The Role of Chicken Delta-Like Protein 1 Expression in Skeletal Muscle Development and Regeneration

Dissertation

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

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2009

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Abstract

Delta-like protein 1 (DLK1) has been studied in muscle development, growth, and regeneration of mammals. The role of DLK1 has been implicated in the muscle hypertrophy observed in DLK1 transgenic mice, callipyge sheep, mouse paternal uniparental disomy (pUPD) 12 and human pUPD14 syndromes. However, no study on DLK1 has been conducted in the avian species. The ultimate goal of this dissertation is to identify the role of chicken delta-like protein 1 (gDLK1) in skeletal muscle development, growth, and regeneration in poultry.

The objectives of the first study were to clone and sequence a full length of chicken DLK1 cDNA, and to investigate the developmental regulation of gDLK1 during embryonic muscle development and postnatal muscle growth. The cloning and sequencing data revealed that gDLK1 contains a total of 1,161 base pairs, encoding 386 amino acids. The similarities of gDLK1 nucleotide and protein sequences were over 50% compared to mammalian species. In addition, chickens only express a full-length gDLK1 in various tissues at different ages without the alternative splicing variants. This suggests that the full-length form of gDLK1 may be sufficient for muscle development in the chicken. The expression of gDLK1 gene in pectoralis major muscle was significantly higher in 13- and 17-d old embryos \((P < 0.05)\), decreased in 1- and 5-d old chicks \((P < 0.05)\), and further decreased in 11- and 33-day old chickens \((P < 0.05)\). The expression pattern of gDLK1 was very similar to the expression patterns of myogenin and Pax7 genes, suggesting a close association with myogenic activities. In conclusion, the
developmental regulation of gDLK1 expression might play an important role in the early stages of muscle tissue development.

The objectives of the second study were to determine gDLK1 mRNA expression during primary muscle cell differentiation and during muscle regeneration after cold injury, as well as to compare gDLK1 mRNA expression during skeletal muscle development in layers and broilers. In the chicken primary muscle cell culture, gDLK1 mRNA expression was significantly increased from 12 h to day 2 ($P \leq 0.05$), when the nascent myotubes were actively formed at days 2-3. During muscle regeneration, the induction of the gDLK1 gene appeared between days 7-10 post-injury ($P \leq 0.05$), when myotubes were actively formed. The expression data of gDLK1 associated with myogenic markers (Pax7, MyoD, and myogenin) and morphological data suggest that gDLK1 may be involved in the late myogenic stages of primary muscle cell differentiation and muscle regeneration. The gDLK1 mRNA in the muscle tissues was very abundant at embryonic ages, but decreased after hatching in both broiler and layer chickens. In addition, the relatively greater expression of the gDLK1 gene in the muscles of broilers compared to layers suggests that gDLK1 may serve as a new selection marker for high muscle growth in chickens. These findings provide new insight into chicken muscle development and regeneration.

Muscle regeneration recapitulates embryonic skeletal muscle development that is governed by the coordinated gene expression of myogenic regulatory factors and chicken gDLK1. Muscular dystrophic (MD) and low score normal (LSN) chickens have a genetic muscle weakness, losing pectoralis major (PM) muscle function during muscle development and growth. Therefore, the objectives for the third study were to compare:
1) the morphological changes, and 2) the expression level and pattern of myogenic regulatory genes (MyoD, Pax7, myogenin, and MRF4) and the gDLK1 gene during muscle regeneration of control, LSN, and MD chickens with those of the control chickens. Both histological analysis and quantitative real-time PCR were used for this comparison and association studies. Histological section showed the delayed and impaired myofiber formation in LSN and MD chickens. A significantly high basal level of MyoD, Pax7, myogenin, and gDLK1 mRNA expression ($P < 0.05$) in LSN chickens suggests that muscle degeneration may have already progressed. LSN chickens had a delayed pattern of myogenin mRNA expression and the maintained expression of Pax7 and MyoD mRNA from D4 to D7 ($P < 0.05$); whereas, MD chickens had a significantly high peak expression of myogenin mRNA, a low peak expression of MRF4 mRNA, and a high peak expression of gDLK1 mRNA ($P < 0.05$). Although both LSN and MD chickens have delayed muscle regeneration, however, distinctive expression of stage-specific myogenic markers suggests that the regenerative mechanisms for their impaired muscles might be different. Understanding morphological and molecular events during muscle regeneration of muscular dystrophic chickens will further aid to identify muscle characteristic genes responsible for muscle developmental disorders.
Dedication

This dissertation is dedicated to God, my wife, sons, and our families for their endless love, support, and encouragement through the years.
Acknowledgments

I would like to express my sincere gratitude to my advisor, Dr. Kichoon Lee, for his mentoring and support over the years. Dr. Lee’s guidance and encouragement enabled me to complete this dissertation.

I would also like to express my deep appreciation to my committee members: Dr. Sandra G. Velleman, Dr. Martha A. Belury, and Dr. Earl Harrison for giving me valuable suggestion and assistance on my dissertation research, and sharing their time and expertise with me over the years. I would like to extend my deep appreciation to the faculties and scientists: Dr. David Latshaw, Dr. Macdonald Wick, and Dr. John M. Reddish for providing valuable experimental materials and comments on my researches over the years. I am very grateful to my three collegues, Dr. Jeffery A. Deiuliis, Dr. Bing Li, and Yeunsu Suh, for their assistance and discussion with research and friendship over the years. I would like to thank with deepest sincerity to all the group members of Dr. Lee and Dr. Velleman: Dr. Sangsu Shin, Julie Serr, Aishlin Lee, Julianne Rutt, Yang Song, Cindy Coy, David Gessmann, Rick Neuhardt, and Dennis Hartzler for their warm assistance, support, and friendship with helping experiments, animal studies, and editing dissertation. I am thankful to the Department of Animal Sciences and the Korean Science and Engineering Foundation (KOSEF) for supporting my education, researches, and stipends over the years.

