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IDENTIFICATION AND MAPPING OF DIFFERENTIALLY EXPRESSED GENES IN FETAL AND POSTNATAL PIG SKELETAL MUSCLE

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IDENTIFICATION AND MAPPING OF DIFFERENTIALLY EXPRESSED GENES IN FETAL AND POSTNATAL PIG SKELETAL MUSCLE

By

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ABSTRACT

IDENTIFICATION AND MAPPING OF DIFFERENTIALLY EXPRESSED GENES IN FETAL AND POSTNATAL PIG SKELETAL MUSCLE

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Fetal myogenesis and postnatal skeletal muscle hypertrophy in growing pigs are critical yet poorly understood processes. Global gene expression analyses can identify key genes and pathways controlling these processes. In addition, integration of gene expression data with genome map information will facilitate identification of genes controlling economically important trait phenotypes. This study was designed to identify differentially expressed genes in developing pig skeletal muscle and locate them on the pig genome map. The specific objectives were: 1) Identify differentially expressed genes in hind limb skeletal muscle of pigs at 60 days of gestation and 7 weeks of age; and 2) Determine the map locations for differentially expressed genes. A combination of differential display RT-PCR, cDNA microarray analysis and oligonucleotide microarray analysis were used to identify differentially expressed genes. In total, over 200 differentially expressed genes were revealed and expression patterns for eight genes were evaluated by relative real time RT-PCR, confirming differential expression for seven of them. Twenty-four genes were mapped to 13 different pig chromosomes using the INRA-University of Minnesota (IMpRH) 7,000 rad radiation hybrid panel. This study represents a first step toward characterizing the transcriptional profile of developing pig skeletal muscle and it improves the porcine-human comparative map.

I dedicate this thesis to my mother, Patricia Rilington whose love and support has kept me going throughout the years. Mom, thank you for being the best mother and role model.

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Literature Review

Introduction

Skeletal muscle is the most abundant tissue in animals and, as meat, it is an economically important food source. The amount of muscle an animal has at market weight is predetermined by the size and number of muscle fibers (reviewed by Novakofski and McCusker, 1993) and fiber number is determined during fetal development (Swatland and Cassens, 1973). Fetal myogenesis is thus an extremely important research topic and many reports discuss the structural changes, contractile proteins and regulatory factors involved in the process of skeletal muscle development. Still, relatively little is known about the complex gene expression patterns associated with this process. Postnatal growth expands the prenatally developed fibers by increasing the diameter and length of the skeletal muscle. Researchers have discovered a number of important genes involved in this process including several growth factors and transcription factors. However, the complete transcriptional profile of developing skeletal muscle is unknown.

Gene expression profiles must be integrated with genome maps to fully understand complex biological mechanisms such as skeletal muscle development. Mapping of differentially expressed genes facilitates integration of genetic variants that affect phenotypic expression of economically important traits. Specifically for pigs, this will lead to maps that will be more informative for

study of biological mechanisms controlling economically important traits such as muscle growth and meat quality. Such integrated maps will also facilitate research using the pig as an animal model for human studies.

Skeletal Muscle Development

Fetal myogenesis

Fetal myogenesis is a complicated process involving coordinated regulation of proliferation and differentiation of myogenic cells. At around day 18 to 20 of gestation in pigs, mesenchymal cells differentiate into committed myogenic precursor cells. These mononucleated proliferating cells migrate from the somites into the growing limb buds to eventually become myoblasts (reviewed by Novakofski and McCusker, 1993). The migrating cells can not become myoblasts until the transcription factor, paired box gene 3 (PAX3) is expressed (Epstein et al., 1996). PAX3 activiates the transcription of c-met, which then interacts with scatter factor (or hepatocyte growth factor, HGF; Dietrich et al., 1999). In order for myogenic precursor cells to become myoblasts, myogenic regulatory factors (MRFs) need to be expressed. PAX3 induces expression of the MRFs, myogenic factor 3 (MYOD1) and myogenic factor 5 (MYF5), which are helix-loop-helix transcription factors. MYOD1 and MYF5 are required at the determination step to commit proliferating precursor cells to the myogenic lineage (Rudnicki et al., 1993). The myoblasts then proliferate, further differentiate into myocytes and mature into myofibers through the action of the MRFs, myogenin (MYOG) and myogenic factor 6 (MYF6; reviewed by Sabourin and Rudnicki, 2000). Myoblast proliferation and

differentiation is also regulated by growth factors. Insulin-like growth factor-I (IGF-I) and IGF- II stimulate myoblast proliferation and differentiation, while fibroblast growth factor (FGF) stimulates myoblast proliferation. Transforming growth factor – β (TGF-β) inhibits FGF and decreases both proliferation and differentiation (reviewed by Florini and Magri, 1989).

Myoblasts proliferate and begin to align with other myoblasts and fuse. The fusing myoblasts form primary myotubes (reviewed by Novakofski and McCusker, 1993). In pigs, production of myotubes or primary fibers begins at approximately 40 days of gestation and primary fibers determine the future size and location of the muscle tissue. At around 50 to 60 days of gestation, secondary fibers form by adhering to the primary fibers (Wigmore and Stickland, 1983). By 70 days of gestation, primary fiber formation has slowed down compared to secondary fiber formation, and by 90 days of gestation secondary fiber formation has also slowed (Beermann et al., 1978) so that at birth (approximately 114 days), the number of muscle fibers in the animal is set.

Postnatal hypertrophy

After birth, muscle growth occurs through hypertrophy by which the muscle increases in size and length. Muscle size is affected by growth factors and exercise, and myogenic satellite cells mediate the postnatal growth of muscle (Schultz, 1989, 1996). Muscle fiber hypertrophy is associated with an increase in DNA content. Because the differentiated myonuclei do not have the ability to synthesize DNA, satellite cells contribute new nuclei by fusing with the growing muscle. Thus, the roles of satellite cells include muscle regeneration,

muscle hypertrophy and postnatal muscle growth (Darr and Schultz, 1987; Grounds, 1998; Grounds and Yablonka-Reuveni,1993; Rosenblatt et al., 1994). The IGFs, FGF, TGF-β and platelet-derived growth factor (PDGF) have all been shown to affect satellite cell proliferation and differentiation. PDGF stimulates satellite cell proliferation (for review see Yablonka-Reuveni, 1995) and FGF stimulates proliferation and depresses differentiation, whereas IGF-I stimulates both proliferation and differentiation, and TGF-β depresses proliferation and inhibits differentiation (Allen and Boxhorn, 1989).

Gene Expression Profiling of Skeletal Muscle Tissue

Several techniques have been developed for evaluating mRNA abundance in tissues or cells. Techniques such as northern blot analysis and real-time reverse transcription PCR (RT-PCR) are effective, but they are limited to examination of only one or a few genes at a time. Thus, screening of hundreds or thousands of genes using these techniques would be time and cost prohibitive. More global approaches are needed to simultaneously examine expression patterns of large numbers of genes. One technique for doing this is differential display RT-PCR (DDRT-PCR; Liang and Pardee, 1992). However, large-scale DDRT-PCR analyses can also be time consuming and expensive. The mapping of the human genome spurred a new generation of gene expression techniques and DNA microarray technologies have emerged as popular methods for identifying differentially expressed genes.

DNA Microarrays

Types of microarrays include different platforms to which the probes are adhered including nylon membranes, glass slides and silicon chips. The probes can either be spotted cDNAs, PCR amplification products, short (25-30mer) oligonucleotides or longer (50-70mer) oligonucleotides. Each of these platform and probe types has been used successfully in many research areas. Microarrays produced by Affymetrix, a short oligonucleotide chip company, have not commonly been used by investigators involved in livestock animal research because chips specific for these species have not been available, although Affymetrix is currently in the process of introducing these products. Other platforms have been very popular for livestock animal research and, as cDNA library and expressed sequence tag (EST) database resources have been developed, both cDNA and long oligonucleotide microarrays have been produced.

Microarrays are heavily integrated into research in many different aspects of the scientific community. Thousands of studies using microarray technologies have been reported. Therefore, the discussion of microarray experiments in this literature review will focus on studies involving gene expression profiling of skeletal muscle. Microarrays have been used to examine skeletal muscle gene expression in humans, mice, rats, zebrafish, cattle and pigs. This research has covered a broad range of subject matter including diseases, exercise and nutritional effects on gene expression. For example, skeletal muscle gene expression profiles have been reported for cancer studies (Basso et al., 2004,

Kappler et al., 2004), Duchenne muscular dystrophy (DMD) studies (Companaro et al., 2002; Muntoni et al., 2002; Noguchi et al., Porter et. al., 2002; Porter et al., 2003a; Porter et al., 2003b; 2003; Winokur et al., 2003;), and studies of hormonal effects (Rome et al., 2003; Sreekumar et al., 2002a, Viguerie et al., 2004; Yang et al., 2002), dietary effects (Linnane et al., 2002, Reverter et al., 2003; Sreekumar et al., 2002b,c) and exercise effects (Carson et al., 2002; Hittel et al., 2003; Mu et al., 2003; Wu et al., 2003).

Additional gene expression profiling studies in humans, mice and rats have identified differentially expressed transcripts between *quadriceps* (white muscle) and *soleus* (red muscle) in mice (Campbell et al., 2001), the effect of neuregulin, a heparin sulfate proteoglycan on primary human myotubes (Jacobson et al., 2004), the effects of forkhead type transcription factor 1 on skeletal mass (Kamei et al., 2004), and metabolic adaptations in skeletal muscle during lactation (Xiao et al., 2004). Other experiments identified candidate genes involved in skeletal muscle injury in mice (Summan et al., 2003), examined the anti-oxidative response of carbonic anhydrase III in skeletal muscle (Zimmerman et al., 2004), and determined effects of reducing temperatures in adult zebrafish (Malek et al., 2004). Zhou et al. (2004) identified distinct gene expression clusters in idiopathic inflammatory myopathies in muscle biopsies. Transcriptional differences were also examined in muscle wasting due to spaceflight (Nikawa et al., 2004, Taylor et al., 2002) and in burn victims receiving anabolic steroid treatment (Barrow et al., 2003). Finally, in a unique application of microarray analysis, Cronin et al. (2004) used an oligonucleotide microarray to

demonstrate that protein-coated poly(L-lactic acid) fibers were a suitable substrate for growing human skeletal muscle cells because expression profiles did not differ from cells grown on standard tissue culture plates.

As discussed above, myoblast differentiation is a critical step in early fetal skeletal muscle development. Tomczak et al. (2004) used expression profiling to examine gene expression during a 12-day time course of differentiating C2C12 myoblasts. The differentiating C2C12 cells progressed through a predictable pattern of myogenic events as the myoblasts ceased proliferating and began differentiating. Tomczak et al. (2004) found that MYF5 expression decreased gradually while MYOD1 transcripts peaked at the onset of differentiation, and MYOG and MYF6 were induced later in the time course. These results were expected because MYF5 and MYOD1 are required at the determination step to commit proliferating myoblasts, whereas MYOG and MYF6 are required for myoblast differentiation. Several transcripts involved in cell-cycle regulation, cell signaling, ion transport, and nucleic acid and protein metabolism exhibited high expression levels in the proliferating myoblasts and decreased over the rest of the time course. Another group of transcripts including genes involved in muscle contraction, muscle development, metabolism, cell signaling, ion transport and transcription were observed to be undectable or lowly expressed during proliferation but to increase progressively throughout the rest of time course. The use of microarrays to examine proliferating and differentiating myoblasts in this study identified both genes known to be involved in muscle development and also previously unknown genes. Moran et al. (2002) also performed a study with

proliferating and differentiating myoblasts that covered a shorter time course. Their results were similar to Tomczak et al. (2004) in that differentially expressed genes fell into functional categories including muscle contraction, cell adhesion, extracellular matrix, cellular metabolism, mitochondrial transport, DNA replication, cell cycle control, mRNA transcription and immune regulation.

The role of growth factors in muscle development was also discussed above. IGF action is critical both for maintaining viability during the transition from proliferating to differentiating myoblasts and for facilitating differentiation. PDGF can sustain cell survival but inhibits differentiation. Kuninger et al. (2004) used microarrays to identify genes induced by IGF-I and PDGF in myoblasts. This study identified 28 muscle-specific genes whose expression was uniquely stimulated by IGF-I including MYOG, several enzymes such as a calcium-dependent ATPase and creatine kinase, numerous transcripts for components of the contractile apparatus such as α-actin, several troponins, myosin heavy and light chains, and tropomyosin, and two sarcoglycans, among others. In contrast, no muscle-specific transcripts were identified among the 41 known genes that were differentially induced by PDGF. Thus, this study begins to define a transcriptional profile of genes induced by IGF-I and PDGF in skeletal muscle.

Transcriptional changes in skeletal muscle associated with aging have been examined in humans, mice and rats. Roth et al. (2002), in a study to determine the influence of age, sex, and strength training (ST) on gene expression patterns in skeletal muscle, identified 50 genes affected by age that represented structural, metabolic, and regulatory gene classes. Welle et al.

(2001) examined gene expression differences in young vs. old skeletal muscle of both mice and men. They identified 17 differentially expressed genes that were similar in mice and men and 32 that were dissimilar. Six were classified as overexpressed in both mice and men, 19 as overexpressed in mice but not in men, 11 as underexpressed in both mice and men, and 13 as underexpressed in mice but not in men. This study demonstrated not only gene expression differences associated with aging, but also species differences in skeletal muscle gene expression patterns.

In 2003, Welle et al. reported a more thorough study that examined gene expression profiles between younger (21-27 yr old) and older men (67-75 yr old). A total of 718 genes were differentially expressed and older muscle was observed to express several hundred more genes than younger muscle. Genes that encode proteins involved in energy metabolism and mitochondrial protein synthesis were expressed at lower levels in older muscle. Genes encoding metallothioneins, high-mobility-group proteins, heterogeneous nuclear ribonucleoproteins and other RNA binding/processing proteins, and components of the ubiquitin-proteasome proteolytic pathway were expressed at higher levels in older muscle. Subsequently this research group conducted a similar study in young and old women (Welle et al., 2004) and the results agreed with those of the men's study. Approximately 1,000 genes were differentially expressed with more genes expressed in older muscle. In addition, over 100 genes involved in energy metabolism were expressed at lower levels in older muscle and over 40

genes encoding proteins that bind to pre-mRNAs or mRNAs were expressed at higher levels in older muscle.

