleman et al., 1999). This transition pattern suggests that different PG may have unique functions during muscle development. Heparan sulfate (HS) PG are low affinity receptors of fibroblast growth factor 2 (FGF2), a potent stimulator of skeletal muscle cell proliferation but an intense inhibitor of cell differentiation (Dollenmeier et al., 1981). Since the HSPG regulate the binding of FGF2 to its high affinity tyrosine kinase receptors (Rapraeger et al., 1991; Yayon et al., 1991), changes in HSPG expression may affect FGF2 signaling transduced to the muscle cells and regulate skeletal muscle cell proliferation and differentiation.

The major HSPG on the cell surface are syndecans and glypicans. The syndecan family is composed of four members, syndecan-1 through 4, that are all found in skeletal muscle. The syndecans have a membrane spanning core protein possessing a highly conserved cytoplasmic domain and transmembrane domain, and a diverse ectodomain to which the GAG chains are attached (Carey, 1997; Rapraeger, 2001). Glypicans, 1 through 6, have a core protein that contains conserved cysteine residues and GAG attachment sites, and are attached to cell plasma membrane through a glycosylphosphatidylinositol anchor (David et al., 1990). Only glypican-1 (glypican) has been reported in skeletal muscle tissue. In vitro studies indicated that syndecan-1, 3, and 4 expression are down-regulated during skeletal muscle cell differentiation (Larraín et al., 1997; Fuentealba et al., 1999), whereas syndecan-2 remains unchanged (Brandan and Larraín, 1998). In contrast, the glypican expression increases significantly during cell differentiation (Brandan et al., 1996). Both syndecans and glypicans mediate FGF2
binding to fibroblast growth factor receptors (FGFR) and regulate FGF2 activity (Steinfeld et al., 1996; Filla et al., 1998). However, little is known about the precise biological functions of syndecans and glypicans during myogenesis. The different expression patterns of syndecan-1 and glypican imply that these two molecules may have functional differences in regulating cellular responsiveness to FGF2 signaling. Therefore, changes in syndecan-1 and glypican expression may affect cell proliferation and differentiation that determine skeletal muscle development and growth. In order to investigate the expression of syndecan-1 and glypican as it relates to muscle development, a selected line of turkey (F) and its randombred control line (RBC2) were used in the current study. The F line turkey was developed from the RBC2 line by selecting for only increased 16-wk body weight (Nestor, 1984). The F line turkeys have a heavier body weight and pectoralis major muscle weight compared to the RBC2 line (Nestor et al., 1987; Liblurn and Nestor, 1991), and was used as a model for faster muscle growth. A previous study (Liu et al., 2002) showed that the F line turkeys had increased HSPG concentration at early embryonic and posthatch developmental stages in the p. major muscle.

Satellite cells are quiescent myogenic cells residing between the basement membrane and plasma membrane of muscle fibers (Mauro, 1961), and are largely responsible for postnatal muscle growth and muscle regeneration (Moss and LeBlond, 1971). Velleman et al (2000) showed that the F line satellite cells have a faster proliferation and differentiation rate than the RBC2 line cells. The RBC2 and F line satellite cells were also used in the present study to investigate syndecan-1 and glypican expression during in vitro cell proliferation and differentiation. In addition, since male
turkeys and chickens have been shown to have heavier body weight and larger muscle mass compared to the corresponding females (Burke and Sharp, 1989; Burke, 1994), the p. major muscle tissue from embryos and turkeys of both sexes, and satellite cells of both sexes were used in the current study to investigate the sex influence on syndecan-1 and glypican expression during myogenesis. The data from the current study provided new information about the expression of syndecan-1 and glypican during muscle growth and development.

5.2 Materials and methods

5.2.1 Animal model

Both the F and RBC2 line turkeys (Nestor, 1984) used in the present study were from flocks maintained at the Ohio Agricultural Research and Developmental Center. The F line was selected for increased body weight at 16-wk posthatch and was developed from the RBC2 line, which has not been selected for any trait and is maintained with little genetic changes observed over generations (Nestor, 1977a; 1977b).

5.2.2 Sample collection and sex identification

Pectoralis major muscle tissue was isolated from 5 individuals of each sex and line at 14, 16, 18, 20, 22, and 24 embryonic days (ED) and at 1, 8, 12, and 16 wk posthatch. The p. major muscle weight was recorded. The muscle was quick frozen in liquid nitrogen and stored at – 70 °C until use. Three replicate sets of samples were collected.
Sex identification of the turkey embryos was performed using a polymerase chain reaction (PCR) method (Liu et al., 2002) adapted from D’Costa and Petitte (1998). The sex of the posthatch birds was identified by dissection.

5.2.3 Cell culture

Satellite cells derived from the *p. major* muscle of 7-wk-old F and RBC2 line turkeys of both sexes (Velleman et al., 2000) were plated on 35 mm gelatin coated plates at a density of 35,000 cells per well. The cells were cultured as described by Liu et al. (2002). At 48 h intervals during cell proliferation and differentiation, the medium was removed from one plate and the cells were rinsed twice with sterile phosphate buffered saline (PBS, 170 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.08). The plates were stored at –70 °C until use.

5.2.4 Extraction of total RNA

Total RNA was extracted from the *p. major* muscle tissue using the method developed by Chomczynski and Sacchi (1987). For the cultured satellite cells, total RNA was extracted according to the RNAqueous™ Small-scale Phenol-free Total RNA Isolation Kit instruction manual (Ambion, Austin, TX).

5.2.5 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

The first strand of cDNA was synthesized using the RETROscript™ First Strand Synthesis Kit according to the instruction manual (Ambion, Austin, TX). In a thin-wall PCR tube, 2.5 µg of total RNA, 100 nM Oligo (dT) primers, and nuclease-free H₂O in a
total volume of 12 µl were mixed, heated at 85 °C for 3 min, and then cooled on ice for 1 min. The RT reaction buffer (10 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl₂, pH 8.3), 10 µM each deoxynucleotide triphosphate (dNTP), 10 IU RNase inhibitor, and 100 IU moloney murine leukemia virus reverse transcriptase were then added to a 20 µl final volume. The RT reaction was carried out at 44 °C for 60 min, followed by a denaturation at 95 °C for 5 min.

Five microliters of the RT product was used as the template to amplify the cDNA. The PCR was performed with 1X PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% TritonX-100, pH 9.0), 1.5 mM MgCl₂, 200 µM each dNTP, 0.45 µM of each primer, and 1.25 IU TaqBead™ hot start polymerase (Promega Corp., Madison, WI) in 50 µl final volume. The syndecan-1 primers were designed based on mouse syndecan-1 cDNA sequence (Saunder et al., 1989) (GenBank # Z22532). The forward primer was 5’-CAACCTGGCCTCCATGAGAC-3’ and the reverse primer was 5’-CCTCCCAGCACTTCCCTTCCT-3’. The amplified 281 base pairs (bp) turkey syndecan-1 cDNA fragment (GenBank # AF416704) has 100% nucleotide homology to the mouse sequence. The glypican primers were developed based on chicken glypican cDNA sequence (Niu et al., 1996) (GenBank # L29089). The forward primer was 5’-CGTGCTGTCATGAAGCTGAT-3’ and the reverse primer was 5’-GCTACTGCAGAGGATTTGGC-3’. The amplified 380 bp turkey glypican cDNA fragment (GenBank # AY150229) has 94% nucleotide homology to the chicken sequence. To amplify the syndecan-1 cDNA fragment, the PCR reaction was started at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 sec, 58.5 °C for 30 sec, 72 °C for 45 sec, and a final extension at 72 °C for 10 min. The glypican cDNA fragment was amplified by 94
°C for 5 min, 35 cycles of 94 °C for 30 sec, 55.5 °C for 30 sec, and 72 °C for 45 sec, with a final extension at 72 °C for 10 min. The cycle numbers for the PCR amplifications were maintained in the logarithmic phase for each primer set. The PCR products were applied to an ethidium bromide containing 1% agarose gel and separated in 1X TBE buffer (89 mM Tris, 89 mM Boric acid, and 2 mM EDTA, pH 8.0) by electrophoresis. The bands were recorded with a Kodak 290 digital camera system and analyzed for optical density with Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY). The syndecan-1 and glypican expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GenBank # U94327).