I thank my family, Soohyun Lee, Ryan Wonha Shin, and Gideon Geonha Shin for
their endless love, support, and encouragement. I thank my friends, Michael D. Cressman, Sagar Thakkar, Rachel Kopec, and Minseok Kim, for their encouragement and help with reading and editing my manuscripts over the years.
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Chapter 1: Introduction

The study of muscle development, developmental defects of muscle, and the association of a candidate gene, delta like protein 1 (DLK1), with them is of great interest in this dissertation. In general, the abnormal regulation of DLK1 expression has been implicated in the muscle phenotypes of callipyge sheep, DLK1-transgenic mice, and human and mouse uniparental disomy (UPD) (Cockett et al., 1996; Georgiades et al., 2000; Davis et al., 2004). These muscle phenotypes are muscle hypertrophy and hypotonia caused by either the interruption of the normal function of imprinted genes or the impairment of their molecular function in the myogenic programming of all species, or both (Georgiades et al., 2000; Taketa et al., 2002; Lin et al., 2007; da Rocha et al., 2009). Therefore, studying DLK1 as one of the imprinted genes associated with genetic muscle disease and muscle development can provide a therapeutic insight for human muscle defects.

Human uniparental disomy (UPD) 14 is an inherited disorder derived from a disruption of the imprinted regions located on the human chromosome 14 (Kurosawa et al., 2002; Cotter et al., 1997). The imprinted regions contain imprinted genes that are expressed in either a maternal allele or a paternal allele of parental origin (da Rocha and Ferguson-Smith, 2004). Those imprinted regions of the human UPD14 are highly homologous to those in the mouse UPD12, resulting in similar developmental defects.
including facial abnormality, abdominal muscular defects, and skeletal anomalies (Georgiades et al., 2000; Villar et al., 2001; Sutton et al., 2003). Human UPD14 and mouse UPD12 show the muscle phenotypes such as muscle hypertrophy in paternal UPD and muscle hypotonia in maternal UPD (Sutton and Shaffer, 2000; Temple et al., 2007), suggesting that an abnormality of imprinted genes can cause the development of UPD muscle phenotypes. To date, DLK1 has been reported to be a candidate gene for expressing these muscular phenotypes in the human and mouse (Gerogiades et al., 2000; Taketa et al., 2002; Lin et al., 2007). Previous studies in callipyge sheep and DLK1-transgenic and knockout mice demonstrated muscle developmental defects, such as muscle myofiber hypertrophy and immuration of myofiber phenotypes (Moon et al., 2002; Davis et al., 2004; White et al., 2008). These suggest that DLK1 may be used as a candidate marker for screening high muscularity among domestic animals. However, no studies have explained the developmental regulation of DLK1 during muscle development and growth in avian species.

Embryonic skeletal muscle development mainly contributes to building muscles and growing them in humans and animals. In general, muscle progenitor cells are migrated, aligned, arranged, and committed into myoblasts. Myoblasts are finally differentiated into multinucleated muscle fibers during embryonic myogenesis (Buckingham et al., 2003; Shi and Garry, 2006). These developmental processes are governed by key molecules, myogenic regulatory transcription factors (MRFs), and paired box (Pax) factors. However, these regulations are so rapid and complicated that the different myogenic stages of embryogenic myogenesis are hardly distinct. Interestingly, regenerative myogenesis of muscle stem cells, also called satellite cells, can
partially overcome the tight and complicated regulation of MRF and Pax genes that
governs embryonic myogenesis. Thus, muscle regeneration in mammals has been widely
used for understanding a number of myogenic mechanisms associated with embryonic
myogenesis and screening an unknown functional genes associated with myogenic
signaling, pathway, and mechanism. However, few studies have shown the association of
DLK1 with embryonic and regenerative myogenesis in the avian species.

The delta-like protein 1 (DLK1) has a glycosylated extracellular domain
containing six-epidermal growth factor (EGF) motifs, a transmembrane domain, and a
small cytosolic domain. It has been studied widely in embryo development, obesity,
human genetic diseases, cancers, stem cells, and ear wound repair (Berends et al., 1999;
Georgiades et al., 2000; Moon et al., 2002; Samulewicz et al., 2002; Lee et al., 2003;
Abdallah et al., 2004; Davis et al., 2004), indicating an embryonic, regenerative, and
myogenic role of DLK1. Callipyge phenotype has a larger longissimus muscle area and
leg scores than normal sheep, indicating muscle hypertrophy. It is an inherited muscular
hypertrophy due to an aberrant imprinted gene expression in chromosome 18 of sheep,
which may be similar to the phenotypes of the human pUPD14 and mouse pUPD12
(Sutton et al., 2003). Previous studies also demonstrated that overexpression of DLK1 in
transgenic mice leads to muscle hypertrophy, recapitulating the callipyge phenotype
(Davis et al., 2004). These data suggest that DLK1 plays an important role in the muscle
development of animals and humans. However, the mechanism by which DLK1
promotes muscle growth and development has not been studied.

Therefore, in this dissertation, we will use the chicken as a model to research the
role of the DLK1 gene in skeletal muscle development. This functional genomic research
will provide, not only a new intervention to improve meat production in animals, but also new strategies in enhancing muscle growth and regeneration associated with genetic muscle diseases-related damage and weakness. Furthermore, the DLK1 gene can serve as a selection marker for superior chicks with high muscle growth, offering potential profits to the chicken industry.

1.1. Hypotheses

1.1.1. Chicken delta-like protein 1 (gDLK1) gene expression is associated with early embryonic muscle developmental stages during embryogenesis.

1.1.2. The high expression of gDLK1 mRNA correlates with a certain specific stage of primary cell and regenerative myogenesis in chickens.