Zhang et al. (2002) observed gene expression patterns in skeletal muscle of young (3 months) vs. old (30 months) rats. The study found 127 differentially expressed genes, among which some genes down-regulated in older muscle were involved in energy metabolism and signal transduction, while some up-regulated genes were related to protein degradation and cell apoptosis. A similar study by Pattison et al. (2003) examined rats of the same ages and identified 682 differentially expressed genes, of which 347 were decreased in older muscle relative to younger muscle with a major category being genes that encode extracellular matrix and cell adhesion proteins. Of the 335 genes increased in older muscle, many were involved in immune response, proteolysis, or stress/antioxidant response. These studies examining aging in skeletal muscle provide insight into genes that may be involved in skeletal muscle development.

To date, only a few studies have been reported involving microarray analysis of pig skeletal muscle. However, the availability of resources for conducting such studies is rapidly increasing. Complementary DNA libraries for pig skeletal muscle have been constructed from adult *biceps femoris* (Davoli et al., 1999) and from an ontogeny of samples from five developmental time points (Yao et al., 2002). These projects have increased the number of ESTs available from pig skeletal muscle. Before pig microarrays became available, Moody et al. (2002) reported successful cross species hybridization using human nylon microarrays with porcine skeletal muscle samples. Zhao et al. (2003) produced a

cDNA nylon macroarray that contained 327 pig ESTs and reported 28 genes that were differentially expressed in pig hind limb skeletal muscle at 75 days of gestation and 1 week of age. Specifically, genes including elongation factor 1 alpha, vimentin, splicing factor arignine/serine rich 12, GABA-A, tubulin, protein phosphatase 2C alpha, several genes encoding ribosomal proteins and several genes of unknown function were more highly expressed at 75 days of gestation, a timepoint when secondary fibers are rapidly forming. Also, glyceraldehyde-3-phosphate dehydrogenase and a gene of unknown function were more highly expressed at 1 week of age when muscle is undergoing rapid hypertrophy. This study gives insight into genes involved in skeletal muscle development.

Bai et al. (2003) reported development of a microarray that included 5,500 clones from two developmentally distinct pig skeletal muscle cDNA libraries and they performed an initial screen of the array with *psoas* and *longissimus dorsi* (LD) muscle RNA from a 22-week-old pig. They found 70 genes that were more highly expressed in the *psoas* and 45 genes that were more highly expressed in the LD, thus identifying, candidate genes influencing muscle phenotypes. Subsequently, da Costa et al. (2004) used this same array to examine the effects of dietary restriction on skeletal muscle gene expression. Twenty genes were more highly expressed in both the LD and *psoas* muscles of pigs fed a low protein and energy diet. Also, thirteen genes were more highly expressed in the *psoas* of pigs fed the restricted diet and 5 were more highly expressed in the LD. The differentially expressed genes affected metabolism, energy, translation and growth. The findings also identified novel genes that have growth modulatory

properties and could play pivotal roles in growth suppression and muscle phenotype determination, which all affect skeletal muscle development. Porcine microarray research has a long way to go to reach the level of research in humans, mice and rats. However, it is likely that in a few years there will be a similar flood of research reports when accessibility to these technologies increases through an increase in the number of pig ESTs and the development of pig microarrays.

Comparative Mapping

Comparative gene mapping utilizes information from species such as human and mouse that have complete genome sequences available to improve the resolution of genome maps for species such as the pig that are not fully sequenced. These maps then aid in the identification of candidate genes for economically important traits such as growth, health, and product quality. In addition, gene expression profiling studies reveal genes involved in the expression of important trait phenotypes. Thus, in order to fully utilize the available information for identifying genes controlling economically important traits, it is important to integrate gene expression data with genome map information.

Development of the porcine-human comparative map has continued to make great advancements over the past decade. A comprehensive study of human-pig conservation using chromosomal painting was reported by Goureau et al. (1996) who used a bidirectional approach in which both human and pig probes were hybridized to metaphase spreads of the opposite species. This

study identified 37 conserved regions between humans and pigs. Following this, a somatic cell hybrid panel was developed (INRA SCHP; Yerle et al., 1996) that allowed for regional localizations of genes on pig chromosomes. Higher resolution maps can be achieved with the use of radiation hybrid (RH) panels. RH panels are constructed by fusing irradiated DNA from a species of interest such as the pig with a rodent cell line to form a panel of stable hybrid cell lines that each contains a different complement of the genome of interest. The most widely used pig RH panel is the INRA-University of Minnesota (IMpRH) 7,000 rad panel (Yerle et al., 1998; Hawken et al., 1999). The first generation porcine whole-genome RH map developed with this panel contained a total of 903 markers (Hawken et al., 1999). More recently, this group has developed a 12,000 rad RH panel that allows for more accurate resolution of gene order to further improve the pig-human comparative map (Yerle et al., 2002).

Many ESTs from cDNA libraries derived from various tissues have been mapped using the INRA SCHP, IMpRH and other panels. These include 67 ESTs from female reproductive tissues (Shi et al., 2001; Tuggle et al., 2003), 182 EST clusters from porcine back fat libraries (Mikawa et al., 2004), and 214 ESTs from a porcine small intestine cDNA library (Cirera et al., 2003). Davoli et al. (2002) reported a first genomic transcript map for pig skeletal muscle that included 125 ESTs derived from their pig *biceps femoris* cDNA library. In efforts to identify positional candidate genes in QTL regions, 20 ESTs were mapped to pig chromosomes 9 and 3 (Middleton et al., 2003) and 28 ESTs were mapped to pig chromosome 10 (Aldenhoven et al., 2003), where QTL for economically

important reproduction and carcass traits have been reported (Hirooka et al., 2001; Malek et al., 2001a,b, Rohrer and Keele 1998a,b; Rohrer et al., 1999; Rohrer 2000; Wada et al., 2000;). Rink et al. (2002) reported the most comprehensive pig EST comparative mapping effort so far by assigning 1,058 EST markers to the IMpRH. Thus, mapping of ESTs to the pig genome map improves the porcine-human comparative map and facilitates the identification of candidate genes for economically important traits.

Summary

Skeletal muscle development is controlled by a complicated biological mechanism. A great deal is known about the structural changes, regulatory genes and growth factors contributing to fetal myogenesis and postnatal hypertrophy. However, relatively little is known about the complexity of gene expression patterns associated with these developmental stages. With the advent of microarray technology, the opportunity for examining these patterns is available.

Numerous studies have used microarray technology to examine gene expression patterns in skeletal muscle of humans, mice and rats. To date, only a few reports have used these technologies to examine gene expression patterns in pig skeletal muscle. However, this is expected to increase in the near future as the availability of porcine EST sequences increases, and cDNA and oligonucleotide microarrays become more accessible. In addition, the integration of gene expression and genetic mapping information will lead to connecting

phenotypic expression to genomic positions, thereby accelerating the discovery of candidate genes.

We hypothesize that growth and development of skeletal muscle tissue is associated with distinct gene expression patterns that are unique to specific developmental stages. This study was designed to identify differentially expressed genes in pig skeletal muscle between pigs at a fetal age corresponding to the initiation of secondary fiber formation and postnatal pigs undergoing rapid muscle hypertrophy. In addition, the study included locating some of these genes on the pig genome map. The specific objectives were to:

1. Identify differentially expressed genes in hind limb skeletal muscles of pigs at 60 days of gestation and 7 weeks of age; and
2. Determine the map locations for differentially expressed genes.

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CHAPTER 2

Differential Gene Expression in Fetal and Postnatal Pig Skeletal Muscle

Abstract

Fetal myogenesis and postnatal skeletal muscle hypertrophy in growing pigs are critical yet poorly understood processes. Global gene expression analyses can be used to increase understanding of these processes by identifying key genes and pathways controlling skeletal muscle development. For this study, three techniques including differential display reverse transcription PCR (DDRT-PCR), a pig skeletal muscle cDNA microarray and a pig 70-mer oligonucleotide microarray were applied to identify differentially expressed genes in hind limb skeletal muscle tissue of pigs at 60 days of gestation and 7 weeks of age. The cDNA and oligonucleotide microarray experiments revealed 35 and 163 genes, respectively, that were differentially expressed between the 60 day fetal and 7 week postnatal samples. The DDRT-PCR experiment also included skeletal muscle tissue from pigs at 105 d of gestation and revealed 16 putatively differentially expressed genes. The genes TTN, MTCO3 and MTND4 were identified by all three techniques to be more highly expressed at 7 weeks of age. Three additional genes TNNC1, TNNC2 and GAPD were identified by both of the microarray platforms to be more highly expressed at 7 weeks of age. Two genes were revealed to be differentially expressed by both DDRT-PCR and the oligonucleotide microarray; COL1A2 was more highly expressed at 60 days of gestation and MYH4 was more highly expressed at 7 weeks of age. Relative real-time RT-PCR was used to validate differential expression of six genes

observed to be significantly differentially expressed by DDRT-PCR, cDNA microarray analysis and/or oligonucleotide microarray analysis. These genes were CNN3, FN1, TTN, TCAP, TPT1 and TNNC1, and significant differential expression was confirmed for all of them except TNNC1. Two additional genes not identified by DDRT-PCR or microarray analysis, TTID and PXN, were also determined to be differentially expressed. Thus, these results provide new information regarding developmental patterns of gene expression in pig skeletal muscle.

Introduction

Although, the physical development of porcine fetal skeletal muscle has been well characterized (http://www.aps.uoguelph.ca/~swatland/ch6_0.htm), the molecular mechanisms controlling this process have not been fully elucidated. In pigs, primary fiber or myotube formation begins at approximately 40 days of gestation and primary fibers determine the future location and size of the muscle tissue. Secondary fiber formation begins at 50 to 60 days of gestation when multinucleated myoblasts align and fuse to form secondary fibers at the surface of existing primary fibers. The formation of primary and secondary fibers is essential for muscle growth because the number of muscle fibers is determined during fetal development (Swatland and Cassens, 1973). Postnatal hypertrophy then increases the length and diameter of these fibers. Thus, the number and size of the fibers determines the amount of muscle an animal has at market weight (for review see Novakofski and McCusker, 1993).

Numerous gene products, including growth factors, binding proteins, receptors, extracellular matrix components, enzymes and transcription factors participate in the coordinated regulation of the myogenic program. Yet relatively little is known about complex gene expression patterns in skeletal muscle and, to date, only a small number of genes have been examined. Muscle specification and differentiation appear to be controlled by a family of basic helix-loop-helix myogenic regulatory factors (MRFs; MyoD, Myf-5, myogenin and Myf-6/MRF4) that transactivate many muscle-specific promoters (for review see Sabourin and Rudnicki, 2000). In addition, a variety of hormones and growth factors are capable of regulating myoblast proliferation and differentiation (for review see Hawke and Garry, 2001). The stimulatory action of insulin-like growth factors-I and –II (IGF) on proliferation and differentiation, mitogenic effects of fibroblast growth factor (FGF), and inhibitory action of transforming growth factor-beta (TGF-β) on muscle cells are well documented (for review see Florini et al., 1991; Florini et al., 1996). Also, hepatocyte growth factor (HGF) has been shown to activate myogenic satellite cells and simulate satellite cell proliferation (Miller et al., 2000), and platelet-derived growth factor (PDGF) stimulates satellite cell proliferation (for review see Yablonka-Reuveni, 1995), whereas myostatin, a member of the TGF-β family, inhibits myoblast proliferation (Thomas et al., 2000).

In order to gain a more complete understanding of the mechanisms controlling myogenesis, a thorough knowledge of the gene products that direct muscle development during different stages of growth is needed. In porcine

skeletal muscle, developmental expression of a few select genes, such as myostatin (Ji et al., 1998) and the IGFs (Gerrard et al., 1998), have been examined. However, we understand little about how complex patterns of gene expression ultimately affect muscle development and growth. Global gene expression analyses can be used to identify key genes involved in this process. Several techniques have been developed for simultaneously evaluating expression patterns of numerous genes, including differential display reverse transcription PCR (DDRT-PCR), cDNA microarrays and oligonucleotide microarrays. All of these techniques allow large-scale gene expression analyses for transcriptional profiling of complex processes such as skeletal muscle development, and each technique offers various benefits and limitations. We have applied all three techniques to examine gene expression differences in pig fetal and postnatal skeletal muscle tissue.

Numerous recent studies involving applications of microarray technologies to evaluate gene expression patterns in skeletal muscle have been reported including several developmental studies evaluating muscle cell differentiation *in vitro* (Kuninger et al., 2004; Moran et al., 2002; Tomczak et al., 2004). To date, few reports have considered normal developmental patterns of fetal and postnatal skeletal muscle or used agricultural species in such analyses. Using a cDNA nylon macroarray, Zhao et al. (2003) identified 28 genes that were differentially expressed between pig skeletal muscle samples at 75 days of gestation and 1 week of age postnatal. Bai et al. (2003) constructed a pig skeletal muscle cDNA microarray and used it in an initial study to examine

differential expression of genes between the *psoas* (a red muscle) and the *longissimus dorsi* (a white muscle) of a 22-week-old pig. Among their results, they identified 22 sarcomeric/structural genes that were more highly expressed in the *longissimus dorsi*. Subsequently, this group used their cDNA microarray to examine nutritional effects on skeletal muscle gene expression (da Costa et al., 2004). Similarly, Reverter et al. (2003) used a bovine cDNA microarray to evaluate nutritional effects on skeletal muscle gene expression in cattle. Our experimental strategy is unique from these previous studies because we have used various techniques to examine characteristics of normal growth and development of pig skeletal muscle tissue during specific developmental stages. Although Moody et al. (2002) reported successful cross-species hybridization of pig skeletal muscle cDNA to human nylon microarrays, the availability of large numbers of porcine ESTs has now made it feasible to develop pig-specific microarray resources including oligonucleotide microarrays. Therefore, our experiment utilized DDRT-PCR, a pig skeletal muscle cDNA microarray and a pig 70-mer oligonucleotide microarray to examine expression patterns of genes in hind limb skeletal muscle tissue of pigs at 60 days of gestation and 7 weeks of age. Our results provide new insights regarding gene expression changes during fetal and postnatal skeletal muscle development that can be used to enhance pig production efficiency, as well as for comparative developmental biology using the pig as a model for other mammalian species.