To adjust the expression of syndecan-1 and glypican to the p. major muscle weight, the amount of total RNA extracted from 0.5 g of muscle tissue was calculated based on the absorbency at 260 nm. The amount of total RNA in the entire p. major muscle was calculated based on the muscle weight. Since each RT reaction used 2.5 µg of total RNA as template, the amount of total RNA in the entire p. major muscle was divided by 2.5 µg to obtain an adjustment factor. The optical density of each PCR product on the agarose gel was multiplied by the adjustment factor to obtain the adjusted relative density based on p. major muscle weight.

5.2.6 In situ hybridization

Pectoralis major muscle was removed from 5 F and 5 RBC2 turkey embryos of each sex at 14, 16, 18, 20, 22, and 24 days of development, and from 5 F and 5 RBC2 turkeys of each sex at 1, 8, 12, and 16 wk posthatch. Samples of the p. major tissue were fixed in 10% formalin for 6 h. After fixing, the muscle samples were dehydrated for 30
min in 50% ethanol, 30 min in 70% ethanol, 30 min in 95% ethanol, 1 h in 100% ethanol with one change at 30 min, and 1 h in xylene with one change at 30 min. The samples were then incubated in paraffin for 1 h with one change at 30 min, and embedded in paraffin. The paraffin embedded muscle samples were then cut into 5 µM thick sections and mounted on precleaned microscope slides (Fisher Scientific, Pittsburgh, PA). For each sample, a series of six slides were prepared.

The PCR amplified syndecan-1 and glypican-1 cDNA fragments were purified by spin columns according to the QIAquick ® PCR Purification Kit instruction manual (QIAGEN Inc., Valencia, CA). The purified cDNA fragments were cloned into a pGEM-T Easy vector (Promega Corp, Madison, WI) according to the instruction manual and transformed into DH 5α cells.

For preparation of digoxigenin (DIG)-labeled riboprobes, plasmid DNA containing inserted syndecan-1 or glypican-1 sequences was extracted using the QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA). The purified plasmid DNA was linearized with the appropriate restriction enzymes and used as template for in vitro transcription of sense and antisense DIG-labeled riboprobes using SP6 and T7 RNA polymerase according to the SP6/T7 Riboprobe Combination System instruction manual (Promega Corp., Madison, WI).

The method of in situ hybridization to detect the temporal and spatial distribution of syndecan-1 and glypican-1 mRNA in p. major tissue from the F and RBC2 lines was adapted from the Nonradioactive In Situ Hybridization Application Manual (Boehringer Mannheim GmbH, Germany). In brief, muscle sections were dewaxed 10 min in xylene for two times, then rehydrated for 5 min in 100% ethanol, 5 min in 95% ethanol, 5 min in
70% ethanol, and two 2 min in RNase-free diethyl pyrocarbonate (DEPC) H$_2$O. After two washing with DEPC-PBS with 5 min, the slides were washed twice with DEPC-PBS containing 100 mM glycine for 5 min. The slides were incubated at room temperature for 15 min in DEPC-PBS containing 0.3% Triton X-100, and washed twice with DEPC-PBS with 5 min. After washing, the tissue sections were permeabilized with 100 µl of RNase-free proteinase K (5 µg/ml) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 20 min at 37 °C. The slides were then washed twice with DEPC-PBS for 5 min, and digested with 20 U DNase I in 50 mM Tris-HCl (pH 7.5) for 30 min at 37 °C. After washing twice with DEPC-PBS for 5 min, the slides were incubated in 100 µl prehybridization buffer [4X SSC (600 mM NaCl and 60 mM Na citrate, pH 7.0) containing 50% formamide] for 30 min at 37 °C. The slides were then overlayed with 100 µl In Situ Hyb buffer (Ambion, Austin, TX) containing 5 ng DIG-labeled riboprobe and incubated overnight at 42 °C. The slides were then washed twice with 2X SSC (300 mM NaCl and 30 mM Na citrate, pH 7.0) for 15 min, and twice with 1X SSC (150 mM NaCl and 15 mM Na citrate, pH 7.0) for 15 min at 37 °C. After two 10 min washes in buffer 1 (100 mM Tris and 150 mM NaCl, pH 7.5), the slides were incubated for 30 min at room temperature in 100 µl of blocking buffer (buffer 1 with 0.1% Triton X-100 and 2% normal sheep serum). The slides were then incubated for 2 h at room temperature in 100 µl buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum, and 1:1000 dilution of sheep anti-DIG alkaline phosphatase conjugate. After two 10 min washes in buffer 1, the slides were incubated for 10 min at room temperature in 100 µl buffer 2 (100 mM Tris, 100 mM NaCl, and 50 mM MgCl$_2$, pH 9.5). The sections were then covered with 200 µl of color solution (2% β-chloroindolylphosphate/nitroblue tetrazolum
and 5 mM levamisole in buffer 2) about 7 h until color development was optimal. The slides were viewed and photographed with an Olympus XI 70 microscope (Melville, KY) equipped with a digital camera and analyzed with Image-Pro Plus software (Media Cybernetics, Sliver Spring, MD). The percentage of muscle fibers that express syndecan-1 and glypican were calculated.

5.2.7 Statistical analysis

A randomized complete block model using the general linear model procedure of SAS software (1985) was used to evaluate the effect of turkey line, sex, and developmental stages on syndecan-1 and glypican expression as measured by semi-quantitative RT-PCR. The means were separated by repeated t-test. Differences were considered significant at $P < 0.05$. The same model was also used to evaluate the line, sex, and age on the percentage of syndecan-1 and glypican expression in muscle fibers detected by in situ hybridization.

5.3 Results

5.3.1 Syndecan-1 expression in turkey p. major muscle

Syndecan-1 expression increased with embryonic development and peaked at embryonic day (ED) 18 in all line-sex subgroups except for RBC2 males which peaked at ED 20 (Figure 5.1). A significant decrease in syndecan-1 expression was detected in both sexes of the F line and RBC2 females at ED 24 compared to ED 18. The F line males had significantly higher syndecan-1 expression at ED 14 and 16 than the RBC2 line males. In contrast, the RBC2 line males expressed more syndecan-1 at ED 20 and 24
than the F line males. No difference was observed for females between the F and RBC2 lines at all embryonic stages studied. Comparing different sexes within lines, the males expressed significantly more syndecan-1 than the females in the RBC2 line from ED 18 through 24. The F line males expressed more syndecan-1 than the F line females at ED 14, 18, 20, and 22.

No significant difference in syndecan-1 expression was detected among all the samples at 1 wk posthatch (Figure 5.2). However, the F line *p. major* muscle of both sexes expressed more syndecan-1 than corresponding muscle of the RBC2 line at 8, 12, and 16 wk posthatch. Comparing within lines, no difference was observed between the sexes in the RBC2 line except at 12 wk posthatch when the males had more syndecan-1 expression than the females. The F line males expressed more syndecan-1 than the females in most of the posthatch stages studied (Figure 5.2).

5.3.2 Syndecan-1 expression in turkey satellite cells

The expression of syndecan-1 increased during satellite cell proliferation in both lines (Figure 5.3). By 96 h after cell differentiation was initiated, a significant decrease in syndecan-1 expression was observed in all cell lines. The F line male cells expressed more syndecan-1 during cell proliferation than the RBC2 line male cells, but no difference was observed during differentiation. The F line female cells also had more syndecan-1 expression than the RBC2 line female cells at 0 h of cell differentiation, but expressed less syndecan-1 at 96 h of cell differentiation.