1.1.3. The high expression of gDLK1 mRNA is implicated for muscle hypertrophy of chickens that have high muscularity.

1.1.4. Muscle weakness chicken models express a distinct expression pattern and level of gDLK1 mRNA associated with morphological changes in muscles during regeneration.

1.2. Specific Aims and Objectives

1.2.1. To identify the correlation of the DLK1 gene expression with myogenic activity during chicken muscle development.

Objective 1. To clone and sequence chicken DLK1 gene (gDLK1) and compare its
nucleotide and deduced protein sequences with mammalian species, and hence investigate the possible alternative splicing of the gDLK1 transcript.

Objective 2. To investigate developmental regulation of the gDLK1 gene in chickens and assess how specific developmental patterns of gDLK1 expression correlate with the specific stages of chicken muscle and adipose development.

1.2.2. To determine the effect of gDLK1 mRNA expression on skeletal muscle development in chickens.

Objective 1. To examine in vitro gDLK1 mRNA expression during chicken fetal myoblast differentiation.

Objective 2. To investigate if the difference in muscularity between broiler- and leghorn-type chickens may correlate with the different levels of gDLK1 mRNA expression during development.

Objective 3. To determine whether the gDLK1 mRNA expression is induced at specific stages of muscle regeneration.

1.2.3. To identify the correlation of the DLK1 gene expression with myogenic activity in chickens with muscular dystrophies.

Objective 1. To compare the muscle morphological changes during the regeneration between control chickens and chickens with muscular dystrophies.

Objective 2. To compare the expression levels and patterns of myogenic marker genes (MyoD, Pax7, myogenin, and MRF4) and gDLK1 gene during the muscle regeneration of control chickens and chickens with muscular dystrophies.
Objective 3. To relate the muscle morphological changes to the different patterns and levels of the mRNA expression of myogenic regulatory factors and gDLK1 during muscle regeneration.
2.1. Delta-like protein 1 (DLK1)

2.1.1. The structure and soluble forms of DLK1

Generally, delta-like protein 1 (DLK1) is a glycosylated protein that has three domains as follows: an extracellular domain, a trans-membrane domain, and a short intracellular domain (Villena et al., 2002). Among them, the extracellular domain of DLK1 contains a signaling peptide, six epidermal growth factor (EGF)-like repeats, and a juxtamembrane region (Smas and Sul, 1993). The EGF-repeats of DLK1 protein contain the conserved spacing of six cysteines, which is characteristic of the EGF motifs and important for protein-protein interaction (Villena et al., 2002). Importantly, it has been reported that two soluble forms, 3EGF (24-25 kDa) and 6EGF (50-60 kDa), of DLK1 protein can be cleaved and generated by a disintegrin and metalloproteinase 17 (ADAM17/ TACE) at the extracellular domain (Smas et al., 1997; Wang and Sul, 2006; Sul, 2009). Of the two EGFs, a large soluble form is known to be bioactive (Mei et al., 2002). The protein of DLK1 belongs to a family of Notch/Delta/Serrate signaling proteins that are involved in cell fate determination due to the high homology as shown in Figure 2.1 (Bray et al., 2008). However, it lacks the DSL-domain that is required for receptor-ligand interaction of the notch family (Tax et al., 1994) and consequently has a shorter intracellular domain (Bray et al., 2008). This indicates that the role of EGF-repeats of DLK1 may be more important for protein-protein interaction. Fifteen years
ago, the protein structure of DLK1 was identified mainly in the mammalian species, but has never been reported in the avian species.

Figure 2.1. Structure of DLK1 and its homology to a family of Notch/Delta/Serrate (Bray et al., 2008). Several members of the Delta-Serrate family of Notch ligands. Comparative structure is shown including the EGF-repeats, signal peptide, extracellular proteolytic cleavage domain and transmembrane domain found in all the ligands in addition to the DSL domain that is missing in mouse DLK1. Jagged 1 = Jag 1, Jagged 2 = Jag 2, Delta 1 = Dll1, Delta like protein 1 = DLK1.

2.1.2. The glycosylation of DLK1 protein

Glycosylation affects the structure and function of many proteins in eukaryotes. The glycosylation of DLK1 protein has been reported in the mammalian species (Smas et al., 1994, 1997). In our previous comparative analysis of DLK1 protein sequence among mammals such as the human, mouse, rat, cow, and pig (Deiuliis et al., 2006), three conserved N-linked glycosylation sites at the 101st, 134th, and 295th amino acid residues were found in all the animals except the pig, which lacked the third glycosylation site (Figure 2.2). Even though the glycosylation of DLK1 protein at the evolutionary
conserved sites is important to modify the protein structure and molecular weight, unglycosylated recombinant DLK1 protein also has an inhibitory role in adipogenic cell differentiation. This suggests that DLK1 protein may have a central role in inhibiting cell differentiation regardless of the glycosylation. The role of DLK1 and the possible effects on protein-protein interactions such as DLK1 dimerization, proteolytic cleavage, and signaling need to be studied further.

2.1.3. Alternative splicing of DLK1, its spliced transcripts, and developmental regulation

Alternative splicing is one of the complex biological functions through which many isoforms of a gene can be generated without an increase in the number of genes in the genome. The various transcripts of DLK1 have been found in different tissues, at different ages, and among different species, possibly due to their different systems of development and evolution. These isoforms are generated by alternative splicing in the fifth axon of DLK1. All isoforms of DLK1 in various tissues of mammals are shown in Table 2.1. The alternative splicing variants of DLK1 in the pig, cow, human, mouse and rat contained consensus sequences shown in the classical model of splicing as summarized (Keller and Noon, 1984; Padgett et al., 1986). The classical model of alternative splicing explains the removal of intronic sequences from a primary transcript pre-mRNA; the DLK1 sequences allow for splicing within the site of 5th axon, which creates splicing variants (Figure 2.3).
Figure 2. The protein sequence comparison of DLK1 full-length form in mammals (Deiuliis et al., 2006). The arrows indicate the N-linked glycosylation sites in the protein. Black shading indicates identical amino acids and gray shading indicates amino acids in the same R groups.
Figure 2. 3. Classical model of primary pre-mRNA splicing (Keller and Noon, 1984; Padgett et al., 1986) and splicing variant sites of DLK1 (Deiuliis et al., 2006).