Materials and Methods

Tissue samples and RNA isolation. Skeletal muscle tissue samples were obtained from the hind limbs of pig fetuses at 60 days of gestation, fetuses at 105 days of gestation and postnatal pigs at 7 weeks of age. To obtain fetal samples, Yorkshire X Landrace crossbred gilts bred to the same boar (n = 3 per gestational stage) were slaughtered in a federally inspected abattoir and fetuses were removed for tissue collection. Three additional gilts were allowed to carry their litters to term (114 days) and one pig from each litter was euthanized at 7 weeks of age for tissue collection. Samples were immediately flash frozen in liquid nitrogen and stored at -80°C. Total RNA from 1.0 g of tissue was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. For fetal samples, tissues from several pigs within each litter were pooled to provide a sufficient sample size, whereas postnatal samples were obtained from individual animals. RNA concentration and quality were determined with an RNA 6000 Pico LabChip® kit using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA) and RNA quality was also assessed by agarose gel electrophoresis.

Northern blot analyses of myogenin and myogenic factor 6. Northern blot analyses were performed to evaluate mRNA abundance of myogenin (MYOG) and myogenic factor 6 (MYF6). Total RNA (30 μg) from each of the 60 days of gestation, 105 d of gestation and 7 weeks of age samples was electrophoresed in 1.2% agarose formaldehyde gels, transferred to nylon membranes (Schleicher and Schuell, Inc., Keene, NH) and UV cross-linked. Probes were generated by

[α-^{32}P]dCTP labeling of cDNAs specific for rat MYOG (gift of W. Wright, The University of Texas Southwestern Medical Center, Dallas TX) or rat MYF6 (gift of S. Konieczny, Purdue University, West Lafayette, IN) using the Multiprime DNA Labeling System (Amersham Pharmacia Biotech, Piscataway, NJ). An 18S rRNA probe was used to adjust for equality of RNA loading. Membranes were prehybridized at 65°C for 2 h with 10 ml of hybridization solution (6X SSC, 5X Denhardt's solution, 0.5% SDS, 0.1 mg/ml sheared salmon sperm DNA). Fresh hybridization solution and denatured probe were added and incubated at 65°C for 18 h. Blots were rinsed and exposed to Kodak X-Omat AR film at -80°C. Signal intensities from autoradiographs were determined by scanning laser densitometry and relative intensities of RNA bands were analyzed by analysis of covariance using a model containing the effect of age along with the 18S rRNA values as covariables.

Differential display reverse transcription-PCR. DDRT-PCR experiments were performed as previously described by our laboratory (Wesolowski et al., 2004) using modifications of published procedures (Liang and Pardee, 1992). A total of eight oligonucleotide primer pairs (3 anchor primers each paired with 1-4 arbitrary primers) were used corresponding to screening of ~5% of all mRNA species present. Following amplification and electrophoresis of the nine RNA samples as described (Wesolowski et al., 2004), fragments that amplified in all three samples of at least one developmental age and were faint or undetectable in the remaining age(s) were excised from the gels, reamplified, cloned and sequenced. Clone sequence identities were determined using the basic local

alignment search tool (BLAST) software and the nonredundant database of GenBank.

cDNA microarray. A normalized porcine skeletal muscle (PoSM) cDNA library was constructed at the Michigan State University Center for Animal Functional Genomics (CAFG) from hind limb skeletal muscle tissue collected at 45 days of gestation, 90 days of gestation, birth, 7 weeks of age and 1 year of age (Yao et al., 2002). A cDNA microarray was constructed in the MSU CAFG using 768 randomly selected clones from the PoSM library. All clones were spotted in triplicate and arrayed in 48 8X8 patches using a Flexys® G3 Robotic Workstation (Genomic Solutions, Inc., Ann Arbor, MI). Quality controls included on the array were 336 positive hybridization controls (bacteriophage Lambda Q gene), 384 blank spots and 48 negatives (10% DMSO). The cDNA microarray screening included only the 60 day gestation and 7 week postnatal samples. Each of the 60 day samples was randomly paired with a 7 week sample. Four cDNA microarray slides were screened. The 60 day gestation samples were labeled with Cy5 and the 7 week postnatal samples were labeled with Cy3 on three of the slides and, for the fourth slide, the dyes were swapped so that the 60 day sample was labeled with Cy3 and the 7 week sample was labeled with Cy5. After analysis, clones that were identified to be differentially expressed were sequenced to determine their identities.

Oligonucleotide microarray. Oligonucleotide microarrays used for this study consisted of 13,297 70-mer oligos (Pig Array-Ready Oligo Set v. 1.0 and Pig Oligo Extension Set v. 1.0, Qiagen, Inc., Valencia, CA) each spotted once on

a single slide. Slides were printed at the University of Minnesota Advanced Genetic Analysis Center and were distributed through the U.S. Pig Genome Coordination Program. Controls included 76 Arabidopsis thaliana gene spots, 17 beta tubulin spots, 17 glyceraldehyde-3-phosphate dehydrogenase spots, 85 heat shock protein gene spots, 69 ribosomal protein gene spots, 112 randomly generated negative control spots and 470 blanks. Like the cDNA microarray, the oligonucleotide microarray was screened with only the 60 day gestation and 7 week postnatal samples. Six oligonucleotide microarray slides were screened. All samples were labeled with both Cy3 and Cy5, and each 60 day gestation sample was randomly paired with two 7 week postnatal samples.

cDNA synthesis, hybridization and scanning. For each sample, 8 µg of total RNA was reverse transcribed with an oligo dt_{18} primer using the Superscript™ Indirect cDNA Labeling System (Invitrogen) according to the manufacturer's instructions. After first-strand synthesis and purification, the cDNAs incorporated amino-modified dUTPs and were labeled with *N*-hydroxysuccinate (NHS) ester Cy3 or Cy5 dyes (Amersham Biosciences, Piscataway, NJ). The labeled cDNAs were purified, combined and concentrated to 10 µl using a microcon spin column (Millipore, Bedford, MA). The concentrated probe was combined with 100 µl of Slide Hyb#3 solution (Ambion, Inc. Austin, TX) and denatured at 70°C for 5 min. Microarray hybridizations took place in sealed hybridization chambers in a GeneTAC™ Hybridization Station (Genomic Solutions) for 18 hours using step-down temperatures ranging from 65°C to 42°C. Following hybridization, the slides were washed twice with

medium stringency buffer and once with high stringency buffer (Genomic Solutions). The slides were rinsed in 2XSSC and deionized water and were dried using centrifugation at 1000xg for 2 min. Fluorescent images were detected by scanning on a GeneTAC™ LS IV Biochip Analyzer (Genomic Solutions). Fluorescence intensity data were collected and background fluorescence was subtracted using the GeneTAC™ Integrator and Analyzer software (Genomic Solutions). Total intensity values for each dye channel were stored as comma-separated values data files and exported into Microsoft Excel spreadsheets for subsequent analysis.

Normalization and statistical analysis of microarray data. The fluorescence intensity data obtained from both microarray platforms was $\log_2$ transformed and LOESS normalized for dye intensities (Yang et al., 2002). For the oligonucleotide microarray, the data for one patch were deleted from the datasets due to a printing error on the slides. This resulted in the loss of oligonucleotides for 292 genes. Statistical analysis included a two-stage mixed model (Wolfinger et al., 2001) using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The first stage used a global normalization with a fixed effect of dye and random effects of array, animal, patch within array and dye*patch(array). The second stage gene specific analysis included fixed effects of age, dye and age*dye and random effects of array and animal within age. False discovery rate (FDR) was calculated as an adjustment for multiple comparison testing (Benjamini et al., 1995) using the SAS procedure PROC

mulitest (SAS Inst. Inc.). In addition, q values for FDR testing were calculated as described by Storey and Tibshirani (2003).

Relative real-time reverse transcription PCR. Relative real-time RT-PCR was used to validate microarray results and examine specific expression patterns of additional related genes. Assays were developed using the nine RNA samples to validate differential expression of six genes observed to be significantly differentially expressed by DDRT-PCR, cDNA microarray analysis and/or oligonucleotide microarray analysis. These genes were calponin 3 (CNN3), fibronectin 1 (FN1), titin (TTN), titin-cap (TCAP), translationally controlled tumor protein (TPT1) and slow troponin C (TNNC1). Assays were also developed for two additional genes that are functionally related to one or more of these genes, titin immunoglobulin domain protein (TTID; also referred to as myotilin) and paxillin (PXN). Primers were designed using Primer Express software v 2.0 (Applied Biosystems, Foster City, CA) and are shown in Table 1. All assays were performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) in the MSU CAFG.

To identify an appropriate control gene for each assay, amplification efficiencies (Livak and Schmittgen, 2001) were determined by performing a SYBR green reaction (as described below) using a serial dilution (4 dilutions) from one of the nine cDNA samples. The cycles to threshold (Ct) were averaged for each dilution for the control and target gene. The averages were subtracted to obtain the delta Ct, after which the log of input of each dilution was plotted against the delta Ct to determine the slope. Efficiencies were considered

acceptable when slopes were < |0.1|. Following the amplification efficiency tests, it was determined that hypoxanthine phosphoribosyltransferase (HPRT) was an appropriate normalizing gene for CNN3, FN1, PAX, TCAP and TTID. HPRT was not suitable for TTN, TPT1 and TNNC1 so the 18S ribosomal RNA gene was used as a control for these genes. The nine samples were assayed in duplicate using SYBR Green PCR Master Mix (Applied Biosystems). Each reaction contained: 1X SYBR Green mix, 300nM of each primer pair, 50ng cDNA (except TTID 200ng) and water for a final volume of 25 µl. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). The ΔCt was computed as explained above and the $\Delta\Delta Ct$ was determined using the average of the 60 d samples as the calibrator. Significance was determined by analyzing Ct values using the PROC MiXED procedure of SAS with a model containing the fixed effect of age, a random effect of pig nested within age and HPRT or 18S Ct values as covariables.

Results

mRNA abundance of myogenin and myogenic factor 6. Results of northern blot analyses for myogenin (MYOG) and myogenic factor 6 (MYF6) are shown in Fig. 1. Hybridization of northern blots with MYOG and MYF6 probes revealed single transcripts of 1.8-kb and 1.6-kb, respectively (data not shown). Abundance of MYOG mRNA was highest at 60 days of gestation and decreased significantly by 105 days of gestation. Abundance of MYF6 mRNA exhibited a pattern opposite that of MYOG such that MYF6 expression was similar at 60 and 105 days of gestation, but increased significantly by 7 weeks of age.

Identification of differentially expressed genes by DDRT-PCR. DDRT-PCR was used to evaluate differences in mRNA transcript abundance in pig skeletal muscle at 60 days of gestation, 105 days of gestation and 7 weeks of age postnatal. Nineteen putatively differentially expressed fragments were excised from the DDRT-PCR gels, reamplified and cloned. Sequencing of these fragments revealed three clones corresponding to TTN and two clones corresponding to cytochrome c oxidase III (MTCO3). Thus, 16 unique genes were identified (Table 2). To minimize the identification of false positives, three samples per age were compared. Only bands that displayed consistent patterns within an age group and differential expression patterns between at least one of the other groups were selected.

Identification of differentially expressed genes by cDNA microarray analysis. A total of 38 clones were found to be differentially expressed between skeletal muscle samples at 60 days of gestation and 7 weeks of age (fold change ≥ 1.5 and $P \leq 0.06$; Table 3). In total, 76 clones were identified to be significantly different at $P \leq 0.06$. For this microarray, we would expect approximately 46 significant differences to occur by chance. Thus, it is likely that some of the observed differences are true differences. In addition to statistical significance, we have also considered only clones with a fold change difference ≥ 1.5. These 38 clones corresponded to 35 genes because multiple clones were significant for two genes (two clones for CDC-like kinase 1 and three clones for cytochrome b). Thirteen genes were more highly expressed at 60 days of gestation and 22 were more highly expressed at 7 wks of age. Approximately 40% of these genes have

unknown identities and approximately 11% are mitochondrial genes. The remainder fall into various functional categories including approximately 14% involved in muscle contraction and 14% that encode enzymes. Differentially expressed genes involved in muscle contraction were more highly expressed at 7 weeks of age and included alpha actin, titin, tropomyosin 4, troponin C slow and troponin C fast.

Identification of differentially expressed genes by oligonucleotide microarray analysis. Mixed model analysis of the oligonucleotide microarrays revealed a total of 193 oligonucleotides with significantly different signal intensities between the 60 day fetal and 7 week postnatal samples (fold change $\geq$ 1.5 and $P \leq 0.05$; Table 4). Sixty-seven of these were significantly different at $P \leq 0.01$. In total, 1135 oligonucleotides were identified to be significantly different at $P \leq 0.05$. For this microarray, we would expect approximately 650 significant differences to occur by chance. Thus, it is likely that some of the observed differences are true differences. In addition to statistical significance, we have also considered only oligonucleotides with a fold change difference $\geq$ 1.5. The FDR was calculated, but there were no genes that had $P < 0.1$, although 5 genes were found at $P = 0.12$. The q values were also calculated as recommended by Storey and Tibshirani (2003) and 5 genes were identified at $q = 0.12$ with only one of these exhibiting a fold change $\geq$ 1.5. Due to the small sample sizes examined in this study, these adjustments may be too strict for this dataset.