For the RBC2 line, female cells expressed more syndecan-1 than the male cells during proliferation and differentiation except at 48 h of cell differentiation (Figure 5.3).
For the F line, female satellite cells had more syndecan-1 expression than the male cells when cell differentiation was activated, but no difference was detected afterward (Figure 5.3).

5.3.3 Glypican expression in turkey p. major muscle

The F line males had significantly more glypican expression at ED 14 and 18 compared to the RBC2 line males (Figure 5.4). No difference in glypican expression was observed between male p. major muscle tissues from the different lines at other embryonic days. The expression of glypican was not different between lines for female embryos except at day 20 when the F line demonstrated a higher glypican expression than the RBC2 line.

The males expressed more glypican than the females in the RBC2 line at ED 20 and 22 (Figure 5.4). An elevation in glypican expression in the males was observed at ED 14, 18, 20, and 22 in comparison to the females in the F line.

No differences between the sexes in glypican expression was measured in the RBC2 line posthatch p. major tissue, whereas the F line males expressed an increased amount of glypican at 12 and 16 wk posthatch compared to the F line females (Figure 5.5). The F line males had more glypican expression than the RBC2 line males in posthatch muscle tissue starting at 8 wk. Only at 16 wk posthatch did the F line females display an increase in glypican expression compared to the RBC2 line females.
5.3.4 Glypican expression in turkey satellite cells

Glypican expression increased with cell differentiation and peaked in all cell lines 48 h after differentiation was induced (Figure 5.6). At 96 h of cell differentiation, a significant decrease in glypican expression was measured in RBC2 and F line satellite cells of both sexes. For male satellite cells, higher glypican expression was observed in the F line at the initiation of cell differentiation and lasted for 48 h compared to the RBC2 line (Figure 5.6). A similar expression pattern was observed during cell differentiation in female satellite cells from the different lines.

Comparing within lines, no sex difference was observed in the RBC2 line satellite cells, except at 0 h of differentiation when the female cells had more glypican expression than the male cells (Figure 5.6). Lower glypican expression was detected in the F line female cells at 48 h of proliferation and 48 h of differentiation as compared to the F line male cells.

5.3.5 In situ hybridization of syndecan-1 and glypican expression in turkey p. major muscle

No significant differences were observed in the percentage of muscle fibers that expressed syndecan-1 mRNA in embryonic p. major muscle as detected by in situ hybridization (Table 5.1). From ED 14 to 24, almost 100% of muscle fibers expressed detectable syndecan-1 mRNA (Table 5.1; Figure 5.7, 5.8, 5.9). No significant line and sex differences were observed. No syndecan-1 mRNA signals were detected in posthatch muscle tissue in all lines (Table 5.1).
Glypican expression in the *p. major* muscle tissue increased with embryonic development (Table 5.2). No glypican expression was detected at ED 14 and 16 in all line-sex subgroups (Figure 5.7). However, at ED 18, about 40% of muscle fibers expressed detectable glypican mRNA signals (Figure 5.8). By ED 24, the glypican expression was detected in almost all muscle fibers (Figure 5.9). No significant line and sex differences were observed. In posthatch *p. major* muscle, about 75% muscle fiber bundles expressed glypican signals at 1 wk posthatch in all lines, and 100% muscle fiber bundles expressed detectable glypican mRNA at 8, 12, and 16 wk posthatch (Table 5.2).

**5.4 Discussion**

During embryogenesis, syndecan-1 expression peaked 2 days earlier than glypican expression in the *p. major* muscle tissue. In agreement with the mRNA expression data measured by semi-quantitative RT-PCR, the glypican mRNA signal was detected later than syndecan-1 in embryonic *p. major* muscle by *in situ* hybridization. These data correlate well with the observation by Larraín et al. (1997) that syndecan-1 expression is down-regulated during muscle cell terminal differentiation and glypican expression increases after differentiation has been triggered (Brandan et al., 1996). During *in vivo* muscle development and growth, the proliferation and differentiation of myoblasts are not distinct phases as during *in vitro* myogenesis. In developing embryonic skeletal muscle tissue, some myoblasts initiate differentiation earlier than others. These cells may express less syndecan-1 but more glypican than those cells still proliferating. The syndecan-1 and glypican expression in the entire muscle tissue may be determined by the relative amount of myoblasts in the proliferation and differentiation
phases. More syndecan-1 may be expressed when most of the myoblasts are still proliferating, and more glypican may be expressed when most cells start differentiation. Thus, the delayed in glypican expression in *p. major* muscle may imply an overall transition of the majority of myoblasts from proliferation to terminal differentiation at those developmental stages.

Both syndecan-1 and glypican interact with FGF2, a strong stimulator of skeletal muscle cell proliferation and a potent inhibitor of cell differentiation. This allows syndecan-1 and glypican to regulate the binding of FGF2 to its signaling receptors on the cell surface (Steinfeld et al., 1996). However, the difference in potential roles for syndecan-1 and glypican in FGF2 signaling remains unknown. Based on the different expression patterns of syndecan-1 and glypican during muscle cell differentiation, Brandan and Larraín (1998) proposed that syndecan may present FGF2 to its high affinity tyrosine kinase receptors on the cell surface to stimulate FGF2 biological effects. In contrast, glypican may sequester FGF2 from the FGFR and block the FGF2 signaling transduced to the muscle cells. In the present study, the F line males had increased syndecan-1 expression at ED 14 and 16 in comparison to that of the RBC2 line males. This may indicate an enhanced FGF2 stimulation in the F line male *p. major* muscle at earlier embryonic stages. As FGF2 inhibits the transcription of myogenin, a transcription factor that is necessary to initiate myotube formation (Brunetti and Goldfine, 1990), enhanced FGF2 signaling will result in an increased or prolonged period of muscle cell proliferation. In support of this hypothesis, Summers and Medrano (1997) showed that high-growth mice have prolonged myoblast proliferation and delayed myotube formation during embryonic development. As a result of increased and prolonged
myoblast proliferation, more myoblasts may be available to contribute to the formation of secondary muscle fibers during embryonic development and result in a faster muscle development associated with hyperplasia in the F line embryos. The higher glypican expression in the F line at ED 18 and 20 compared to the RBC2 line may indicate a larger number of myoblasts that enter cell differentiation.

After a slight decrease in syndecan-1 expression compared to the first peak at ED 18 in the F line male and ED 20 in the RBC2 male, a second peak of syndecan-1 expression was observed which is accompanied by a significant decrease in glypican expression at ED 22 and 24 in F line males and RBC line males, respectively. These results may suggest a new wave of FGF2 stimulation in muscle tissue before hatch that may active satellite cell populations for the massive muscle growth that occurs by hypertrophy in the postnatal turkeys. The second peak of syndecan-1 expression appeared earlier in the F line males, which may imply that satellite cell proliferation was activated earlier in the F line than in the RBC2 line males.

A continued increase in syndecan-1 and glypican expression was detected in the entire F line *p. major* muscle during posthatch growth compared to the RBC2 line. The significant difference between syndecan-1 expression in the F and RBC2 lines appeared earlier than that of glypican expression. A reasonable potential explanation is that higher syndecan-1 expression in the F line muscle tissue may allow more FGF2 be presented to FGFR and more satellite cell proliferation is stimulated. As a result, more satellite cells would be available to fuse with the existing muscle fibers through muscle hypertrophy. This is supported by the results of Lilburn and Nestor (1991) that that the *pectoralis major* muscle weight in the F line is significantly higher with an average of 0.882 g at
hatch compared to 0.652 g in the RBC2 line, and the differences continued at 4, 8, 12, and 16 wk posthatch.