(A) In the classical model, the 5’ splice site contains the consensus sequence A/CAG in the 5’ exon followed by GTA/GAGT at the exon-intron junction. The splicing machinery recognizes this sequence and cleaves after the A/CAG. A branching point shows the sequence CTA/GAC/T is then usually found in the intron sequence 20-50 base pairs from the 3’ splice site. The branching site contains an obligatory adenosine residue near 3’ splicing site that is required for formation of the lariat structure and transesterification reactions. The structure and reactions are responsible for the excision of the intron sequence. The 3’ splice site in the classical model contains NCAG in the 3’ end of the intron followed by a G residue in the adjacent exon of the intron-exon junction. (B) DLK1 nucleotide sequence comparison across mammalian species. The known 5’ (arrow) and 3 (diamond) splice site in the pig (GenBank #: DQ309460), cow (AF181466), human (BC007741), mouse (BC052159), and rat (NM_053744) are illustrated. Black shading represents identical nucleotide sequences among them.
Figure 2. 3
Table 2. 1. Variant isoforms of DLK1 by alternative splicing in various tissues among mammalian species.

<table>
<thead>
<tr>
<th>Mammalian Species/isoforms of DLK1</th>
<th>Adipose tissue (adipocytes)</th>
<th>Muscle</th>
<th>Heart</th>
<th>Liver</th>
<th>Lung</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>A</td>
<td></td>
<td>A</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>A, B, C, C2, D, D2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>A, C2, E</td>
<td>C2</td>
<td>C2</td>
<td></td>
<td>C2</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>A, C2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The human has only the full length form (A) of DLK1 identified in adipose, liver, and kidney tissues (Deiuliis et al., 2006). The A form of DLK1 gene was highly induced in liver and kidney tissues of the fetus and absent in the tissues as adults. In the case of the mouse, six iso-forms of DLK1-A, -B, -C, -C2, -D, and -D2 forms were identified in adipose tissues (Smas et al., 1994, 1997). When alternative spliced variants of DLK1, two soluble isoforms of DLK1, and a DLK1 cleavage enzyme (ADAM17/ TACE) have been identified, questions arised about which splicing variants or isoforms are bioactive. Mei et al. (2002) demonstrated that the larger soluble form (6EGF) of DLK1 inhibited adipogenesis, indicating that the 6EGF form derived from DLK1-A and -B was bioactive, but the smaller form (3EGF) derived from DLK1-C and -D was not bioactive during murine adipocyte differentiation.

In the pig, our previous data found that B and C2 transcripts of DLK1 were identified in the adipose, muscle, heart, liver, spleen, and intestine (Deiulliis et al., 2006). Additionally, a recent study showed that DLK1-A and -C were also expressed in
preadipocytes and adipose tissues of the pig (Samulin et al., 2009). All four isoforms were developmentally down-regulated in preadipocytes and adipose tissues at different ages of the pig. Among them, the DLK1-C2 form was highly expressed in those cells and tissues of the pig, expecting that the C2 form may play a dominant role in adipose tissue development.

In cattle, A, C2, and E spliced forms of DLK1 were identified (Fahrenkrug et al., 1999; Minoshima et al., 2001; Vuocolo et al., 2003). The DLK1-A and -C2 genes were highly induced in fetal adipose tissue while only the DLK-C2 gene was maintained in neonate and adult adipose tissue. The expression of the DLK1-C2 gene was predominant in the fat depots of tissues such as cardiac fat, kidney fat, omental fat, genital fat, and the muscles containing longissimus dorsi skeletal muscle, semi-tendinosus skeletal muscle, cardiac muscle, and the placenta (Vuocolo et al., 2003). This indicates that the bovine DLK1-C2 form may be required for the development of these tissues.

In normal sheep and callipyge sheep, which have a muscle hypertrophy phenotype, the major expression of DLK1-C2 form and the minor expression of DLK1-A form were shown (Davis et al., 2004). Normally, the A form expression was decreased after birth, showing a developmental regulation of a full-length form of DLK1 while the C2 form expression still remained at 8 weeks old. This indicates that for the skeletal development of sheep, the conditions such as decreasing the level of the A form expression and maintaining the C2 form expression may be required. However, information has not been reported in the avian species regarding DLK1 expression, alternative transcripts, and developmental regulation.
2.1.4. A specific role of DLK1 in various cell types during their differentiation.

DLK1 has been known to play a critical role in determining cell fates during development (Laborda et al., 2000). To date, the role of DLK1 and its cleaved proteins has been identified in several developmental studies, including adipogenesis (Smas et al., 1993 and 1997; Mei et al., 2002), hematopoiesis (Moore et al., 1997; Li et al., 2005), chondrogenesis and osteogenesis (Abdallah et al., 2004; Wang and Sul, 2009), and adrenal gland and neuroendocrine cell differentiation (Okamoto et al., 1998; Jensen et al., 1999; Ansell et al., 2007). Following DLK1 studies will help us to understand the distinct role of DLK1 in different cell types and their differentiation.