Of the 193 significantly different oligonucleotides, 109 were observed to be more highly expressed at 7 weeks of age, while the remaining 84 were more

highly expressed at 60 days of gestation. The 193 oligonucleotides corresponded to 163 unique genes (89 more highly expressed at 7 weeks of age and 74 more highly expressed at 60 days of gestation). Two genes, glyceraldehyde-3-phosphate dehydrogenase (GAPD) and ribosomal protein S18 (RPS18) that were spotted in multiple locations on the microarray were found to be significantly different. Fourteen spots containing an oligonucleotide specific for GAPD were more highly expressed at 7 weeks of age (range of fold changes 1.71-3.02, $P \leq 0.04$). Similarly, 11 spots corresponding to an oligonucleotide specific for RPS18 were more highly expressed at 60 days of gestation (range of fold changes 1.56-1.97, $P \leq 0.03$). Seven genes observed to be more highly expressed in the 7 week samples were found to have two significant oligonucleotides corresponding to each gene present on the microarray. One of these was a second oligonucleotide for GAPD and the others included MTCO3, MYOZ1, PDLIM7, PYGM, RPS4X and TTN. While the presence of multiple oligonucleotides for the same gene was unexpected, the fact that two independent oligonucleotides for the same gene yielded significant results adds confidence that these genes were truly differentially expressed. Approximately 42% of the differentially expressed genes have unknown identities and five genes more highly expressed in the 7 week samples are mitochondrial genes. The remainder fall into various functional categories including approximately 9% involved in muscle contraction and 19% that encode enzymes. Most differentially expressed genes involved in muscle contraction were more highly expressed at 7 weeks of age, although

myosin heavy polypeptide 3 (MYH3) and myosin light polypeptide 4 (MYL4) were more highly expressed in the 60 day fetal samples.

Confirmation of differential expression. Six genes were selected from the DDRT-PCR and microarray experiments based on their functional roles in skeletal muscle structure and contraction for validation using relative real-time RT-PCR. Two additional genes that were functionally related to the differentially expressed genes were also selected for evaluation. Although the microarray experiments did not include the samples obtained from pigs at 105 days of gestation, these samples were included in the relative real-time RT PCR analyses in order to reveal additional information regarding the developmental expression patterns of the selected genes.

Assays were developed for four genes involved in muscle contraction (Fig. 2). Titin (TTN) was observed to be differentially expressed on both microarray platforms and also several TTN clones were obtained in the DDRT-PCR experiment. Slow troponin C (TNNC1) was observed to be differentially expressed on both microarray platforms and titin-cap (TCAP) was observed to be differentially expressed on the oligonucleotide microarray. Titin immunoglobulin domain protein (TTID) was also evaluated. Statistical analyses of the microarray data did not reveal TTID to be significantly differentially expressed at the cutoff thresholds of a fold change ≥ 1.5 and $P \leq 0.05$. However, on the oligonucleotide microarray, TTID exhibited a 1.27-fold higher expression in the 7 week postnatal samples at $P = 0.06$. In addition, TTID is functionally related to TTN and TCAP in that they are all proteins of the skeletal muscle Z-disc so we chose to further

evaluate TTID. Relative real-time RT-PCR analyses confirmed the microarray and DDRT-PCR results for TTN and TCAP and also revealed that TTID expression was significantly increased in the 7 week samples. Evaluation of the 105 day fetal samples indicated that TTID expression was intermediate between the 60 day fetal and 7 week postnatal samples, whereas TTN expression at 105 days was similar to the 60 day samples and TCAP expression at 105 days was similar to the 7 week samples. Thus, even though the products of these genes are functionally related, inclusion of the 105 day gestation samples revealed subtle differences in the expression patterns for these genes. Expression of TNNC1 appeared to be higher in the 7 week postnatal samples (105 day vs. 7 week $P = 0.06$). However, large sample-to-sample variation in TNNC1 mRNA abundance for the 7 week samples limits this interpretation.

Assays were developed for three genes involved in cytoskeletal structure (Fig. 3). Calponin 3 (CNN3) and fibronectin 1 (FN1) were observed by DDRT-PCR to be more highly expressed in the 60 day fetal samples and their expression patterns were confirmed by relative real-time RT-PCR. Paxillin (PXN) was also evaluated because of its functional relationship to FN1. Unlike FN1, PXN mRNA abundance was not found to be different between the 60 day fetal and 7 week postnatal samples, but PXN expression in the 105 day fetal samples was significantly higher than the 60 day and 7 week samples.

A final gene that was selected for validation was translationally controlled tumor protein 1 (TPT1). Abundance of TPT1 mRNA was significantly higher in the 7 week postnatal samples confirming the cDNA microarray results.

Expression of TPT1 in the 105 day samples was found to be intermediate between the 60 day fetal and 7 week postnatal samples.

Discussion

We have used three different approaches to examine transcriptional profiles in a set of samples obtained from hind limb skeletal muscle tissue of pigs at 60 days of gestation, 105 days of gestation and 7 weeks of age postnatal. The most comprehensive technique that we used was screening of a 13,000 member 70-mer pig oligonucleotide microarray. In addition, we used a relatively small cDNA microarray that contained 768 cDNAs derived from a pig skeletal muscle specific cDNA library (Yao et al., 2002) and we conducted a DDRT-PCR experiment with a limited number of primer combinations. While comparisons between these platforms are limited by the small size of the cDNA microarray and DDRT-PCR experiments, we were able to identify some of the same genes using two or more of the techniques.

All of the techniques were able to reveal genes with relatively large differences in mRNA abundance, but they were less robust for identifying genes with more subtle differences in mRNA abundance. Three genes were revealed by all three techniques to be more highly expressed at 7 weeks of age. Two of these were the mitochondrial genes MTCO3 and MTND4, indicating differences in energy metabolism between the 60 day fetal and 7 week postnatal samples. The sarcomeric protein TTN was also discovered to have higher mRNA abundance at 7 weeks of age by all three techniques. Three additional genes were revealed by both of the microarray platforms (TNNC1, TNNC2 and GAPD)

and two genes were revealed by both DDRT-PCR and the oligonucleotide microarray (COL1A2 and MYH4).

Only a few reports in the literature have considered comparisons of various microarray platforms and most of these have involved comparisons of cDNA or long oligonucleotide microarrays with Affymetrix GeneChip arrays. Wang et al. (2003) compared 70-mer oligonucleotides and cDNAs for the same genes printed on the same glass slide and they reported a correlation coefficient of 0.80 with approximately 8% of the genes examined showing discordant results. Park et al. (2004) systematically compared an Affymetrix array, a custom cDNA array and custom oligonucleotide arrays. They concluded that in general Affymetrix and cDNA arrays agreed fairly well, but that the long oligonucleotide arrays were less concordant. Also, they noted that highly expressed genes gave fairly similar results on all of the platforms, but lowly expressed genes were much more variable. Our study using both a 70-mer oligonucleotide microarray and a cDNA microarray was not designed as a systematic comparison of the two platforms as were the Wang et al. (2003) and Park et al. (2004) studies, but our results appear to agree with these studies in that we were able to identify differences in some highly expressed genes using both platforms.

While several reported studies have used DDRT-PCR to identify differentially expressed genes in skeletal muscle samples, to our knowledge no previous studies have been reported that examined the same samples with both DDRT-PCR and microarray analyses. Other laboratories have used DDRT-PCR to successfully identify differentially expressed genes involved in skeletal muscle

development (Cho et al., 2000; Janzen et al., 2000; Levin et al., 2001, McDaneld et al., 2004) and we have used DDRT-PCR to identify differentially expressed genes in developing pig fetuses (Wesoloski et al., 2004). The disadvantage of DDRT-PCR vs. microarray approaches is clearly that it is a much more time consuming technique to perform. However, DDRT-PCR does have some advantages. Gene discovery with DDRT-PCR does not require prior knowledge of gene or EST sequences as is needed for construction of microarrays (Stein and Liang, 2002). In addition, DDRT-PCR allows direct comparisons to be made between more than two samples at a time and it may be more sensitive for detection of relatively low abundance transcripts. Even with a limited number of primer combinations, we were able to identify 16 putatively differentially expressed genes, five of which were also revealed by one or both of the microarray platforms.

Zhao et al. (2003) used a cDNA nylon macroarray containing 327 ESTs to examine differential gene expression in pig fetal and postnatal skeletal muscle. Twenty-eight genes were identified in this study to be differentially expressed between 75 day fetal and 1 week postnatal skeletal muscle samples. The present study extends these observations to include evaluation of higher density microarrays and additional developmental ages of pigs. The Zhao et al. (2003) study observed differential expression for several ribosomal protein genes and we also observed differences in many ribosomal protein genes pointing toward the key role of protein synthesis mechanisms in muscle development. Zhao et al. (2003) also observed higher expression of GAPD in the 1 week postnatal

samples than in the 75 day fetal samples, which agrees with our results from both microarray platforms indicating that GAPD mRNA abundance was greater in the 7 week postnatal samples than in the 60 day fetal samples. Identification of an appropriate housekeeping gene for use as a control in gene expression analyses such as real time RT-PCR is critical and GAPD is frequently used for this purpose. We have previously observed that GAPD is not a suitable control for evaluating developing skeletal muscle tissue (unpublished data), and our results as well as those of Zhao et al. (2003) support this observation.

We initially evaluated our samples by examining mRNA abundance of two myogenic regulatory factor (MRF) genes that we predicted to be differentially expressed in developing pig skeletal muscle. The MRFs are members of the basic helix-loop-helix family of transcription factors and their expression is specific to skeletal muscle (for review see Sabourin and Rudnicki, 2000). In our study, relative abundance of MYOG mRNA was highest in pig skeletal muscle at 60 days of gestation, whereas abundance of MYF6 mRNA was highest at 7 weeks of age. Our results agree with reports of developmental expression patterns for these genes in mice and rats (Bober et al., 1991; Hinterberger et al., 1991) providing evidence that expression of these genes is developmentally regulated.

We selected six genes from the DDRT-PCR and microarray analyses and two additional genes for further evaluation using relative real time RT-PCR. Of the four genes that had been identified by microarray analyses (TTN and TNNC1 identified by both platforms, TCAP identified only on the oligo array and TPT1

identified only on the cDNA array), three were validated using relative real-time RT-PCR. The results indicated that the magnitude of the fold changes observed with the real time RT-PCR assays was much greater than had been observed with the microarrays, pointing to the greater sensitivity of real time RT-PCR for detecting differences in mRNA abundance. This appears to be a common observation when genes identified by microarray analyses are confirmed by real time RT-PCR (Park et al., 2004). The only gene whose expression pattern was not confirmed was TNNC1. The real time RT-PCR results for this gene indicated a tendency toward higher expression in the 7 week postnatal samples, but large sample-to-sample variation among the 7 week samples limited the interpretation of the results. Northern blot analysis of human fetal and adult TNNC1 revealed a weak signal in the fetal tissue and abundant signal in the adult tissue (Gahlmann et al., 1988) which agrees with the microarray results for pig TNNC1 in the present study. Two genes that were observed to be differentially expressed only by DDRT-PCR (CNN3 and FN1) were confirmed by real time RT-PCR analyses and two additional genes (TTID and PXN) selected for their functional relationship to the other genes were also confirmed to be differentially expressed.

Clearly sarcomeric proteins are essential for muscle function and the Z-disc is an important contractile component (for review see Faulkner et al., 2001). Several genes whose products are a part of the Z-disc structure were observed to be more highly expressed in the 7 week postnatal samples: ACTA1, CAPZA2, FLNC, PDLIM3, TTN, TCAP and TTID. These proteins are all linked together through a complex network of interactions. TCAP interacts with TTN (Gregorio et

al., 1998), CAPZA2 is an F-actin Ca^{2+} independent capping protein and PDLIM3 interacts with α-actinin 2 (Klaavuniemi et al., 2004). TTID is a thin filament associated protein that interacts with α-actin (Salmikangas et al., 2003) and its expression has been reported to increase throughout skeletal muscle development in mice (Mologni et al., 2001), which agrees with our results for developing pig skeletal muscle. Several contractile protein genes were also identified to be differentially expressed including MYH4, which exhibited higher mRNA abundance in the 7 week postnatal samples in both the DDRT-PCR experiment and the oligonucleotide microarray. In contrast, MYH3 and MYL4 were more highly expressed in the 60 day gestation samples, which is supported by the literature indicating these are embryonic genes (Ontell et al., 1993).

FHL1 from the four and half LIM family is reported to be expressed in skeletal muscle and to have elevated mRNA expression in postnatal growth (Morgan and Madgwick, 1995), which is in agreement with our oligonucleotide microarray results for FHL1. PYGM is a muscle glycogen phosphorylase that was found to be more highly expressed in the 7 week postnatal samples on the oligonucleotide array. This gene also appeared to be more highly expressed in the 7 week samples on the cDNA microarray ($P = 0.08$), however, the fluorescence intensity of the 60 day gestation samples was below background levels, which likely affected the analysis. This expression pattern agrees with results reported for humans in which fetal PYGM mRNA is not seen until 80-100 days of gestation (Omenn and Cheung, 1974; Miranda et al., 1985). PXN is a cytoskeletal protein involved in actin membrane attachment sites, cell adhesion,

focal adhesion and regulating the response to fibronectin (Hagel et al., 2002), and our results provide information regarding the expression patterns of PXN and FN1 in developing pig skeletal muscle.

Despite its name, TPT1 has many roles in different tissue types. It is regulated by growth signals, developmental factors and stress conditions, and it is involved in cell growth, apoptosis and microtubule stabilization (Bommer et al., 2004). Hu et al. (2003) used northern blot analysis to show that TPT1 is expressed mainly in heart and skeletal muscle. In addition, Bryne et al. (2005) found TPT1 to be more highly expressed in skeletal muscle of diet restricted Brahman steers. Our results demonstrating increased mRNA abundance during pig skeletal muscle development provide additional information regarding expression of this gene.