The satellite cells isolated from F line p. major muscle of both sexes displayed higher syndecan-1 expression during cell proliferation. This result correlates well with previous reports that the F line satellite cells proliferate faster than the RBC2 line satellite cells (Velleman et al., 2000). It is likely that more syndecan-1 in the F line cells enhances FGF2 signaling in those cells. Since FGF2 strongly stimulates cell proliferation, more FGF2 signaling in the F line satellite cells will result in an increase in cell proliferation rate.

Previous reports indicated that male turkey and chicken embryos have a larger skeletal muscle mass than the females (Burke and Sharp, 1989; Burke, 1994). In the present study, increased syndecan-1 expression was measured in the F and RBC2 line male p. major tissue in most of the embryonic developmental stages studied compared to the females. This may suggest that more myoblasts are produced by hyperplasia in the embryonic male p. major tissue. Elevated glypican expression was also measured at ED 20 and 22 in the male embryos from both lines compared to the females. These data suggest that in males the myoblasts enter terminal differentiation earlier than in the females. As a result of enhanced proliferation and differentiation of the myoblasts, the males may have increased embryonic skeletal muscle formation.

The expression of syndecan-1 and glypican is transcriptionally regulated by FGF2 signaling. Jaakkola et al. (1997) reported that the 5’ flanking region of the syndecan-1 gene contains an FGF-inducible response element. Once activated by FGF2, the transcription of the syndecan-1 gene is significantly increased. On the other hand,
glypican mRNA expression is inhibited dramatically by FGF2 treatment (Brucato et al., 2002). In skeletal muscle, it is likely that the expression of FGF2 determines the transcription levels and the relative ratio of syndecan-1 and glypican. We propose that the differential expression of syndecan-1 and glypican regulates the intensity of FGF2 signaling transduced to the muscle cells that will, in part, modulate muscle cell proliferation and differentiation. Hence, FGF2, FGFR, and cell surface HSPG including syndecan-1 and glypican form a regulatory loop that is critical in the regulation of skeletal muscle development and growth.
References


Figure 5.1 Reverse transcription polymerase chain reaction analysis of syndecan-1 gene expression in F and RBC2 line pectoralis major muscle at different embryonic developmental days (ED). The relative expression of syndecan-1 mRNA was normalized with GAPDH and adjusted with the weight of p. major muscle. Bars represent the standard error of the mean. Adjusted relative densities with no common letter were significantly different ($P < 0.05$).
Figure 5.2 Reverse transcription polymerase chain reaction analysis of syndecan-1 gene expression in F and RBC2 line pectoralis major muscle at different posthatch stages. The relative expression of syndecan-1 mRNA was normalized with GAPDH and adjusted with the weight of p. major muscle. Bars represent the standard error of the mean. Adjusted relative densities with no common letter were significantly different ($P < 0.05$).
Figure 5.3 Reverse transcription polymerase chain reaction analysis of syndecan-1 gene expression in F and RBC2 line satellite cells during proliferation (P) and differentiation (D). The relative expression of syndecan-1 mRNA was normalized with GAPDH. Bars represent the standard error of the mean. Adjusted relative densities with no common letter were significantly different ($P < 0.05$).
Figure 5.4 Reverse transcription polymerase chain reaction analysis of glypican gene expression in F and RBC2 line *pectoralis major* muscle at different embryonic developmental days (ED). The relative expression of glypican mRNA was normalized with GAPDH and adjusted with the weight of *p. major* muscle. Bars represent the standard error of the mean. Adjusted relative densities with no common letter were significantly different \((P < 0.05)\).
Figure 5.5 Reverse transcription polymerase chain reaction analysis of glypican gene expression in F and RBC2 line pectoralis major muscle at different posthatch stages. The relative expression of glypican mRNA was normalized with GAPDH and adjusted with the weight of p. major muscle. Bars represent the standard error of the mean. Adjusted relative densities with no common letter were significantly different (P < 0.05).
Figure 5.6 Reverse transcription polymerase chain reaction analysis of glypican gene expression in F and RBC2 line satellite cells during proliferation (P) and differentiation (D). The relative expression of glypican mRNA was normalized with GAPDH. Bars represent the standard error of the mean. Adjusted relative densities with no common letter were significantly different ($P < 0.05$).
Figure 5.7 *In situ* hybridization for syndecan-1 and glypican in *pectoralis major* muscle from F and RBC2 line turkeys at embryonic day 14. A-D) RBC2 line male; E-H) F line male; A and E) syndecan-1 antisense probe; B and F) syndecan-1 sense probe; C and G) glypican antisense probe; and D and H) glypican sense probe. Bar represents 60 µm.
Figure 5.8 *In situ* hybridization for syndecan-1 and glypican in *pectoralis major* muscle from F and RBC2 line turkeys at embryonic day 18. A-D) RBC2 line male; E-H) F line male; A and E) syndecan-1 antisense probe; B and F) syndecan-1 sense probe; C and G) glypican antisense probe; and D and H) glypican sense probe. Bar represents 60 µm.
Figure 5.9 *In situ* hybridization for syndecan-1 and glypican in *pectoralis major* muscle from F and RBC2 line turkeys at embryonic day 24. A-D) RBC2 line male; E-H) F line male; A and E) syndecan-1 antisense probe; B and F) syndecan-1 sense probe; C and G) glypican antisense probe; and D and H) glypican sense probe. Bar represents 60 µm.
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Table 5.1 Percent muscle fibers expressing syndecan-1 mRNA in *pectoralis major* muscle at different embryonic developmental days (ED) and posthatch stages detected by *in situ* hybridization.

\textsuperscript{a, b} Percentage with no common superscript are significantly differently different ($P<0.05$).
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Table 5.2 Percent muscle fibers expressing glypican mRNA in *pectoralis major* muscle at different embryonic developmental days (ED) and posthatch stages detected by *in situ* hybridization.

<sup>a-d</sup> Percentage with no common superscript are significantly differently different (*P*<0.05).
CHAPTER 6

EXPRESSION OF FIBROBLAST GROWTH FACTOR 2 AND ITS RECEPTOR DURING SKELETAL MUSCLE DEVELOPMENT FROM TURKEYS WITH DIFFERENT GROWTH RATES

Abstract

Fibroblast growth factor 2 (FGF2) is a key regulator of muscle cell proliferation and differentiation. To address how FGF2 and fibroblast growth factor receptor 1 (FGFR1) expression influences skeletal muscle development and growth, *pectoralis major* muscle was isolated at embryonic days 14, 16, 18, 20, 22, and 24, and at 1, 8, 12, and 16-wk posthatch from a turkey line (F) selected only for increased 16-wk body weight and its genetic control line (RBC2). The FGF2 and FGFR1 expression was measured by semi-quantitative reverse transcription polymerase chain reaction. The F line males expressed more FGF2 at embryonic days 14 and 16, and had more FGFR1 expression at embryonic day 18 than the RBC2 line males. The expression of these two genes was down-regulated at embryonic day 22 in the F line males compared to the RBC2 line males. Although no FGF2 expression was detected in posthatch muscle tissue, the F line turkeys expressed more FGFR1 at 8, 12, and 16-wk posthatch. During myogenic satellite cell proliferation, the F line cells had higher FGF2 and FGFR1 expression than the RBC2 line cells. The satellite cell responsiveness to FGF2 treatment was evaluated by the ability of the cells to proliferate. The male satellite cells were more responsive to FGF2 stimulation than the female cells in both lines. These results suggest
that the F line turkeys have increased FGF2 signaling that affects muscle cell proliferation and differentiation, and leads to an enhancement in muscle development and growth rate.