A cDNA microarray determined that DLK1 was highly expressed in preadipocytes, while it was absent in adipocytes after differentiation, suggesting that DLK1 has a role in adipogenesis (Smas et al., 1993). To understand the functions of the DLK1 gene, its spliced variants, and their proteins, genetic modification (overexpression and knockout) of DLK1 and their derivatives have been studied in adipocytes and adipose tissue during development (Smas et al., 1993 and 1997; Sul et al., 2000; Moon et al., 2002; Lee et al., 2003). In fact, an inhibitory role of DLK1 has been displayed in preadipocyte differentiation, called adipogenesis (Sul et al., 2000), as well as, in fat tissues of animals (Moon et al., 2002; Lee et al., 2003). Overexpression of DLK1 inhibited adipocyte differentiation (Smas et al., 1993); whereas, knockdown of DLK1 enhanced adipogenesis in murine 3T3-L1 cells (Smas et al., 1999). Likewise, the DLK1-transgenic mouse showed that overexpression of DLK1 (pref-1/hFc) reduced the mass and size of fat by inhibiting adipogenesis and caused an impairment of glucose intolerance and insulin sensitivity (Lee et al., 2003; Figure 2.4A); whereas, a DLK1-
knockout mouse showed that absent expression of DLK1 increased the size and mass of fat by accelerating adiposity (Moon et al., 2002; Figure 2.4B). These strongly suggest that DLK1 negatively regulates adipocyte or adipose tissue development of animals.

Figure 2.4. Histological analysis of adipose tissue and cell size distribution in DLK1-transgenic (A) or -knockout (B) mice (Lee et al., 2003; Moon et al., 2002). Left panel (Renal white adipose tissues), Paraffin-embedded sections of adipose tissue from 10-week-old male mice were stained with hematoxylin and eosin. Scale bar = 50 μm. Right panel (Distribution of the adipocyte volume). The volume of at least 300 cells per sample (mean of four mice per group) was determined.

DLK1 was highly expressed in osteoblastic cells of fetal bone and decreased in adult bone, suggesting a role of DLK1 in skeletal development (Abdallah et al., 2004). Overexpression of DLK1 did not affect the proliferation rate of human mesenchymal stem cells (hMSC), but inhibited the further developmental processes of bone formation. In addition, mineralization, important for bone formation, was inhibited by DLK1 overexpression, resulting in the decrease in the ratios of osteoblast/bone surface and osteoid surface/bone surface, osteoid perimeter, osteoid area, and osteoid width. Also,
continued expression of DLK1 inhibited maturation of osteoblasts by inhibiting bone marker gene expressions [alkaline phosphatase (ALP), collagen type I (Col1), and osteocalcin (OC)]. Conditional media containing a large soluble form of DLK1, which regulates cell proliferation or differentiation, showed maintenance of the progenitor cell population of osteoblasts (Abdallah et al., 2004). Recently, the inhibitory role of DLK1 in MSC fate to chondrocyte maturation and differentiation of osteoblasts was determined by promoting expression of Sox9, required for early chondrogenesis (Akiyama et al., 2004; Wang and Sul, 2009).

DLK1 expression was found in patients with myelodysplastic syndromes (MDS), which results from the impairment of hematopoietic stem cell differentiation (Hofmann et al., 2002; Langer et al., 2004). In addition, it was suggested that DLK1 expression correlated with hematopoietic supporting activity of the stem cells (Moore et al., 1997). Moore et al. (1997) observed that a soluble form of DLK1 did not affect proliferation of stem cells, but a full-length form of DLK1 increased stem cell populations by promoting “cobblestone area” (CSA) colony formation, suggesting that a full-length form of DLK1 may have a role in proliferating hematopoietic stem cells. In the study of Li et al., (2005), overexpression of DLK1 inhibited both the differentiation and proliferation of human promyelocytic HL-60 cells. Cleaved soluble forms of DLK1 did not inhibit hematopoietic cell differentiation, suggesting that an intracellular domain of DLK1 may be important for the function (Li et al., 2005).

It has been reported that DLK1 is also expressed in a number of endocrine cells, such as somatotroph cells of the pituitary gland (Lärsen et al., 1996), β cells of the pancreas (Tornehave et al., 1993), leydig cells of the testis, and the theca interna and the
Hilus cells of the ovary (Jensen et al., 1999), suggesting that DLK1 may have an endocrine function. Ansell et al. (2007) demonstrated that DLK1 negatively regulated the promoter region of the growth hormone (GH), suppressing GH expression at the gene level in somatotroph GH3 cells. Taken together, DLK1 has various functions for cell differentiation based on different cell types and characteristics, but mainly inhibits cell differentiation.
2.2. Mammalian genetic disorders and DLK1

2.2.1. Imprinted gene, DLK1.

Genomic imprinting is a normal process that regulates a subset of genes to be developmentally expressed from one of two parental alleles on a chromosome (da Rocha and Ferguson-Smith, 2004). Some imprinted genes, maternally expressed genes (MEGs), are expressed from the maternal allele of inherited chromosomes while others, paternally expressed genes (PEGs), are expressed from the paternal allele of inherited chromosomes, suggesting that maternal and paternal genomes do not have the same function. In addition, this means that both functions of maternal and paternal genomes are essential for normal development and growth (da Rocha and Ferguson-Smith, 2004).

To date, 70 imprinted genes containing MEGs and PEGs have been identified in humans. One of the PEGs among them is delta-like protein 1 (DLK1). The imprinted gene is located in chromosome 14 of the human (Berends et al., 1999), chromosome 12 of the mouse (Schmidt et al., 2000), chromosome 18 of the sheep (Takeda et al., 2006), chromosome 21 of the cow (Minoshima et al., 2001), and chromosome 7 of the pig (Table. 2.2). Although the DLK1 gene is expressed in the paternal allele of those mammalian chromosomes, the regulation and mechanism of the paternally imprinted gene DLK1 are complicated with other genetic factors. Dr. Ferguson-Smith’s group has extended great effort on verifying the role of imprinted genes and their mechanisms by studying epigenetics (da Rocha and Ferguson-Smith, 2004; Ogata et al., 2008; da Rocha et al., 2009). There are two general regulation theories as follows: 1) the DLK1 gene expression is regulated by the negative feedback function of a gene cluster from the maternal allele of the chromosomes; 2) epigenetic modifications, such as DNA
methylation at CpG dinucleotides and translational modifications of core histones (methylation and acetylation), may be involved in the regulation of imprinted regions containing DLK1 gene (Ogata et al., 2008). Recently, at least six among 52 microRNAs in the DLK1/DIO3 region of paternal alleles of mouse chromosome 12 were shown to negatively regulate DLK1 (Hagan et al., 2009). This suggests that another mechanism is involved for regulation of imprinted genes and interaction between alleles of parental origin. Taken together, these suggest that the paternal expression of DLK1 is tightly regulated for mammalian tissue development and growth. Therefore, observing phenotypes of genetic disorders related to abnormal expression of DLK1 in mammals allows us to predict the role of DLK1 in normal tissue development and growth.