Microarray technologies have been integrated into many scientific disciplines and the increasing availability of genomics resources for various species will continue to increase the effectiveness of these approaches for deciphering complex gene expression patterns and regulatory mechanisms. This study reports the application of three approaches for identifying differentially expressed genes in pig fetal and postnatal skeletal muscle. In total, over 200 genes were identified and expression patterns for eight genes were evaluated by relative real time RT-PCR. Further elucidation of the roles of these genes, including those genes not previously known to be expressed in skeletal muscle and the genes of unknown function is of future interest. These results provide new information regarding developmental patterns of gene expression in skeletal

muscle and can be used to increase our understanding of normal growth processes and the consequences of molecular disorders in the pig and other mammalian species.

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Table 1. Real-time RT-PCR primer sequences[1]

Gene symbol	Gene name	Primer sequence
18S	18S ribosomal RNA[2]	Forward: 5'-CGGCTACCACATCCAAGGAA-3'
		Reverse: 5'-GCTGGAATTACCGCGGCT-3'
CNN3	calponin 3, acidic	Forward: 5'-ATGGTATGAAGCCACATGACATATTT-3'
		Reverse: 5'-CCTGGGTCATGTTCCCATTC-3'
FN1	fibronectin 1	Forward: 5'-GGCAGTTGATAAGAGGAATTTGGT-3'
		Reverse: 5'-CTAAACAGTTGTCTTTCCGCAGTAGTAA-3'
HPRT	hypoxanthine	Forward: 5'-CTGGCAAAACAATGCAAACCT-3'
	phosphoribosyltransferase	Reverse: 5'-AAGCTTGCAACCTTGACCATCT-3'
PXN	paxillin	Forward: 5'-ACACCCAGCCTCTGCTATGAG-3'
		Reverse: 5'-GGAAACAGATAGGGCTGGTGTT-3'
TTN	titin	Forward: 5'-AAGGACTCTTTCATGGAGGACATG-3'
		Reverse: 5'-TCGTCTCAGTCAGTCCAAATGTCT-3'
TCAP	titin-cap	Forward: 5'-CCCAGCGCATTCCAGATC-3'
		Reverse: 5'-GGCCCCGAACAGATTTCAG-3'
TTID	titin immunoglobulin domain	Forward: 5'-CATTAAGCTAAGGAACACTGAGATCATC-3'
	protein (myotilin)	Reverse: 5'-TGGCACTGCTTTCTAAATACTGTTCT-3'
TNNC1	troponin C, slow	Forward: 5'-ATCCTTCATGCACCGAACCA-3'
		Reverse: 5'-TGATTGACGAGGTGGATGAAGAC-3'
TPT1	tumor protein,	Forward: 5'-CCACCATCAGCGTCAAACAT-3'
	translationally controlled 1	Reverse: 5'-CCTACCTCAGCGAGGAGATGA-3'

[1]Primers were designed using Primer Express v 2.0 software (Applied Biosystems, Foster City, CA) and amplification conditions were 50°C for 2 min., 95°C for 10 min. and for 40 cycles 95°C for 15 sec. and 60°C for 1 min.

[2]18S primers were graciously provided by Dr. Susan Ewart of the The Molecular Respiratory and Equine Genetics Laboratory at Michigan State University.

Table 2. Differentially expressed genes observed by differential display reverse transcription PCR (DDRT-PCR)[1].

Clone[2]	Insert size (bp)	GenBank ID	TIGR TC report	Gene name (gene symbol)[3]	DDRT-PCR gel[4]
41M2-1	494	CF106688	TC181503	annexin A2 (ANXA2)	1>2>3
51M41	405	CB826594	TC163159	calponin 3, acidic (CNN3)[5,6]	1>2>3
54M77	400	CB826601	singleton	cardiomyopathy associated 3 (CMYA3)	1>2=3
51M44E	486	CB826597	TC180810	collagen, type I, alpha 2 (COL1A2)[6,7]	1=2>3
53M64D	295	CB826602	TC181259	cytochrome c oxidase III (MTCO3)[7,8]	1<2<3
41M6-1	544	CB826595	TC162457	fibronectin 1 (FN1)[5]	1=2>3
54M71-2	377	CB826603	TC163178	heat shock 70kDa protein 5 (HSPA5)	1<2=3
51M44B	417	CB826598	TC182174	janus kinase 1 (JAK1)	1=2>3
53M63G	425	CB826599	singleton	KIAA0373 gene product[9]	1=2<3
41M7	459	CB826592	TC165147	myosin, heavy polypeptide 4, skeletal muscle (MYH4)[6,7]	1=2<3
54M71-1	383	CB826593	TC189479	myosin, heavy polypeptide 8, skeletal muscle, perinatal (MYH8)	1<2>3
53M64B	164	CB826600	TC181077	NADH dehydrogenase 4 (MTND4)[7]	1<2<3
52M55	569	CB826591	singleton	nebulin (NEB)[6]	1<2<3
54M71-3	338	CB826596	TC185696	ras homolog gene family, member E (ARH3)	1=2<3
41M8-1	420	CX244545	TC182705	S100 calcium binding protein A11, calgizzarin (S100A11)	1=2<3
11M7A-1	307	CB826590	singleton	titin (TTN)[5,6,7,8]	1<2<3

[1]Primers used for DDRT-PCR were obtained from the U.S. Swine Genome Coordinator, and primer sequences are available at http://www.genome.iastate.edu/resources/ddprimer.html.
[2]Primers used to obtain each clone are indicated by the first two digits of the clone name (1st digit anchor primer; 2nd digit arbitrary primer).
[3]Predicted identities were determined by comparison of clone sequences to entries in the GenBank database.
[4]Relative pattern of mRNA abundance observed on DDRT-PCR gels. 1=60 d of gestation; 2=105 d of gestation; 3=7 wks of age.
[5]Expression pattern confirmed by relative real-time RT-PCR analysis.
[6]Expression pattern confirmed by northern or dot blot analysis (data not shown).
[7]Gene found to be significantly differentially expressed on cDNA microarray and/or oligonucleotide microarray.
[8]Multiple fragments identified as same gene.
[9]Gene name not approved by the HUGO Gene Nomenclature Committee.

Table 3. Differentially expressed genes observed on the cDNA microarray[1]

GenBank ID	TIGR TC report	Gene symbol	Gene Name	Fold change	P-value
		Genes more highly expressed at 7wks of age			
Calcium binding					
BM190036	TC181355	TPT1[2]	tumor protein, translationally controlled 1	1.61	0.054
Cell proliferation					
BM190535	TC163308	CLK1	CDC-like kinase 1	2.34	0.042
BM190623	TC163308	CLK1	CDC-like kinase 1	2.37	0.059
Enzymes					
BM190413	TC163277	ALDOA	fructose 1,6 diphosphate aldolase	1.70	0.058
BM190100	TC162668	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	3.43	0.055
BM190228	TC183161	PPT2	palmitoyl-protein thioesterase 2	1.65	0.049
BM190306	TC185631	RPE	ribulose-5-phosphate-3-epimerase	1.89	0.047
Extracellular matrix					
BM190576	TC181877	COL12A1	collagen, type XII, alpha 1	1.80	0.026
Mitochondrion					
BM190103	TC181361	MTCYB	cytochrome b	2.47	0.038
BM190124	TC181361	MTCYB	cytochrome b	1.91	0.042
BM190070	TC181361	MTCYB	cytochrome b	2.55	0.043
BM190061	TC181259	MTCO3[3]	cytochrome c oxidase subunit 3	2.09	0.053
BM190298	TC182398	NDUFA4	MLRQ subunit of NADH: ubiquinone oxidoreductase	2.83	0.052
BM190105	TC181077	MTND4[3]	NADH dehydrogenase subunit 4L	1.64	0.037

Table 3. cont.

GenBank ID	TIGR TC report	Gene symbol	Gene Name	Fold change	P-value
Myofibrillar contractile proteins					
BM190045	TC161973	ACTA1	skeletal alpha actin gene	2.38	0.053
BM190276	TC163842	TTN[2,3]	titin	4.44	0.053
BM190246	TC181436	TPM4	tropomyosin 4	2.85	0.037
BM190114	TC183088	TNNC1[2,3]	troponin C, slow	1.86	0.035
BM190123	TC163161	TNNC2[3]	troponin C2, fast	3.35	0.053
Ribosome Structure					
BM189988	TC181344	RPL7A	ribosomal protein L7a	1.49	0.060
Unknown					
BM190088	TC178050			1.60	0.046
BM190109	TC167023			1.7	0.048
BM190265	TC164066			2.72	0.057
BM190277	TC165141			2.18	0.050
BM190591	TC181362			2.67	0.054
Genes more highly expressed at 60 d of gestation					
Amino acid transport					
BM190337	TC183061	SLC38A2	solute carrier family 38, member 2	4.09	0.054

Table 3. cont.

GenBank ID	TIGR TC report	Gene symbol	Gene Name	Fold change	P-value
Enzymes					
BM190440	TC164022	NANS	N-acetylneuraminic acid synthase (sialic acid synthase)	2.09	0.033
Transcription regulation					
BM190400	TC186996	HIRIP3	HIRA interacting protein 3	1.74	0.045
Signal Transduction					
BM190445	TC167127	LEPREL2	leprecan -like 2 protein	1.89	0.050
Unknown					
BM190083	TC188844			2.49	0.042
BM190187	TC197893			1.63	0.035
BM190190	TC193222			1.60	0.039
BM190206	BM190206			1.43	0.052
BM190376	TC166111			1.46	0.060
BM190464	TC188352			1.74	0.044
BM190468	BM190468			1.82	0.037
BM190472	TC166425			1.50	0.030
BM190666	TC162067			4.04	0.043

[1]Genes listed exhibited fold changes ≥ 1.5 ($P \leq 0.06$). Genes are categorized by function and grouped by age at which expression was highest.
[2]Relative real-time RT-PCR was performed for validation of microarray results.
[3]Gene also found to be significantly differentially expressed on the oligonucleotide array.

Table 4. Differentially expressed genes observed on the oligonucleotide microarray[1]

TIGR TC report[2]	Gene symbol	Gene name	Fold Change	P- value
		Genes more highly expressed at 7 weeks of age		
Calcium binding				
NP494962	CASQ1	calsequestrin 1 (fast-twitch, skeletal muscle)	2.54	0.011
Cell growth and/or maintenance				
TC182018	FHL1	four and a half LIM domains 1	2.28	0.015
TC181858	PDLIM7	PDZ and LIM domain 7 (enigma)	4.75	0.036
TC181857	PDLIM7	PDZ and LIM domain 7 (enigma)	3.92	0.012
TC181327	UBC	ubiquitin C	2.52	0.001
Cytoskeleton				
TC164139	CAPZA2	capping protein (actin filament) muscle Z-line, alpha 2	1.60	0.046
TC166176	MYOZ1	myozenin 1	3.72	0.003
TC166176	MYOZ1	myozenin 1	2.53	0.003
TC166052	PDLIM3	PDZ and LIM domain 3	1.80	0.018
DNA repair				
TC163452	RAD23A	RAD23 homolog A	1.61	0.006
Enzymes				
TC182601	ARL2	ADP-ribosylation factor-like 2	1.54	0.004
TC163277	ALDOA	aldolase A, fructose-bisphosphate	11.18	0.001
TC194373	ALOX12	arachidonate 12-lipoxygenase	1.66	0.005
TC164323	ATPIF1	ATPase inhibitory factor 1	1.55	0.005

Table 4. cont.

TIGR TC report[2]	Gene symbol	Gene name	Fold Change	P- value
TC191298	FARP1	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived))	1.61	0.023
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	3.02	0.000
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	2.47	0.006
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	2.25	0.014
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	2.08	0.040
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	2.07	0.010
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	2.03	0.009
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	1.99	0.013
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	1.97	0.031
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	1.97	0.030
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	1.93	0.016
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	1.91	0.019
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	1.77	0.012
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	1.77	0.014
CF359258	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	1.71	0.023
NP275743	GAPD[3]	glyceraldehyde-3-phosphate dehydrogenase	2.31	0.031
TC163151	LDHA	lactate dehydrogenase A	3.03	0.042
TC182611	NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa	1.64	0.009
TC162798	P17.3[4]	neuronal protein 17.3	1.81	0.023
TC164637	PGM1	phosphoglucomutase 1	1.69	0.002
TC181879	PGK1	phosphoglycerate kinase 1	2.45	0.021
TC185107	PGAM2	phosphoglycerate mutase 2 (muscle)	5.57	0.002
TC185184	PYGM	phosphorylase, glycogen; muscle (McArdle syndrome, glycogen storage disease type V	3.37	0.016
TC185184	PYGM	phosphorylase, glycogen; muscle (McArdle syndrome, glycogen storage disease type V	2.10	0.008
TC198557	PITRM1	pitrilysin metalloproteinase 1	1.56	0.039
TC170654	LYK5[4]	protein kinase LYK5	1.54	0.012
BP159064	PTPN6	protein tyrosine phosphatase, non-receptor type 6	1.74	0.006
TC162821	PKM2	pyruvate kinase, muscle	1.94	0.021
TC163975	HSPC051[4]	ubiquinol-cytochrome c reductase complex (7.2 kD)	1.99	0.010
TC182529	UQCRC1	ubiquinol-cytochrome c reductase core protein I	2.19	0.014

Table 4. cont.