6.1 Introduction

During early embryonic development, presumptive myoblasts migrate to the appropriate sites for skeletal muscle formation and give rise to myoblasts. The myoblasts proliferate, align with each other, and eventually fuse to form multinucleated myotubes that develop into mature muscle fibers (Swartz et al., 1994). Another group of myogenic cells that reside between the basement membrane and the plasma membrane of the muscle fibers, the satellite cells, are the primary source of mononucleated cells that contribute to support postnatal muscle hypertrophy and muscle regeneration (Mauro, 1961; Moss and LeBlond, 1971). The complex processes of myogenesis and muscle growth are precisely regulated by a number of extrinsic regulators including growth factors such as transforming growth factor-β (TGF-β), epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor 2 (FGF2) (Dodson et al., 1996).

Fibroblast growth factor 2 is one of the major growth factors involved in the regulation of muscle growth. It is a potent stimulator of skeletal muscle cell proliferation and an intense inhibitor of differentiation (Dollenmeier et al., 1981). One biological effect of FGF2 during myogenesis is to inhibit the transcription of myogenin, a transcription regulatory factor required for the initiation of myotube formation (Brunetti and Goldfine, 1990). By suppressing myogenin expression, FGF2 maintains the skeletal muscle cells in a state of proliferation.
The FGF2 signal transduction pathway is mediated by high-affinity cell surface tyrosine kinase receptors. Four fibroblast growth factor receptors (FGFR), FGFR through 4, have been reported. The FGFR1 is the most widely studied one and is the major type found in muscle cells (Sogos et al., 1998). The tyrosine kinase domain of the FGFR1 is required for the inhibition of muscle cell differentiation (Kudla et al., 1998). An in vivo study indicated that FGFR1 signaling is necessary for maintaining skeletal muscle mass and normal muscle fiber organization (Flanagan-Steet et al., 2000). The availability of FGFR1 plays a critical regulatory role during myogenesis (Scata et al., 1999). Therefore, changes in FGFR1 transcription will have a direct influence on muscle development and growth.

In addition to FGFR, cell surface heparan sulfate proteoglycans (HSPG) are also required for FGF2 signaling. The cell surface HSPG, syndecans and glypicans, function as low affinity co-receptors of FGF2 that regulate the binding of FGF2 to FGFR and the activity of FGF2 (Rapraeger et al., 1991; Yayon et al., 1991). The combination of FGF2, FGFR, and HSPG expression determines the intensity of FGF2 stimulation, and regulates cell responsiveness to the FGF2 signal.

The expression of FGFR and HSPG is regulated by FGF2. Hodik et al. (1997) reported that an avian FGF receptor gene expression is induced by FGF2 in chicken satellite cells. The syndecan-1 gene has an upstream enhancer that is activated only by FGF (Jaakkola et al., 1997). With FGF2 treatment, the expression of syndecan-1 is up regulated whereas syndecan-2, -4, and glypican mRNA levels are down-regulated (Bansal et al., 1996). In skeletal muscle, changes in FGF2 concentration may influence FGFR and HSPG expression, which will ultimately alter the intensity of the FGF2 signal transduced to the
muscle cells and regulate muscle cell growth. Although the role of FGF2 in regulating \textit{in vitro} muscle cell proliferation and differentiation is well known, the relationship between the expression of FGF2 and \textit{in vivo} muscle growth has not been established. It was hypothesized that differences in the expression of FGF2 and its receptors in muscle tissue will affect cell proliferation and differentiation \textit{in vivo}, which can lead to changes in skeletal muscle development and growth rate.

To address how FGF2 and FGFR expression changes are related to skeletal muscle development and growth, a turkey line (F) selected only for increased 16-wk body weight (Nestor, 1984) and its randombred control line (RBC2) from which the F line was derived were used in the present study. The F line turkey has a heavier body weight and heavier \textit{pectoralis major} muscle weight (Nestor et al., 1987; Lilburn and Nestor, 1991). Myogenic satellite cells derived from the F line have increased proliferation and differentiation rates than the RBC2 cells (Velleman et al., 2000). Therefore, the F line turkey represents a faster muscle growth model and the RBC2 line is the genetic control.

The expression of FGF2 and FGFR1 in \textit{p. major} muscle and satellite cells from F and RBC2 lines were measured in the current study. In addition, satellite cell responsiveness to increasing concentrations of FGF2 was also measured. These data showed the influences of FGF2 and FGFR1 expression as it relates to muscle development and growth.
6.2 Materials and methods

6.2.1 Animal model

The F line turkey was selected only for increased body weight at 16-wk posthatch and was developed from the randombred control RBC2 line (Nestor, 1984). The RBC2 line has not been selected for any trait and little genetic change has been expected or observed (Nestor, 1977a; 1977b). Both the F and RBC2 line turkeys are maintained at the Ohio Agricultural Research and Developmental Center.

6.2.2 Sample collection

_Pectoralis major_ muscle tissue was collected from 5 RBC2 and 5 F male and female embryos at 14, 16, 18, 20, 22, and 24 days of development, and from 5 RBC2 and 5 F line turkeys of each sex at 1, 8, 12, and 16-wk posthatch. Three sets of sample were collected.

To identify the sex of turkey embryos, a PCR method (Liu et al., 2002) adapted from D’Costa and Petitte (1998) was used. The sex of the posthatch birds was identified by dissection. The _p. major_ muscle weight was recorded. The muscle tissue was quick frozen in liquid nitrogen and stored at –70 °C until use.

6.2.3 Cell culture

Satellite cells derived from the _p. major_ muscle of 7-wk-old F and RBC2 turkeys of each sex (Velleman et al., 2000) were plated on 35 mm gelatin-coated plates at a density of 35,000 cells per well. The cells were cultured as described by Liu et al. (2002). At 48 h intervals during cell proliferation and differentiation, the medium was removed
from one plate and the cells were rinsed twice with sterile phosphate buffered saline (PBS, 170 mM NaCl, 3 mM KCl, 10 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$, pH 7.08). The plates were stored at –70 °C until use.

Satellite cells used for the FGF2 responsiveness assay were plated on 16 mm gelatin-coated plates at a density of 15,000 cells per well. After a 24 h attachment in plating medium (10% chicken serum, 5% horse serum, 1% antibiotic/antimycotic, and 0.1% gentamicin in Dulbecco’s Modified Eagle Medium), the cells were rinsed twice with serum-free defined medium (McFarland et al., 1991). The cells were then cultured in defined medium [24] containing 0, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 ng/ml of FGF2. The medium was changed daily until 96 h after plating. At 96 h of cell proliferation, the defined medium was removed from the plates and the cells were rinsed twice with sterile PBS. The plates were stored at –70 °C until use.

6.2.4 Extraction of total RNA

Total RNA was extracted from $p$. major muscle tissue using a single step method developed by Chomczynski and Sacchi (1987). For the cultured satellite cells, total RNA was extracted according to the RNAqueous™ small-scale phenol-free total RNA isolation kit instruction manual (Ambion, Austin, TX).

6.2.5 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

The first strand of cDNA was synthesized according to the RETROscript™ First strand synthesis Kit for RT-PCR instruction manual (Ambion, Austin, TX). In brief, 2.5 µg total RNA, 100 nM Oligo (dT) primers, and nuclease-free H$_2$O up to 12 µl were
mixed in a thin-wall PCR tube, heated at 85 °C for 3 min, and cooled on ice for 1 min. The reverse transcription (RT) reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl$_2$), 10 µM of each deoxynucleotide triphosphate (dNTP), 10 IU RNase inhibitor, and 100 IU moloney murine leukemia virus reverse transcriptase were then added to a final 20 µl of volume. The RT reaction was carried out at 44 °C for 60 min, followed by a denaturation at 95 °C for 5 min.