Table 2. The location of imprinted gene DLK1 in chromosome of the human, mouse, sheep, cow, and pig.

<table>
<thead>
<tr>
<th>Human</th>
<th>Mouse</th>
<th>Sheep</th>
<th>Cow</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome 14q32.2</td>
<td>Chromosome 12E-F1</td>
<td>Chromosome 18</td>
<td>Chromosome 21q24</td>
<td>Chromosome 7</td>
</tr>
<tr>
<td>Ideogram</td>
<td>Ideogram</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The diagrams were obtained in National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). No diagrams of the sheep, cow, and pig were printed.
2.2.2. Genetic disorders in mammals and DLK1

Analyzing abnormal phenotypes of both maternal and paternal uniparental disomy (UPD) in mammals has provided clues to identify the function and regulation of imprinted genes (Kurosawa et al., 2002; Sutton et al., 2003). In addition, consideration of UPD phenotypes associated with aberrant imprinted gene expression may contribute to approaching and understanding the development of severe human malformation syndromes, including Prader-Willi syndrome, Angelman syndrome, Beckwith-Wiedemann syndrome, and possibly Russell-Silver syndrome (Hagan et al., 2009).

The uniparental disomy (UPD), one of the genetic disorders, results in overexpression or absent expression of one of the parentally imprinted genes (Ogata et al., 2008). If both paternal alleles are inherited in chromosome 14, called paternal UPD14 (pUPD14/ upd(14)pat), PEGs are overexpressed, whereas if both maternal alleles are inherited in chromosome 14, called maternal UPD14 (mUPD14/ upd(14)mat), PEGs are not expressed (MEGs are overexpressed) by different mechanisms as shown in Figure 2.5 (da Rocha and Ferguson-Smith, 2004).

In addition, imprinted regions of human UPD14 are homologous with those of mouse UPD12, resulting in a pattern of similar phenotypes in mUPD12 or pUPD12 mice to those of humans (Sutton et al., 2003). This indicates that the aberrant expression of one imprinted gene can result in the severe developmental phenotypes of the human and mouse UPD as shown in Table 2.3 and Figure 2.6.
Figure 2. 5. Genomic imprinting defect caused by different mechanisms such as UPDs, imprinted gene mutation, and ICR mutation (da Rocha and Ferguson-Smith, 2004).

Figure 2. 6. Defects of developmental growth of both pUPD12 and mUPD12 at embryonic day 15.5 (A) and at embryonic day 18.5 (B). Scale bars, 1 mm (Georgiades et al., 2000).
Table 2. 3. Phenotypes of uniparental disomy (UPD) in the human and mouse.

<table>
<thead>
<tr>
<th>Genetic disorder</th>
<th>Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>pUPD14/Upd(14)pat</td>
<td>Polyhydramnios, thoracic and abnormal wall defects, growth retardation, severe developmental delay, characteristic dysmorphism, and mental retardation (Sutton and Shaffer, 2000)</td>
</tr>
<tr>
<td>mUPD14/Upd(14)mat</td>
<td>Prenatal and postnatal growth retardation, hypotonia, joint laxity, motor delay, early onset of puberty, and minor dysmorphic features of the face, hands, and feet (Sutton and Shaffer, 2000; Temple et al., 2007)</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>pUPD12/Upd(12)pat</td>
<td>Early death after 16 days gestation, placentomegaly, muscle overgrowth, cardiomyopathy, and skeletal defects, including delayed ossification centers in the sternum, thin ribs with abnormal sternal attachments, and a bell-shaped thorax (Georgiades et al., 2000; Villar et al., 2001; Sutton et al., 2003)</td>
</tr>
<tr>
<td>mUPD12/Upd(12)mat</td>
<td>Growth retardation, postnatal death, defect of skeletal muscle fiber maturation, and defect of neural crest-derived middle ear ossicles (Georgiades et al., 2000)</td>
</tr>
</tbody>
</table>

2.2.3. Dysregulation of DLK1 gene expression and muscular phenotypes in animal studies.

Defects of growth and development in mammalian species have been described and implicated with an imprinted gene, DLK1. Recently, DLK1 has been studied as a leading factor for muscular phenotypes in animals, such as transgenic mice and callipyge sheep. Thus, it is of interest to link the dysregulation of DLK1 gene expression with muscular phenotypes in animals.