TIGR TC report[2]	Gene symbol	Gene name	Fold Change	P- value
BI344086	UQCRC2	ubiquinol-cytochrome c reductase core protein II	1.57	0.004
Extracellular matrix				
NP276599	COL10A1	collagen, type X, alpha 1(Schmid metaphyseal chondrodysplasia)	1.78	0.003
Immune response				
TC164126	C1QA	complement component 1, q subcomponent, alpha polypeptide	1.52	0.037
TC181534	HLA-A	major histocompatibility complex, class I, A	3.02	0.021
Mitochondrion				
TC181259	MTCO3[3]	cytochrome c oxidase III	3.52	0.006
TC181259	MTCO3[3]	cytochrome c oxidase III	2.72	0.016
TC181595	MTND2	NADH dehydrogenase 2	3.91	0.000
TC181775	MTND3	NADH dehydrogenase 3	2.55	0.002
TC181077	MTND4[3]	NADH dehydrogenase 4	2.43	0.031
Myofibrillar contractile proteins				
TC183327	CRYAB	crystallin, alpha B	1.76	0.004
TC165117	FLNC	filamin C, gamma (actin binding protein 280)	3.31	0.003
TC180257	MYBPC2	myosin binding protein C, fast type	3.35	0.024
TC182003	HUMMLC2B[4]	myosin light chain 2	2.80	0.026
TC165147	MYH4	myosin, heavy polypeptide 4, skeletal muscle	8.98	0.020
TC164383	MYL1	myosin, light polypeptide 1, alkali; skeletal, fast	3.89	0.013
TC163842	TTN[3,5]	titin	3.69	0.004
TC163841	TTN[3,5]	titin	3.34	0.024
TC168093	TCAP[5]	titin-cap (telethonin)	7.88	0.005
TC183088	TNNC1[3,5]	troponin C, slow	1.66	0.026
TC163161	TNNC2[3]	troponin C2, fast	4.83	0.001
AW657610	TNNT1	troponin T1, skeletal, slow	2.30	0.050

Table 4. cont.

TIGR TC report[2]	Gene symbol	Gene name	Fold Change	P- value
TC198053	TNNT3	troponin T3, skeletal, fast	3.01	0.007
Ribosome structure				
TC181000	RPL10A	ribosomal protein L10a	1.55	0.037
TC181500	RPL6	ribosomal protein L6	1.54	0.012
TC162859	RPS4X	ribosomal protein S4, X-linked	2.42	0.006
TC162859	RPS4X	ribosomal protein S4, X-linked	2.01	0.006
TC181569	RPS7	ribosomal protein S7	2.23	0.017
TC181617	RPLP1	ribosomal protein, large, P1	2.05	0.014
Signal transduction				
BP439453	B2M	beta-2-microglobulin	2.02	0.029
TC166317	F2RL1	coagulation factor II (thrombin) receptor-like 1	1.76	0.010
TC163759	EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	1.80	0.026
Translation				
TC130780	EEF1A2	eukaryotic translation elongation factor 1 alpha 2	9.38	0.001
TC162959	HSPB1	heat shock 27kDa protein 1	3.65	0.004
Transporter				
TC163709	ATP5J2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2	1.92	0.012
TC183143	ATP2A1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	5.49	0.001
TC194111	STK23	serine/threonine kinase 23	2.56	0.016
TC182256	SLC25A4	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator)	1.79	0.016
TC163102	SURF2	surfeit 2	1.52	0.020
TC181180	VDAC1	voltage-dependent anion channel 1	1.89	0.031

Table 4. cont.

TIGR TC report[2]	Gene symbol	Gene name	Fold Change	P- value
Unknown function				
TC171259	FAM16AX	family with sequence similarity 16, member A, X-linked	1.54	0.017
BM658731			1.55	0.003
TC162710			1.71	0.013
TC162719			1.65	0.020
TC163805			2.03	0.001
TC165785			1.69	0.002
TC166001			2.14	0.013
TC166330			1.52	0.036
TC168215			1.53	0.034
TC169791			1.59	0.002
TC171081			2.19	0.028
TC176101			2.04	0.001
TC178030			1.67	0.005
TC180903			1.95	0.000
TC181362			1.77	0.040
TC181891			1.63	0.040
TC182018			2.34	0.023
TC183447			1.53	0.020
TC184525			2.61	0.016
TC184896			1.61	0.023
TC185430			1.59	0.028
TC186477			1.61	0.001
TC189021			1.68	0.021
TC196973			1.68	0.000
TC197564			1.78	0.002
TC198089			2.14	0.001

Table 4. cont.

TIGR TC report[2]	Gene symbol	Gene name	Fold Change	P- value
		Genes more highly expressed at 60 d of gestation		
Apoptosis				
TC180964	HSPA1B	heat shock protein 70.2 gene, promoter	1.51	0.009
Cell growth and/or maintenance				
CK455405	FTH1	ferritin, heavy polypeptide 1	1.72	0.038
Cytoskeleton				
TC165807	BIN3	bridging integrator 3	1.57	0.033
TC183282	HIP1	huntingtin interacting protein 1	1.51	0.012
Enzymes				
TC185809	AKR1CL2	aldo-keto reductase family 1, member C-like 2	1.78	0.001
TC168198	A4GALT	alpha 1,4-galactosyltransferase	1.80	0.001
TC194827	ALS2CR7	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 7	1.51	0.003
TC163055	CTSC	cathepsin C	1.55	0.017
TC181654	GBGT1	globoside alpha-1,3-N-acetylgalactosaminyltransferase 1	1.88	0.022
TC185008	KATNB1	katanin p80 (WD repeat containing) subunit B 1	1.53	0.012
TC165037	POLDIP2	polymerase (DNA-directed), delta interacting protein 2	1.52	0.001
TC183190	PPP1R14B	protein phosphatase 1, regulatory (inhibitor) subunit 14B	2.34	0.007
TC178701	RECQL4	RecQ protein-like 4	1.80	0.004
TC166639	B3GNTL1	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase-ike 1	1.78	0.036
TC184039	URKL1	uridine kinase-like 1	2.41	0.019
Extracellular matrix				
TC180812	COL1A1	collagen, type I, alpha 1	1.51	0.022

Table 4. cont.

TIGR TC report[2]	Gene symbol	Gene name	Fold Change	P- value
TC180810	COL1A2	collagen, type I, alpha 2	1.63	0.016
Immune response				
TC181541	TRB@	T cell receptor beta locus	1.55	0.005
NP275341	TRDD3	T cell receptor delta diversity 3	1.56	0.033
Myofibrillar contractile proteins				
TC167400	MYH3	myosin, heavy polypeptide 3, skeletal muscle, embryonic	3.64	0.003
TC168323	MYL4	myosin, light polypeptide 4, alkali; atrial, embryonic	1.53	0.011
Ribosome structure				
TC165797	MTA1	metastasis associated 1	1.69	0.038
TC166729	NOLA2	nucleolar protein family A, member 2 (H/ACA small nucleolar RNPs)	1.60	0.016
TC181715	RPS18	ribosomal protein S18	1.56	0.030
TC181715	RPS18	ribosomal protein S18	1.60	0.000
TC181715	RPS18	ribosomal protein S18	1.72	0.001
TC181715	RPS18	ribosomal protein S18	1.78	0.005
TC181715	RPS18	ribosomal protein S18	1.79	0.018
TC181715	RPS18	ribosomal protein S18	1.80	0.000
TC181715	RPS18	ribosomal protein S18	1.80	0.001
TC181715	RPS18	ribosomal protein S18	1.85	0.002
TC181715	RPS18	ribosomal protein S18	1.88	0.021
TC181715	RPS18	ribosomal protein S18	1.92	0.010
TC181715	RPS18	ribosomal protein S18	1.97	0.001
TC180909	RPS5	ribosomal protein S5	1.69	0.006
Signal transduction				
TC166317	APOB	apolipoprotein B (including Ag(x) antigen)	1.53	0.017

Table 4. cont.

TIGR TC report[2]	Gene symbol	Gene name	Fold Change	P- value
TC166966	AVP	arginine vasopressin (neurophysin II, antidiuretic hormone, diabetes insipidus, neurohypophyseal)	2.08	0.004
TC191992	EPO	erythropoietin	1.71	0.026
Transcriptional regulation				
TC184976	CRSP	Calcitonin receptor-stimulating peptide	2.27	0.002
TC162852	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1.72	0.019
NP275174	IPF1	insulin promoter factor 1, homeodomain transcription factor	1.51	0.021
TC167760	TIF1	transcriptional intermediary factor 1	1.59	0.040
Unknown function				
TC186341	C14orf32	chromosome 14 open reading frame 32)	1.50	0.007
TC182961	FLJ10420	hypothetical protein FLJ10420	1.71	0.030
TC163512	FLJ12681[4]	hypothetical protein FLJ12681	1.73	0.012
TC164687	FLJ22955	hypothetical protein FLJ22955	2.59	0.049
BM658515	HSPC268[4]	hypothetical protein HSPC268	1.50	0.017
TC165883	KIAA0157	KIAA0157	1.55	0.014
TC184707	DKFZp566C0424[4]	putative MAPK activating protein PM20,PM21	1.68	0.023
TC168563	MGC9564	similar to RIKEN cDNA 1110002C08 gene	1.90	0.050
BF709307			1.55	0.008
BG695754			2.06	0.027
TC162585			1.54	0.025
TC163366			1.63	0.009
TC163691			1.64	0.008
TC164785			2.08	0.029
TC165520			1.74	0.023
TC165903			1.70	0.028
TC165907			2.00	0.001

Table 4. cont.

TIGR TC report[2]	Gene symbol	Gene name	Fold Change	P- value
TC166287			1.68	0.006
TC167679			2.59	0.001
TC170235			1.53	0.016
TC170998			1.68	0.014
TC171040			2.85	0.035
TC181653			1.57	0.037
TC182331			1.50	0.017
TC182517			2.22	0.002
TC182890			1.55	0.006
TC183320			1.51	0.049
TC183468			1.53	0.016
TC183805			1.51	0.021
TC183842			2.00	0.001
TC184338			1.88	0.038
TC184875			1.57	0.011
TC184949			2.06	0.002
TC185603			1.51	0.014
TC186667			1.77	0.008
TC186782			1.60	0.038
TC186890			1.80	0.025
TC188584			1.64	0.001
TC190478			1.63	0.031
TC191072			1.60	0.014
TC191161			1.54	0.007
TC199217			1.51	0.015

[1]Genes listed exhibited fold changes ≥ 1.5 ($P \leq 0.05$). Genes are categorized by function and grouped by age at which expression highest.

[2]The TIGR Tentative Consensus sequence numbers from which oligonucleotides were designed.

[3]Gene also found to be significantly differentially expressed on the cDNA microarray.

[4]Gene symbol and name not approved by HUGO Gene Nomenclature Committee.

[5]Relative real-time RT-PCR was performed for validation of microarray results.

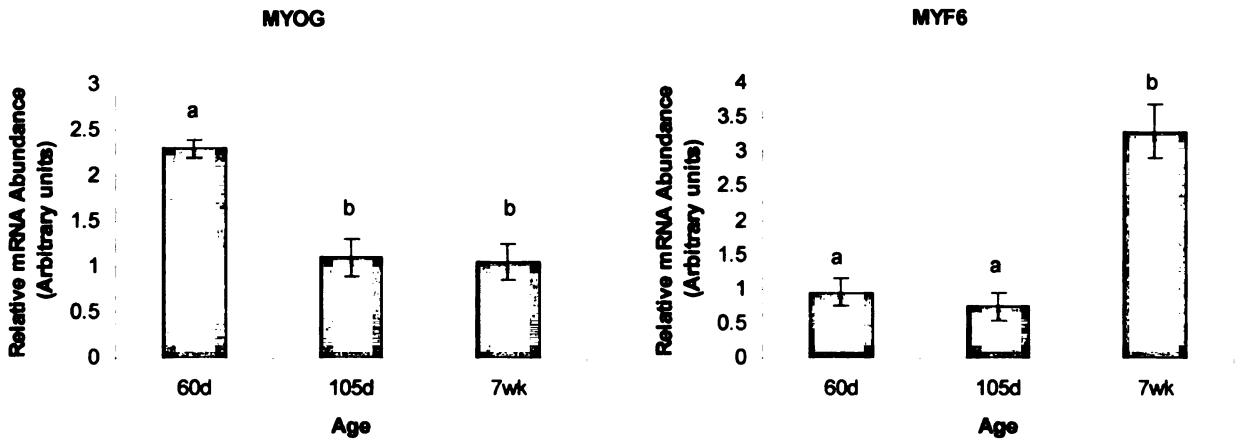

Figure 1. Relative mRNA abundance of myogenin (MYOG) and myogenic factor 6 (MYF6) in hind limb skeletal muscle of pigs at 60 d of gestation, 105 d of gestation or 7 wks of age postnatal (n=3 per age). Total cellular RNA was used in northern blot analyses and results of densitometric analyses of northern blots probed with a MYOG or MYF6 cDNA are presented. The data were obtained by scanning laser desitometry and are expressed in density units ± S.E. Bars with different superscripts are significantly different (MYOG, $P < 0.02$; MYF6 $P < 0.001$).