Five microliters of the RT product was used as the template to amplify the cDNA. The polymerase chain reaction (PCR) was performed with 200 µM of each dNTP, 1X PCR reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, and 0.1% Triton X-100), 1.5 mM MgCl$_2$, 0.45 µM of each primer, and 1.25 IU TaqBead™ hot start polymerase (Promega Corp., Madison, WI) in a final 50 µl of volume. The FGF2 primers were adapted from Kocamis et al. (2001). The amplified 270 base pair (bp) turkey FGF2 cDNA fragment has 97% nucleotide homology to the chicken sequence. The FGFR1 primers were adapted from Mitchell et al. (1999). The amplified turkey FGFR1 cDNA fragment is 462 bp in size and has 99% nucleotide homology to the chicken sequence.

To amplify the FGF2 fragment, the PCR reaction was started at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 45 sec with a final extension at 72 °C for 10 min. The FGFR1 fragment was amplified by 94 °C for 5 min, then 32 cycles at 94 °C for 30 sec, 52.5 °C for 45 sec, 72 °C for 45 sec, and a final extension at 72 °C for 10 min. The cycle numbers for the PCR amplifications were maintained in the logarithmic phase for each primer set. The PCR products were applied on an ethidium bromide containing 1% agarose gel and electrophoresed in 1X TBE
buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.0). The bands were recorded with a Kodak 290 digital camera system and analyzed for optical density (OD) with Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY). The FGF2 and FGFR1 expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GenBank # U94327).

To adjust the expression of FGF2 and FGFR1 to *p. major* muscle weight, the amount of total RNA extracted from 0.5 g muscle tissue was calculated based on the absorbency ratio at 260 and 280 nm. The amount of total RNA in the entire *p. major* muscle was calculated based on the muscle weight. Since each RT reaction used 2.5 µg total RNA as the template, the amount of total RNA in the entire muscle tissue was divided by 2.5 µg to obtain an adjustment factor. The OD of each PCR product on the agarose gel was multiplied by the adjustment factor to obtain the adjusted relative density based on muscle weight.

**6.2.6 FGF2 responsiveness assay**

The method of FGF2 responsiveness assay was adapted from McFarland et al. (1995) by the analysis of DNA concentration using Hoechst 33258 fluorochrome (Sigma-Aldrich Corp., St. Louis, MO). In brief, the cells in each well were treated with 200 µl of trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA) in filtered 1X TNE buffer (10 mM Tris, pH 7.4, 1 mM EDTA, and 2 M NaCl) for 7 min at room temperature. The reaction was stopped with 20 µl of 10X trypsin inhibitor (48 IU/ml) and the plates were frozen at –70 °C for 1 h. The suspension was transferred to a “VIS-UV grade” methacrylate
cuvette (Life Science Product, Inc., Frederick, CO) containing 1.8 ml of working dye solution (0.2 µg/ml Hoechst 33258 fluorochrome in filtered 1X TNE), mixed well and incubated in the dark at room temperature for 1 h. The fluorescence was measured in a Perkin-Elmer 650-10LC fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) with excitation at 365 nm and emission at 460 nm. A range of 0.1 to 1.2 µg double stranded calf thymus DNA was used as the standard. There were 5 replications of each FGF2 treatment with each line, and the entire assay was repeated 3 times.

6.2.7 Statistical analysis

A randomized complete block model using the general linear model procedure of SAS (1985) was used to evaluate the effects of turkey line, sex, and developmental stages on FGF2 and FGFR1 expression in muscle tissue and satellite cells as determined by semi-quantitative RT-PCR. The means were separated by repeated $t$-test. This model was also used to evaluate the effects of turkey line, sex, and FGF2 concentration on satellite cell proliferation as measured by DNA concentration assay. The means were separated by line, age, and treatment. Differences were considered significant at $P < 0.05$.

6.3 Results

6.3.1 FGF2 expression in turkey p. major muscle

Fibroblast growth factor 2 expression increased with embryonic development in all lines and peaked at day 20 (Figure 6.1). The F line males expressed more FGF2 than
the RBC2 line males at embryonic days 14 and 16. However, more FGF2 expression was detected in the RBC2 line males at days 22 and 24 compared to the F line males. There were higher concentrations of FGF2 in the RBC2 line females at embryonic day 14, but the F line females had significantly higher FGF2 expression at embryonic day 16. No differences were detected between RBC2 and F line females at later embryonic stages.

Higher FGF2 expression was observed in RBC2 line males at embryonic days 20, 22, and 24 than the RBC2 line females (Figure 6.1). The F line males expressed significantly more FGF2 than the F line females at embryonic days 14, 20, and 22.

After peaking at embryonic day 20, FGF2 expression was down-regulated with development. There was no detectable FGF2 expression in the posthatch muscle tissue in all lines (data not shown).

6.3.2 FGF2 expression in turkey satellite cells

The expression of FGF2 increased during satellite cell proliferation and continued until 48 h after cell differentiation was initiated (Figure 6.2). Both the F line male and female satellite cells expressed more FGF2 during proliferation than the RBC2 line cells. No difference was found between the RBC2 and F line male satellite cells during differentiation, but the RBC2 line female cells expressed more FGF2 at 48 h and 96 h of differentiation than the F line female cells.

No significant difference between the sexes in FGF2 expression was detected in the F line satellite cells (Figure 6.2). A similar result was observed in the RBC2 line satellite cells except that the RBC2 line female cells had more FGF2 expression at 48 h of differentiation than the RBC2 line male cells.
6.3.3 FGFR1 expression in turkey p. major muscle

The F line males had significantly higher FGFR1 expression than the RBC2 line males at embryonic day 18, whereas the RBC2 line males expressed more FGFR1 at embryonic days 22 and 24 (Figure 6.3). Differences in FGFR1 expression in the females were detected at embryonic days 14 and 22, when the RBC2 line females expressed significant more FGFR1. Both the F and RBC2 line males had more FGFR1 expression than the females starting at embryonic days 14 and day 16, respectively. The higher FGFR1 expression in males remained until embryonic day 22.

The F line males had significantly higher FGFR1 expression than the RBC2 line males at 8, 12, and 16-wk posthatch, and the F line females expressed more FGFR1 than the RBC2 line females at 8 and 16-wk posthatch (Figure 6.4). No significant difference in FGFR1 expression was measured between different sexes in the RBC2 and F line muscle tissue in most of the posthatch stages studied.

6.3.4 FGFR1 expression in turkey satellite cells

The F line male satellite cells expressed significantly more FGFR1 at the initiation of differentiation (0D) than the RBC2 line male cells, and the elevated expression lasted until 48 h of differentiation (Figure 6.5). At 96 h of cell differentiation, the RBC2 line male cells expressed more FGFR1 than the F line male cells. No difference in FGFR1 expression was found between the RBC2 and F line female satellite cells during proliferation, but the RBC2 line female cells expressed more FGFR1 at 48 h of differentiation than the F line female cells.
The F line female satellite cells expressed more FGFR1 at 48 h of proliferation than the F line male cells, and the F line male cells had more FGFR1 expression at 0 h and 48 h of differentiation (Figure 6.5). No difference was detected between the sexes in RBC2 line satellite cells until 96 h of differentiation, when the RBC2 line male satellite cells had more FGFR1 expression.

6.3.5 Satellite cell responsiveness to FGF treatments

Satellite cell proliferation increased with FGF2 treatment in a dose-dependent manner in all lines (Figure 6.6). Both the RBC2 and F line male satellite cells were more sensitive to increasing concentrations of FGF2 compared to the female cells. No difference between the RBC2 and F line males was observed except at 0.1 ng/ml FGF2. However, the F line female satellite cells proliferated faster than the RBC2 line female cells with 0.1, 0.5, 5, and 10 ng/ml of FGF2.