As shown in Figure 2.6 and Figure 2.7, embryos of UPD12 mice have defects in
body growth and muscle development (Georgiades et al., 2000). In UPD12 mice, the pUPD12 embryo showed a larger myofiber cross-sectional area and a high number of myofibers with central nuclei when compared to the normal embryo; whereas, mUPD12 mice showed a small size and mass of myofiber cross-sectional area. Georgiades et al. (2000) suggested that myofibers with central nuclei of pUPD12 may be due to retention of myofiber maturation with the development of muscles. It was also suggested that fewer immature myofibers shown in muscles of mUPD12 may be caused by the defects of secondary myogenensis, including myofiber formation, elongation, and maturation. Moreover, pUPD12 mice also exhibited cardiomyopathy whose phenotypes increased the mass and size of the heart, increased ventricular diameter, thinner, less compact myocardium, and deep intertabeular recesses compared to wild type mice (Villar et al., 2001). These muscle phenotypes in UPD mice associated with imprinted regions including DLK1 and those muscle phenotypes in callipyge sheep associated with the locus of CLPG including DLK1 suggest that the regulation of DLK1 plays an important role in muscle development in animals (Cockett et al., 1996; Georgiades et al., 2000; Taketa et al., 2000; Lin et al., 2007).
Figure 2.7. Muscle morphology of normal (A,D,G), mUPD12 (B,E,H) and pUPD12 (C,F,I) embryos at embryonic day 18.5 (Georgiades et al., 2000). The myofibers were stained with the myofiber specific antibody MY32 (A-C). Comparable sections through the diaphragm and (D-F) forearm muscles. (G-I) High power views of the extensor pollicis longus muscle of normal (G), mUPD12 (H) and pUPD12 (I). ecrl, extensor carpi radialis longus; epl, extensor pollicis longus; fds, flexor digitorum superficialis; r, radius; u, ulna. Scale bars, 0.05 mm (A); 0.2 mm (D), 0.0125 mm (G).

In the case of callipyge sheep, polar overdominance on chromosome 18 of sheep causes an inheritable muscular hypertrophy, called callipyge phenotype as shown in Figure 2.8 (Cockett et al., 1996). This muscular hypertrophy phenotype is primarily due to myofiber hypertrophy. Muscle tissue sectioning by histology between callipyge and normal sheep showed a larger diameter and a greater population of fast-twitch glycolytic
(FG) muscle fibers than slow-twitch oxidative (SO) fibers, suggesting that myofiber changes in callipyge sheep were highly associated with FG muscle fibers rather than SO fibers (Carpenter et al., 1996). These phenotypes were greater in 8-week-old lambs than in 2-week-old lambs, indicating a gross phenotype during post-natal development of callipyge sheep (Carpenter et al., 2000).

Figure 2. Callipyge sheep (number 1 and 3). Number 2 and 4 indicate normal sheep (Cockett et al., 1996).

The callipyge locus on ovine chromosome 18 was homologous to those imprinted region on the murine chromosome 12 and human chromosome 14. Of the callipyge locus, high expression of DLK1 mRNA in callipyge sheep was determined during fetal and post-natal skeletal muscle development compared to normal sheep (Perkins et al., 2006; Fleming-Waddell et al., 2007; White et al., 2008). In addition, DLK1 protein was
localized in myofibers, indicating an association of DLK1 with the muscular hypertrophy phenotype of callipyge sheep (White et al., 2008). Taken together, paternally expressed gene DLK1 may be involved in the post-natal myofiber hypertrophy of callipyge sheep.

Supportively, this callipyge phenotype was recapitulated in muscles of transgenic mice where DLK1 protein was ectopically over-expressed (Davis et al., 2004). The increase in muscle mass and average myofiber diameters were clearly shown in muscles of transgenic mouse lines. In addition, a majority of myofibers with central nuclei in transgenic mice suggested a similar phenotype of myofiber immaturation to that of pUPD12. This indicates that DLK1 may play an important role in myofiber formation or maturation although its mechanism still remains unknown.

Furthermore, deletion of the unmethylated intergenic, parental-origin-specific differentially methylated region (IG-DMR) on the maternal chromosome of mice resulted in switching epigenotype (maternal into paternal) via activation of the imprinted genes such as DLK1, RTL1, and DIO3 (Lin et al., 2007). At embryonic day 19 of the IG-MDR knockout mice, immature myofibers and myofiber hypertrophic phenotypes including myofibers with central nuclei are shown in Figure 2.9. The myofiber hypertrophy has been characterized in the callipyge sheep and pUPD12 mice. This indicates that PEGs are normally suppressed by maternally expressed non-coding RNAs or microRNAs such as IG-MDR for muscle development. In addition, this suggests that suppression of DLK1 may be normally required for myofiber maturation during muscle development in animals.

Taken altogether, abnormal expression of an imprinted gene DLK1 causes defects of muscle and heart development and exhibits myofiber immaturities (myofibers with
centered nuclei) in callipyge sheep, UPD12 mice, DLK1-transgenic mice, and IG-DMR knockout mice.

Figure 2.9. The immature and hypertrophic muscle phenotypes in IG-DMR knockout mouse embryos (Lin et al., 2007). (A,C,E) Normal muscle muscularity at embryonic day 19. (B,D,F) The muscle phenotypes of IG-MDR/+ embryos at E19. The myofibers were stained with myofiber-specific antibody MY-32. Cross-sections of the forelimb (A, B), the extensor carp radialis longus (E, D), and the extensor pollicis longus muscle (E, F). epl, extensor pollicis longus; ecrl, extensor carpi radialis longus; r, radius; u, ulna.
2.2.4. DLK1 protein localized in embryonic myofibers in mammalian species.

To date, DLK1 has been implicated in myofiber phenotypes when it is abnormally expressed and regulated in mammalian species. However, the protein expression of DLK1 in myofibers in mammals has not yet been discussed. Therefore, immunostaining of DLK1 in muscles provides a critical clue to DLK1’s effect on myogenic activity during myotube formation. According to a study for the immunostaining of DLK1 in human skeletal muscles at fetal week 8 to 16 (Figure 2.10A; Floridon et al., 2000), DLK1 protein was localized in myotubes with central nuclei, but not localized in myotubes with peripheral nuclei, and cardiac and smooth muscles, suggesting that DLK1 may have a role in the maturation of myofibers.