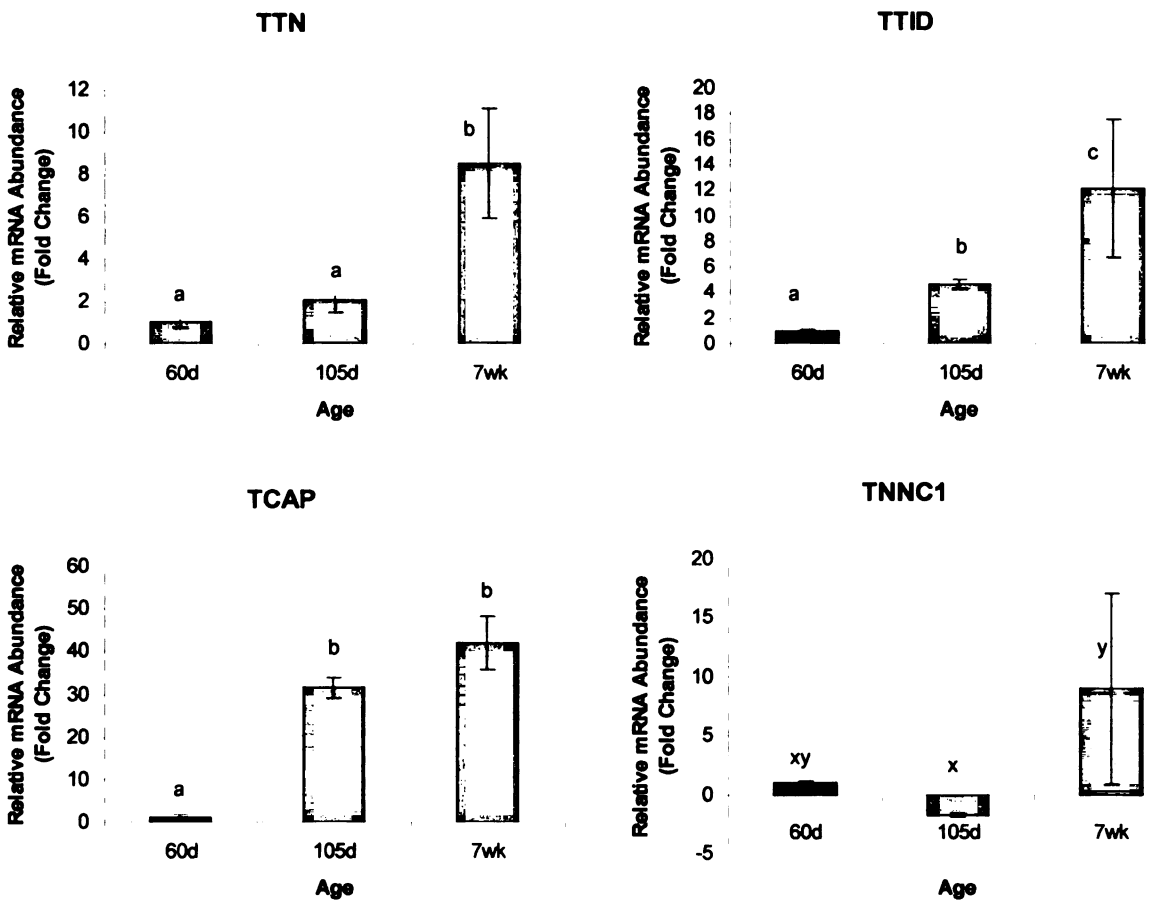

Figure 2. Relative mRNA abundance of genes encoding contractile proteins. Relative real time RT-PCR was performed using total RNA obtained from pigs at 60 d of gestation, 105 d of gestation and 7 wks of age postnatal (n=3 per age). Fold changes relative to HPRT (TCAP and TTID) or 18S rRNA (TTN and TNNC1) and the 60 d samples were calculated by the $2^{-\Delta\Delta Ct}$ method and significance was determined by mixed model analysis (see Materials and Methods). Bars with different superscripts are significantly different (a,b,c $P \leq 0.05$; x,y *P=0.06*).

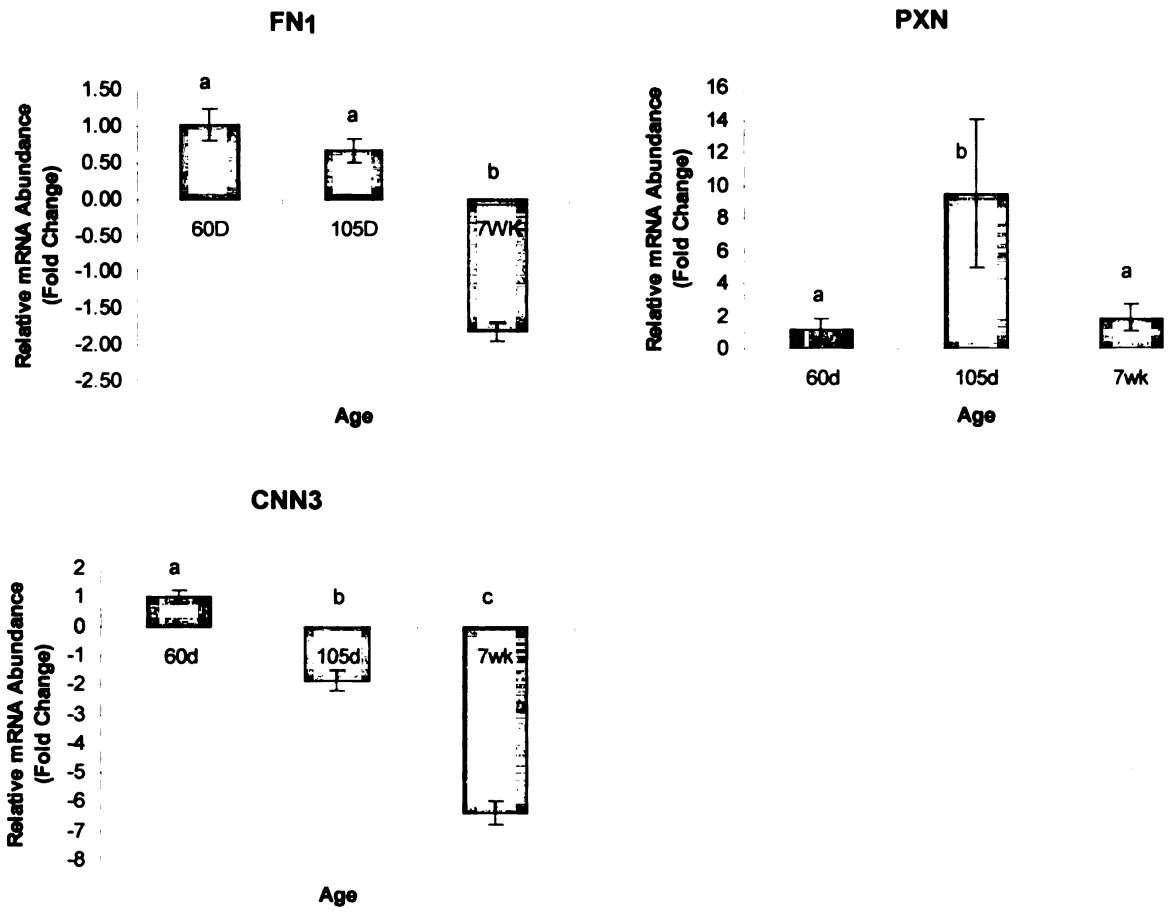

Figure 3. Relative mRNA abundance of genes encoding cytoskeletal proteins. Relative real time RT-PCR was performed using total RNA obtained from pigs at 60 d of gestation, 105 d of gestation and 7 wks of age postnatal (n=3 per age). Fold changes relative to HPRT and the 60 d samples were calculated by the $2^{-\Delta\Delta Ct}$ method and significance was determined by mixed model analysis (see Materials and Methods). Bars with different superscripts are significantly different ($P \leq 0.05$).

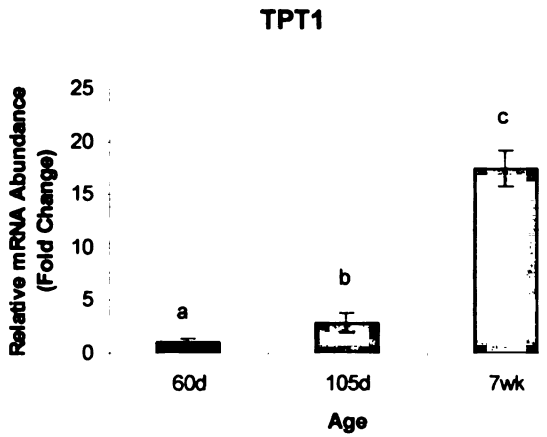

Figure 4. Relative mRNA abundance of TPT1. Relative real time RT-PCR was performed using total RNA obtained from pigs at 60 d of gestation, 105 d of gestation and 7 wks of age postnatal (n=3 per age). Fold changes relative to 18S rRNA and the 60 d samples were calculated by the $2^{-\Delta\Delta Ct}$ method and significance was determined by mixed model analysis (see Materials and Methods). Bars with different superscripts are significantly different ($P \leq 0.004$).

CHAPTER 3

Mapping of Porcine Skeletal Muscle ESTs

Summary

Radiation hybrid (RH) mapping of 24 expressed sequence tags (ESTs) derived from porcine skeletal muscle is reported. These ESTs were observed to be differentially expressed in skeletal muscle tissue from pigs at 60 days of gestation or 7 weeks of age postnatal using either a cDNA microarray or differential display reverse transcription PCR. The IMpRH panel was used for mapping and the ESTs were assigned to 13 different pig chromosomes. Nineteen of these assignments were at LOD score ≥ 5.79 (15 > 8.6). Twenty-two of the ESTs correspond to genes of known identity and all of these mapped to the expected porcine-human comparative map locations. The mapping of ESTs in this study contributes to characterization of the pig skeletal muscle transcriptome and further improves the porcine-human comparative map.

Keywords: skeletal muscle, ESTs, radiation hybrid mapping, pig

Introduction

Development of high resolution genome maps for species such as the pig is facilitated by comparative gene mapping, which utilizes information from species such as human and mouse that have complete genome sequences available. These maps then aid in the identification of candidate genes for economically important traits. In addition, current applications of global gene

expression profiling techniques such as differential display reverse transcription PCR (DDRT-PCR) and DNA microarrays are also revealing genes involved in the expression of important trait phenotypes. Thus in order to fully utilize the available information for identifying genes controlling economically important traits, it is important to integrate gene expression data with genome map information. A first step toward achieving this goal is to map genes identified by expression profiling studies. For the present study, we used a pig-rodent radiation hybrid (RH) panel (Yerle et al. 1998; Hawken et al. 1999) to map expressed sequence tags (ESTs) that were observed to be differentially expressed in skeletal muscle from pigs at 60 days of gestation and 7 weeks of age postnatal (Rilington et al., in preparation).

Materials and Methods

A total of 24 oligonucleotide primer pairs for use in the PCR were designed from sequences of cDNA clones derived from either a porcine skeletal muscle cDNA library (18 ESTs; Yao et al. 2002) or a porcine skeletal muscle differential display experiment (6 ESTs; Rilington et al., in preparation) using the OLIGO 5.1 primer analysis software (Molecular Biology Insight Inc., Cascade, CO). The PCR was performed using 25 ng genomic DNA in 10 µL reactions containing 1 X PCR buffer (Promega, Madison, WI), 1.5 or 2.0 mM $MgCl_2$, 150 µM of each dNTP, 0.25 or 0.5 µM of each primer and 0.2 units of *Taq* DNA polymerase (Promega, Madison, WI). The PCR profiles included an initial denaturation of 3 min at 94°C followed by 30 cycles of 94°C for 1 min, 53°- 63°C for 1 min, 72°C for 1 min and a final extension of 72°C for 10 min. The PCR

products were visualized on 1% agarose gels with 0.4 μg/ml of ethidium bromide. The GenBank accession numbers of the clones, primer sequences, PCR conditions and observed PCR product sizes are shown in Table 1.

The ESTs were mapped using the INRA-University of Minnesota 7,000-rad porcine RH (IMpRH) panel (Yerle et al. 1998; Hawken et al. 1999) using the same PCR profile except that 12.5 ng of hybrid DNA was used. The IMpRH panel was screened twice for each EST and products were visualized on 1- 3% agarose gels. Each of the 118 hybrids was scored as positive, negative or ambiguous, and two-point analysis of RH data was performed using the IMpRH server mapping tool as outlined by Milan et al. (2000; http://imprh.toulouse.inra.fr/).

Results and Discussion

A total of 24 ESTs were mapped for this study (Table 2). These included four ESTs on SSC5, three ESTs each on SSC2 and SSC15, two ESTs each on SSC1, SSC3, SSC9 and SSC14, and one EST each on SSC4, SSC11, SSC12, SSC13, SSC17 and SSCX. Twenty-two of the ESTs had significant similarities to genes of known identity and all of these mapped to their expected porcine-human comparative map locations (http://www.toulouse.inra.fr/lgc/pig/compare/compare.htm). Eleven of the 24 ESTs had previously been mapped through candidate gene or EST studies in ours or other laboratories using physical or genetic mapping techniques, including seven previous RH map assignments. The results of the present study help to confirm these previous assignments, as well as add 13 new assignments.

Nineteen of the 24 map assignments were with LOD scores ≥ 5.79 (15 > 8.6). However, the remaining five assignments were with LOD scores < 4.5, and thus must be considered as tentative. Four of these five assignments were consistent with expected comparative map locations and two of these had previously been mapped in other laboratories. Thus, there is evidence that these assignments are likely to be correct. An EST of unknown identity (PigESTB) was tentatively assigned to SSC9 (LOD = 4.35). Further study will be needed both to determine the identity of this EST and to confirm its map position.

We report here the mapping of 24 ESTs to 13 pig chromosomes. Davoli et al. (2002) reported a first genomic transcript map for pig skeletal muscle that included 125 markers. While three of the ESTs mapped in the present study were included on the Davoli et al. map, the other 21 ESTs represent new contributions to the pig skeletal muscle transcript map. The ESTs mapped in the present study were observed to be differentially expressed in pig skeletal muscle tissue at 60 days of gestation or seven weeks of age postnatal. Thus, placing these ESTs on the pig genome map not only helps to improve the porcine-human comparative map, but also contributes to the characterization of the pig skeletal muscle transcriptome. Integration of genome map information with gene expression profiling data is an important step toward identifying the genes controlling economically important trait phenotypes.