6.4 Discussion

*Pectoralis major* muscle from the F line turkeys expressed more FGF2 at embryonic day 16 than the RBC2 line for both males and females. These results suggest that more FGF2 is available earlier during embryonic development in the F line to stimulate muscle growth. Since FGF2 is a strong stimulator of muscle cell proliferation but an inhibitor of differentiation (Dollenmeier et al., 1981), it is conceivable that the F line myoblasts proliferate faster than the RBC2 line cells. The results from the *in vitro* studies support this hypothesis in that the F line satellite cells had an increased proliferation rate compared to the satellite cells derived from the RBC2 line (Velleman et
al., 2000). As a result, more myoblasts are generated to support faster muscle growth through hyperplasia in the F line. It was also observed that the FGFR1 expression in the F line males was significantly higher than that in RBC2 line males at embryonic day 18. This result indicates that the intensity of FGF2 signal transduced to muscle cells has the potential to be higher in the F line muscle. This is supported by the results of Summers and Medrano (1997) that showed high-growth mice having prolonged myoblast proliferation during embryonic development. At embryonic days 22 and 24 in males, the F line samples had reduced expression of FGF2 and FGFR1 compared to the RBC2 line samples. These data suggest that a decrease in FGF2 signaling at later embryonic stages may allow myoblasts to differentiate at a faster rate in the F line since the inhibitory effect of the FGF2 on cell differentiation is limited by the lower concentration of FGF2 and FGFR1.

The increase in FGF2 and FGFR1 expression in F line males compared to the RBC2 line males began at embryonic days 14 and 18, and is associated with changes of HSPG expression. It was previously reported (Liu et al., 2002) that HSPG expression in the F line p. major muscle is increased significantly at embryonic days 14 through 18 compared to that in the RBC2 line samples in both males and females. These are the same stages that FGF2 and FGFR1 expression are increased. Since FGF2, FGFR1, and HSPG are key components of FGF2 signal transduction, the coordinated fashion of FGF2, FGFR1, and HSPG expression in the F line muscle is suggestive of an overall increase in FGF2 stimulation at those embryonic developmental stages that will result in prolonged myoblast proliferation during muscle development.
The fact that male *p. major* muscle from both lines expressed more FGFR1 than the females during embryonic development may explain, partially, the reason that males have larger skeletal muscle mass than females (Burke and Sharp, 1989; Burke, 1994). In addition, the HSPG levels in males were significantly higher than in females at embryonic days 14 and 16 in the RBC2 line and at embryonic days 14 through 20 in the F line (Liu et al., 2002). The higher concentrations of FGFR1 and HSPG in the male *p. major* tissue may result in an increase in the FGF2 stimulation. More FGF2 signaling in the male tissue will enhance muscle cell proliferation, which is required for muscle hyperplasia.

More FGF2 was expressed in the F line satellite cells than the RBC2 line cells during proliferation. Coordinated with FGF2 expression, the F line male satellite cells have increased HSPG expression during proliferation (Liu et al., 2002). Thus, more FGF2-FGFR-HSPG signal complexes may form and the overall FGF2 stimulation in the F line satellite cells be enhanced. Hence, the increase in FGF2 signaling will stimulate more cell proliferation as previously reported for the F line satellite cells (Velleman et al., 2000).

Differences in male and female satellite cells in responsiveness to FGF2 stimuli may also be due to the intensity of the FGF2 signal received by those cells. Previous data showed that the male satellite cells from both lines had more HSPG expression than the female cells during cell proliferation and differentiation, and no differences were observed in F and RBC2 line satellite cells within the same sex (Liu et al., 2002). Since the HSPG are the co-receptors of FGF2, and formation of FGF2-FGFR-HSPG complexes is a requirement for FGF2 signaling (Rapraeger et al., 1991; Yayon et al., 1991), the
amount of HSPG on the cell surface may directly affect the amount of FGF2 signaling complex formed, and in turn the intensity of FGF2 signal transduction. The male satellite cells, by increasing HSPG expression, are capable of forming more FGF2 signaling complexes and presenting more FGF2 to its receptors. Therefore, the male satellite cells are more sensitive in response to FGF2 treatment than the female cells.

Although no FGF2 expression was detected in the posthatch *p. major* muscle, FGFR1 expression in the F line was much higher than that in the RBC2 line. The muscle may synthesize less FGF2 with muscle development. However, the muscle cells may still receive FGF2 that was synthesized at earlier developmental stages or by other cells. The increased FGFR1 expression during posthatch muscle development in the F line may suggest a continued increase in FGF2 signaling to the muscle cells, and this signaling may activate more satellite cells to proliferate. As a result, the *p. major* muscle may grow faster in the F line due to muscle hypertrophy. Since all fibroblast growth factors share four FGFR for signal transduction (Szebenyi and Fallon, 1999) and changes in FGF1 level have been related to FGFR1 expression (Düsterhöft and Pette, 1999), it is also possible that the increase in FGFR1 expression in the F line may indicate a functional overlap of FGFR1 in signal transduction pathways involved with other FGF family growth factors.

In conclusion, the differences in the FGF and FGFR1 expression in *p. major* muscle, together with the expression of HSPG, may directly influence muscle cell proliferation and differentiation, and result in differences in muscle growth properties.
Reference


Figure 6.1 Reverse transcription polymerase chain reaction analysis of FGF2 gene expression in RBC2 and F line *p. major* muscle at different embryonic developmental days (ED). The relative expression of FGF2 mRNA was normalized with GAPDH and adjusted to the weight of *p. major* muscle. Bars represent the standard error of the mean. Adjusted relative densities with no common letter were significantly different (*P* < 0.05).
Figure 6.2 Reverse transcription polymerase chain reaction analysis of FGF2 gene expression in RBC2 and F line satellite cells during proliferation (P) and differentiation (D). The relative expression of FGF2 mRNA was normalized with GAPDH. Bars represent the standard error of the mean. Relative densities with no common letter were significantly different ($P < 0.05$).
Figure 6.3 Reverse transcription polymerase chain reaction analysis of FGFR 1 gene expression in RBC2 and F line *p. major* muscle at different embryonic developmental days (ED). The relative expression of FGFR1 mRNA was normalized with GAPDH and adjusted to the weight of *p. major* muscle. Bars represent the standard error of the mean. Adjusted relative densities with no common letter were significantly different (*P* < 0.05).
Figure 6.4 Reverse transcription polymerase chain reaction analysis of FGFR 1 gene expression in RBC2 and F line *p. major* muscle at different posthatch stages. The relative expression of FGFR1 mRNA was normalized with GAPDH and adjusted to the weight of *p. major* muscle. Bars represent the standard error of the mean. Adjusted relative densities with no common letter were significantly different ($P < 0.05$).
Figure 6.5 Reverse transcription polymerase chain reaction analysis of FGFR 1 gene expression in RBC2 and F line satellite cells during proliferation (P) and differentiation (D). The relative expression of FGF2 mRNA was normalized with GAPDH. Bars represent the standard error of the mean. Relative densities with no common letter were significantly different ($P < 0.05$).
Figure 6.6 F and RBC2 satellite cell responsiveness to increasing concentrations of FGF2. The cell responsiveness was measured by the DNA concentration. Bars represent the standard error of the mean. DNA concentrations with no common letter were significantly different ($P < 0.05$).
CHAPTER 7

SUMMARY

Major results from the current study:

- The F line male and female satellite cells expressed more FGF2 and syndecan-1 during proliferation than the RBC2 line cells, and had faster proliferation rates.
- The F line male satellite cells expressed more glypican during differentiation compared to the RBC2 line male cells, and had an increased differentiation rate.
- The F line male embryos had more FGF2 and syndecan-1 expression at early embryonic stages than the RBC2 line males.
- The F line turkeys of both sexes expressed more syndecan-1 and glypican during posthatch stages compared to the RBC2 line turkeys.
- The male embryos from both the F and RBC2 lines had more syndecan-1 and glypican expression than the females.

The objective of the current study was to develop an understanding about how heparan sulfate proteoglycans (HSPG), especially cell surface HSPG syndecan-1 and glypican, are involved in the regulation of skeletal muscle development and growth. Since the HSPG are involved in the regulation of fibroblast growth factor 2 (FGF2) signaling, and their expression is affected by FGF2, the expression of FGF2 and
fibroblast growth factor receptor 1 (FGFR1) with regard to muscle growth was also investigated.

To address these questions, a turkey line (F) genetically selected only for increased 16-wk body weight (Nestor, 1984) and an unselected randombred control line (RBC2) from which the F line was derived were used. The F line turkeys are heavier in body weight and posthatch *pectoralis major* muscle weight compared to the RBC2 line turkeys (Nestor at al., 1987; Liburn et al., 1991). A significant increase in the *p. major* muscle weight was measured in the F line for both sexes as early as from embryonic day 16 and continued until 16-wk posthatch. In addition to the differences in *in vivo* muscle development and growth between the F and RBC2 lines, increased cell proliferation and differentiation rates were measured in the F line myogenic satellite cells as compared to the RBC2 line cells (Velleman et al., 2000). Therefore, the F line turkeys represent a faster muscle growth model than its genetic control RBC2 line.

Cell surface HSPG, syndecans and glypicans, are low-affinity co-receptors for FGF2. They regulate the binding of FGF2 to its high-affinity tyrosine kinase receptors and affect FGF2 activity (Raprager et al., 1991; Yayon et al., 1991). Considering that FGF2 is one of the key growth factors involved in the regulation of myogenesis by stimulating muscle cell proliferation and inhibiting cell differentiation, changes in FGF2 signaling will lead to cell behavior changes that result in differences in muscle development and growth. Since the formation of a HSPG-FGF2-FGFR complex is a requirement for FGF2 stimulation (Raprager et al., 1991; Yayon et al., 1991), the cell surface HSPG expression is critical in regulating the FGF2 signaling in muscle tissue. *In vitro* studies indicated that the cell surface HSPG have unique expression patterns during
myogenesis. Syndecan-1, 3, and 4 expressions are down-regulated whereas syndecan-2 remains unchanged during cell terminal differentiation (Larraín et al., 1997; Fuentealba et al., 1999; Brandan et al., 1998). Glypican-1 (glypican) is the only member of the glypican family found in skeletal muscle and its expression is up-regulated during cell differentiation (Brandan et al., 1996). Based on the different expression patterns of syndecans and glypican, Brandan and Larraín (1998) proposed that the syndecans may present FGF2 to its receptors to stimulate biological effects of FGF2, whereas glypican may sequester the interaction of FGF2 with FGFR to block FGF2 signaling that is transducted to muscle cells.

The expression of syndecan-1 and glypican is regulated by FGF2 at the transcription level (Jaakkola et al., 1997; Brucato et al., 2002). The syndecan-1 gene has an FGF-inducible responsiveness element in its enhancer region, and its expression is up-regulated by FGF2 stimulation (Jaakkola et al., 1997). Unlike syndecan-1, the glypican gene expression is significantly inhibited by FGF2 treatment (Brucato et al., 2002). Based on these facts, a model of FGF2 and HSPG in regulating muscle development is proposed from the results of the current study (Figure 7.1). According to this model, increased expression of FGF2 will result in elevated expression of syndecan-1 but decreased glypican expression. In muscle cells during proliferation, more syndecan-1 may exist on the cell surface than glypican. As a result, more FGF2 will be presented to the FGFR by syndecan-1. The continuation of FGF2 signaling will maintain the cells in a state of proliferation. With the decrease in FGF2 expression, less syndecan-1 will be expressed and more glypican will be synthesized. The FGF2 is sequestered from the
FGFR by binding to glypican. Thus, the inhibitory effect of FGF2 on cell differentiation is limited, and cell differentiation and myotube formation are allowed to proceed.

The results from both the in vitro and in vivo studies support the proposed model. In myogenic satellite cell culture, more FGF2 was expressed in the F line during cell proliferation. Coordinated with the FGF2 expression, the F line satellite cells had higher syndecan-1 expression during proliferation and displayed a faster proliferation rate than the RBC2 line cells. The F line male satellite cells expressed more glypican during cell differentiation, with no differences in syndecan-1 and FGF2 expression compared to the RBC2 line male satellite cells. Accordingly, the F line male satellite cells had increased differentiation. The expression of FGF2 in p. major muscle tissue from the F line males increased at embryonic day 14. Correspondingly, the syndecan-1 expression increased at the same time compared to the RBC2 line males. This increased syndecan-1 expression may allow more FGF2 binding to FGFR to form the HSPG-FGF2-FGFR complexe that is required for FGF2 signaling. Enhanced FGF2 signaling may lead to a faster or prolonged myoblast proliferation in the F line muscle tissue similar to the high-growth mice (Summers et al., 1997). As a result, more myoblasts may be produced to support the formation of secondary muscle fibers and result in a heavier p. major muscle weight associated with hyperplasia. No FGF2 expression was detected in the posthatch p. major muscle tissue. However, the FGF2 stimulation may continue during posthatch stages since FGF2 molecules produced earlier or by cells other than the muscle cells may be accumulated in the p. major tissue. The increased FGFR1 expression may imply a continued FGF2 signaling during posthatch muscle growth. Unlike in vitro myogenesis in which the cell proliferation and differentiation are distinctively separated, a part of the
muscle cells in skeletal muscle tissue are differentiating whereas other cells remain in proliferation at a given developmental stage. Thus, relative amounts of syndecan-1 and glypican expressed in the muscle tissue may decide the ratio of muscle cells undergoing proliferation and differentiation. Both syndecan-1 and glypican were expressed at high concentrations in the F line posthatch *p. major* tissue. It is possible that more satellite cells in the F line posthatch muscle tissue are activated to proliferate by increased syndecan-1 expression that leads to an enhancement of FGF2 signaling. At the same time, more cells are induced to differentiate as the FGF2 signaling is sequestered by the increased glypican expression in those cells. As a result, the F line turkeys may have a faster muscle growth through hypertrophy.

Burke (1994) reported that the male turkey embryos have a heavier muscle mass than the females. A similar result was observed in both the F and RBC2 line turkeys of different sexes used in the current study. This also can be explained partially by the presented model of FGF2 and HSPG in regulating muscle growth. Although no significant differences in FGF2 expression was detected in the *p. major* muscle tissue of the different sexes, the males from both the F and RBC2 lines expressed significantly more syndecan-1 and glypican during embryonic development than the females. More syndecan-1 may present and stimulate more FGF2 signaling for cell proliferation. And more glypican expression can sequester the FGF2 and allow an increase in myoblast differentiation.

The FGF2 regulates not only the syndecan-1 and glypican expression but also influences the expression of the transcription factor myogenin. Myogenin is a muscle specific transcription factor that is critical for the onset of myoblast differentiation and
myotube formation (Venuti et al., 1995). By inhibiting myogenin expression, FGF2 inhibits myotube formation and keeps the cells for proliferation. The increased expression of FGF2 in the F line *p. major* muscle at embryonic day 14 may also have a direct influence on muscle cells to prolong the cell proliferation and delay the differentiation.

Taken together, changes in cell surface HSPG syndecan-1 and glypican expression were related to differences in muscle development and growth. Cell surface HSPG play critical roles in regulating FGF2 signal transduction pathway and are involved in the regulation of skeletal muscle myogenesis through a FGF2-dependent manner.
Reference


Figure 7.1 Model of FGF2 and cell surface HSPG syndecan-1 and glypican regulate skeletal muscle proliferation and differentiation.
LIST OF REFERENCES


197