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Table 1. Primer sequences and PCR amplification conditions for porcine skeletal muscle ESTs

Accession number	Gene/ EST identity	Gene/ EST symbol	Primers 5′ → 3′[1]	Annealing Temperature (°C)	Observed PCR product size (bp)
CB826594	Acidic calponin	*CNN3*	F-GCAACTGGATAGAGGAGGTG R-ATGTCAATGGTGGTGTGGAA	59	2200
CF106688	Annexin A2	*ANAX2*	F-ACACCAAGGGCGACTACCA R-AGAGGAGAAAGCCAGGCAACT	62	200
CB826597	Collagen type 1 alpha 2	*COL1A2*	F-GAAGGAGTTACCACCAAGGA R-CAAGGATAGGCAGGCGAGAT	62	1100
CB826595	Fibronectin 1	*FN1*	F-CCTGCTCTCCAAAGTGGTTC R-TTCTAGGCAATTACCAAAGTC	57	300
BM190400	HIRA interacting protein 3	*HIRIP3*	F-GCTCCTCTTCCTCTTCCACC R-CCTCTTCCCGCTGCTCCTTC	59	500
BM190666	Hyothetical protein HSPC111	*HSPC111*[2]	F-GATACCCCAAAACAGATTCG R-CCCTCCTGCCCTTAAATCCT	55	200
BM190195	Leprecan-like 2	*LEPREL2*	F-CCCCCACATCCTTCTCACTT R-TCCCTGCACCCCCACCATCT	57	200
BM190067	Myoglobin	*MB*	F-ACCCTGCCCACGCCTCCTTC R-GTCCCTGCCAGCCTCCAAAC	62	150
BM190440	N-acetylneuraminic acid synthase (sialic acid synthase)	*NANS*	F-AATCGGAAAGCCTTGGAGAG R-CAGAGGCAGTGAAGAAGATG	57	150
BM190116	O-linked N-acetylglucosamine transferase	*OGT*	F-GAGGAATGATGTGCTATGTA R-TAATGCTATCCAGTAACAGA	60	450
BM190028	Paxillin	*PXN*	F-TGGGTACAAGTGGAGACATC R-AGGGCTGGTGTTCTGGGTCA	59	250
BM190280	Phosphorylase, glycogen; muscle (McArdle syndrome, glycogen storage disease type V)	*PYGM*	F-AAGCGCTGGTTGATCTCATA R-CGGCTGGAGTGGGAGAAGG	60	400
BM190472	Protein tyrosine kinase 9	*PTK9*	F-ACCTGATAAACCTGCTGTAA R-AAGGTGCATATATCGTTAGA	57	200
CB826596	Ras homolog gene family, member E	*ARHE*	F-TTTAATGAGGACACGGAAAC R-TCTCACCGCTTGCTCTAC	57	200
BM190045	Skeletal muscle alpha-actin	*ACTA1*	F-TGTGTCGGTTTGGGCAGCAG R-AGCGCAAGTACTCCGTGTGG	59	250
BM190337	Solute carrier family 38, member 2	*SLC38A2*	F-AGTGTAATGGAATCAAATGT R-GCATGTATTTATTTTAGTTC	55	200
CB826590	Titin	*TTN*	F-TGACTGGAATCCCTACACCT R-AGCGGGTACTACTTCTTCAC	59	700

Table 1. cont.

Accession number	Gene/ EST identity	Gene/ EST symbol	Primers 5′ → 3′[1]	Annealing Temperature (°C)	Observed PCR product size (bp)
BM190108	Titin immunoglobulin domain protein (myotilin)	*TTID*	F-AAATGTGCAAGAGTCAAAGG R-AGATGTAAATAAGAAAGATG	53	800
BM190107	Titin-cap (telethonin)	*TCAP*	F-CGCTGTCTTTGCTCCCTTCC R-TTCGTCGCTCCCTGTCTCGT	59	250
BM190123	Troponin C, fast	*TNNC2*	F-GCCATCGTTGTTCTTGTCTC R-AAGGGGAAGAGTGAGGAGGA	63	350
BM190114	Troponin C, slow	*TNNC1*	F-GCCATCATTGTTCTTGTCAC R-CAAAGGAAAGTCTGAGGAGG	59	450
BM190036	Tumor protein, translationally controlled 1	*TPT1*	F-AATGCCTCCGCTCCAAAGAA R-ACGGGCTGTGTCTGGAGGTG	59	600
BM190265	Unknown	PigESTA[3]	F-AGGGCTTGGGATGGCTATTT R-AGTTCCCTGGTGGTTTAGTG	63	350
BM190601	Unknown	PigESTB[3]	F-TCTCCCTTCCCTTGGCTCTG R-CTTCTCGGCGTGGTCATCTG	59	500

[1]Amplification conditions included 1.5 mM $MgCl_2$ and 0.5 µM each primer except ANAX2 2.0 mM $MgCl_2$, and TNNC2, PYGM and PigESTA primer concentrations 0.5 µM forward and 0.25 µM reverse.
[2]Symbol not approved by the HUGO Gene Nomenclature Committee.
[3]EST does not significantly match any gene of known identity in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/).

Table 2. Radiation hybrid mapping results for porcine skeletal muscle ESTs

Gene symbol	RH assignment	Closest linked marker	LOD score	Human map location	Human genome position[1]	Previous assignments[2]
ACTA1	14	SWR346	8.67	1q42.1	Chr1:225,873,730-225,876,576	Tosser-Klopp et al. (1998) (S) Archibald (1995) (L) Kapke et al. (1996) (L)
ANXA2[3]	1	S0122	3.19	15q21-q22	Chr15:58,426,826-58,477,465	Karnuah et al. (2001) (R) Wintero et al. (1998) (S)
ARHE	15	SW2072	6.62	2q23.3	Chr2:151,150,216-151,169,672	
OGT	X	SW949	3.33	Xq13.1	ChrX:70,536,001-70,576,693	
CNN3	4	S0097	22.41	1p22-p21	Chr1:95,074,555-95,104,684	
COL1A2	9	SWR915	8.81	7q22.1	Chr7:93,668,523-93,705,195	Karnuah et al. (2001) (R) Cirera et al. (2003) (S & R) Johansson et al. (1994) (L)
FN1[3]	15	SW2083	13.55	2q34	Chr2:216,051,092-216,060,578	Robic et al. (1999) (R) Looft et al. (2000) (L)
HIRIP3	3	SW1443	8.7	16p12.1	Chr16:29,911,817-29,914,888	
HSPC111	2	S0226	3.44	5q35.3	Chr5:175,743,554-175,748,959	
LEPREL2	5	SW963	16.26	12p13	Chr12:6,808,111-6,819,271	
MB	5	AC02	5.79	22q13.1	Chr22:34,327,311-34,337,876	Lahbib-Mansais et al. (2000) (S & R)
NANS	1	SSC11E11	8.64	9p24.1-p23	Chr9:97,898,567-97,924,912	
PTK9	5	SWR1974	20.48	12q12	Chr12:42,473,792-42,486,445	
PXN	14	SW295	10.8	12q24.23	Chr12:119,110,976-119,166,229	Farber et al. (2003) (R)

Table 2. cont.

Gene symbol	RH assignment	Closest linked marker	LOD score	Human map location	Human genome position[1]	Previous assignments[2]
PYGM	2	SW256	17.2	11q12-q13.2	Chr11:64,270,703-64,283,946	Davoli et al. (2000) (S) Fontanesi et al. (2003) (L)
SLC38A2	5	SW1200	4.48	12q	Chr12:45,038,238-45,052,822	Cirera et al. (2003) (S & R)
TCAP	12	SW943	25.02	17q12	Chr17:35,075,127-35,076,332	
TNNC1	13	SSC24F05	9.12	3p21.1	Chr3:52,460,157-52,463,097	
TNNC2	17	SW2431	6.6	20q13.12	Chr20:43,885,264-43,889,358	Zambonelli et al. (2000) (C)
TTID	2	SW1879	9.17	5q31	Chr5:137,231,463-137,251,430	Davoli et al. (2002) (S)
TTN	15	SW1263	7.03	2q31.2	Chr2:179,216,226-179,497,655	Davoli et al (2002) (S) Lahbib-Mansais et al. (2000) (S & R) Bertani et al. (1999) (L & S)
TPT1	11	SSC6E09	19.17	13q	Chr13: 44,810,794-44,813,204	
PigESTA	3	SW251	8.85	--------	--------	
PigESTB	9	SSC8B04	4.35	--------	--------	

[1]Numbers indicate position of gene in bp from p-terminus on indicated human chromosome based on the May 2004 assembly using the University of California Santa Cruz Genome Browser (http://genome.ucsu.edu).

[2]Method of mapping Somatic cell hybrid panel (S), Linkage (L), Radiation hybrid panel (R), Cytogenetic (C).

[3]Regionally assigned using a somatic cell hybrid panel (Robic et al. 1996; Yerle et al. 1996). ANXA2, SSC1q11-q17; FN1, SSC15q23-q26. Risk of error less than 0.1%.

CHAPTER 4

Summary and Recommendations for Future Research

Enhancing pork quality and production efficiency are major concerns for pig producers. The advent of new technologies such as large scale gene expression microarrays and high resolution gene maps can lead quickly to candidate genes for economically important phenotypic traits allowing for a faster turn around to gene tests that could improve pork quality. The market weight of an animal is directly linked to the amount of muscle fibers and the size of the fibers that the animal has. The fiber number is determined before the birth of the animal and the size is due to postnatal hypertrophy. A great deal is known about the structural changes, regulatory genes and growth factors involved in skeletal muscle development. However, relatively little is known on the cascade of events controlling fetal myogenesis and postnatal hypertrophy.

Skeletal muscle is the most abundant tissue in an animal's body and it is regulated by complex biological mechanisms. Thus, to begin to understand gene expression patterns during the growth process requires a technology that simultaneous determines expression of the numerous genes involved. Microarray technology allows researchers to screen large biological systems in one experiment. Not only is understanding the gene expression of a system important, but beginning to integrate these large amounts of data into other areas of the biological system is also important. Searching for candidate genes controlling important traits can be a long process. However, taking the data acquired from microarrays and mapping the identified genes allows for an easier

search. Linking gene expression data and genetic maps connects the phenotypic expression to economically important traits.

This study was designed to identify differentially expressed genes in developing pig skeletal muscle and locate them on the pig genome map. The specific objectives were: 1) Identify differentially expressed genes in hind limb skeletal muscles of pigs at 60 days of gestation and 7 weeks of age; and 2) Determine the map locations for differentially expressed genes.

To achieve Objective 1, a combination of differential display reverse transcription PCR, cDNA microarray analysis and 70-mer oligonucleotide microarray analysis were used. A total of 214 genes were found to be differentially expressed in developing pig hind limb skeletal muscle using these techniques. Three genes were identified with all three techniques and five other genes were common to two of the techniques. Results of this study provide a unique set of differentially expressed genes involved in skeletal muscle development. Some of these genes have previously been functionally characterized in skeletal muscle of other species. However, many of the genes have not been evaluated in skeletal muscle. For example, translationally controlled tumor protein 1 (TPT1) has been reported to be expressed in skeletal muscle, but there is no report on its role or importance in muscle development. Thus, we were able to provide information about the expression pattern of TPT1 in developing skeletal muscle.

Relative real-time RT-PCR confirmed that titin (TTN), titin-cap (TCAP) and TPT1 were more highly expressed in pig skeletal muscle at 7 weeks of age. Titin

immunoglobulin domain protein (TTID) was also more highly expressed in the 7 week samples, in agreement with information in the literature indicating that expression of TTID increases throughout mouse development. Troponin C1 (TNNC1) was observed to be differentially expressed on both microarray platforms. This result was not confirmed by relative real time RT-PCR, but the large animal-to-animal variation may have affected the statistical analysis. Thus, the expression pattern of TNNC1 is still inconclusive and it should be repeated with a different set of animals. Abundance of fibronectin 1 (FN1) and calponin 3 (CNN3) mRNA was confirmed to be highest at 60 days of gestation. Evaluation of paxillin (PXN) indicating expression to be higher at 105 days of gestation than at 60 days of gestation or 7 weeks of age provides additional information about expression of cytoskeletal genes in developing skeletal muscle.

To achieve Objective 2, 24 genes were selected from the DDRT-PCR and cDNA microarray experiments conducted for Objective 1 and they were localized on a pig radiation hybrid (RH) map. These genes were assigned to 13 different pig chromosomes and those of known identity (22 of the 24) mapped to the expected porcine-human comparative map locations. Not only does mapping of these genes help to improve the porcine-human comparative map, but since they were observed to be differentially expressed in hind limb skeletal muscle of pigs at 60 days of gestation and 7 weeks of age, they represent new contributions to the pig skeletal muscle transcript map.

In summary, this project represents a first step toward characterizing the transcriptional profile of developing pig skeletal muscle. Thus, there are several

considerations for future research efforts. During fetal myogenesis, there are distinct structural changes in skeletal muscle, and postnatally muscle hypertrophy rapidly increases. Therefore, for future microarray studies it would be prudent to include more developmental ages in the evaluation. The addition of more ages at critical times of fiber formation and hypertrophy would give a clearer understanding of the skeletal muscle development process. It is also recommended that these studies include more animals at each age in order to improve the power of the statistical analyses.

Both the cDNA microarray and 70-mer oligonucleotide microarray platforms appear to work well for evaluating transcriptional profiles of developing skeletal muscle. The cDNA microarray used for this study contained only 768 clones so it was only a small representation of the pig genome and what genes could possibly be expressed in skeletal muscle. Therefore, it is recommended to expand this array to include more clones in order to improve the comprehensiveness of gene expression profiles. Although the currently available pig oligonucleotide microarray contains over 13,000 oligonucleotides, the expressed sequence tag (EST) collection that was available when these oligonucleotides were designed contained very few ESTs derived from skeletal muscle. Thus, as the number of skeletal muscle ESTs increases, future oligonucleotide sets will contain a better representation of genes expressed in muscle and future studies will benefit from this improved resource. In addition to improved microarray resources, future studies to profile gene expression patterns

in skeletal muscle will benefit from application of the latest approaches for microarray screening and data analysis.

Transcriptional profiling of pig skeletal muscle tissue will provide a wealth of information, but it is important that genes identified by microarray analyses be further studied at both the mRNA and protein levels in order to fully characterize their functions. In addition, the map positions of these genes should be determined and integrated into quantitative trait loci (QTL) studies for muscle growth traits. This will not only allow all of the available information to be used to improve pig production, but the resulting comparative map information will provide basic fundamental knowledge about mammalian skeletal muscle development.