Figure 2.10. DLK1 protein is localized in the membrane of multinucleated myotubes with centrally located nuclei. (A) Skeletal muscles in human fetuses are shown from week 8 to 16 (Floridon et al., 2000). (B) Skeletal muscles in the mouse are shown from embryonic day 17.5 (our unpublished data).
In addition, our unpublished data showed that DLK1 was also localized in membranes of myofibers with central nuclei in the mouse muscles at embryonic day 17.5 (Figure 2.10B). Recently, Andersen et al. (2009) further confirmed that DLK1 was expressed in human fetal myofibers at a gestational age from 12 to 23 weeks. This clearly suggests that DLK1 is strongly associated with myofibers and may act on a negative role in maturation of myofibers.
2.3. Skeletal muscle development and regeneration

2.3.1. Skeletal muscle development

Skeletal muscles are formed originally from the paraxial mesoderm during vertebrate embryogenesis (Buckingham et al., 2003). Through segmentation and specialization of the somite during embryogenesis, dermomyotome (the origin of skin, skeletal muscle of the trunk and limbs), myotome, and sclerotome (the origin of skeleton) are formed (Shi and Garry, 2006). Dermomyotomal cells migrate in a dorsal fashion to produce the epaxial myotome (the origin of trunk and back muscle) and in a ventral fashion to produce the hypaxial myotome (the origin of limb muscle) (Ordahl et al., 2000). The progenitor cells from dermomyotome and myotome migrate and adopt a skeletal muscle fate in a coordinated fashion governed by molecular programs containing myogenic signals and myogenic specific regulatory genes (Buckingham et al., 2003; Shi and Garry, 2006).

In general, skeletal muscle development is a multi-step process, called myogenesis, as follows: myogenic progenitor cell (MPC) activation and proliferation during which MPC become myoblasts, cell cycle withdrawal, alignment and rearrangement of myoblasts, cell differentiation (elongation and fusion), myotube formation, and finally myofiber maturation (Figure 2.11). These developmental processes, accompanied by morphological and biological changes of myogenic cells, are governed by a complex orchestration of signals that are mediated by myogenic regulatory transcription factors (MRFs) (Buckingham, 2006), the extracellular matrix (ECM) (Velleman, 1999), and growth factors (McFarland, 1999). Although the developmental stages during animal embryonic myogenesis are overlapped and complicated, myogenic
regulatory factors (MRFs), molecular indicators, could allow us to know which stages of myogenesis have progressed.

**Muscle cell differentiation**

![Diagram of muscle cell differentiation](image)

**Figure 2.11. The summary of muscle cell differentiation, their stages, and their regulatory transcription factors.** MPC: myogenic precursor cells.

2.3.2. The regulation of myogenic regulatory factors (MRFs) during myogenesis

MRFs are a family of basic helix-loop-helix (bHLH) transcription factors and play a critical regulatory role in the process of embryonic skeletal muscle development (Buckingham et al., 2003 and 2006; Shi and Garry, 2006). They consist of myogenic differentiation 1 (MyoD/Myod1), myogenic determination factor 5 (Myf5), myogenin (Myog), and myogenic regulatory factor 4 (MRF4/Myf6) (Davis et al., 1987; Pinney et al., 1988; Rhodes and Konieczny, 1989; Wright et al., 1989; Bryson-Richardson and Currie, 2008). As shown in Figure 2.11, a sequential expression of MRFs promotes myogenesis where myoblasts are fused together and formed into multinucleated
myofibers. Previous studies suggested that the gene or protein expression of MyoD and Myf5 are indicated as early stages of myogenesis, such as specification to myogenic lineage, cell cycle exit, and initial differentiation of myoblasts (Rudnicki et al., 1993; Kablar et al., 1998). Following their expression, myogenin is induced at the fusion of myoblasts and the formation of myotube with central nuclei (Hasty et al., 1993; Nabeshima et al., 1993). Finally, the gene or protein expression of MRF4 indicates a late stage of myogenesis (terminal differentiation of the muscle cells), promoting to form functional myofibers with peripheral nuclei (Patapoutian et al., 1995).

Paired-box (Pax) transcription factors also play a critical role in embryonic myogenesis. High and broad expression of Pax3 in the developing mouse embryo suggested a role in embryogenesis (Shi and Garry, 2006). The mutant mice by disruption of the Pax3 gene exhibited somite defects such as defects of segmentation and loss of the hypaxial dermomyotome, resulting in migration failure of the muscle progenitors and absence of limb muscle (Goulding et al., 1994; Bober et al., 1994). Absence of Pax3 and Myf5 in mice resulted in lack of body wall and limb muscle (Tajbakhsh et al., 1997). These suggest that Pax3 is genetically upstream of the MRF members and has a regulatory role in the initiation of myogenesis. Recent knockout and knock-in studies have demonstrated that Pax7 can replace the role of Pax3, but embryos without Pax7 have normal muscles during embryogenesis (Relaix et al., 2004; Mansouri et al., 1996), suggesting that Pax7 may not be required for embryonic muscle development. Instead, Pax7 is important for specification and activation of satellite cells (Seale et al., 2000). These muscle stem cells are located in the basal lamina of myofibers and play an essential role in muscle growth and regeneration after the muscle injury or exercise.
(Schultz and McCormick, 1994; Zhao and Hoffman, 2004; Parise et al., 2006). Pax7 null mice developed normally, but they lack activation, proliferation, and fusion of satellite cells, resulting in a failure to postnatal muscle growth (Seale et al., 2000). Taken together, myogenic regulatory transcription factors are expressed during embryogenesis and the coordination of MRF expression regulates embryonic muscle development (Buckingham et al., 2003 and 2006; Shi and Garry, 2006).

2.3.3. Skeletal muscle regeneration

To understand a complicated myogenic program during embryogenesis, muscle regeneration in the muscle of animals has been performed to recapitulate embryonic myogenesis (Goetsch et al., 2003, Porter et al., 2003; Turk et al., 2005). Unlike embryonic myogenesis, regenerative myogenesis has an advantage to observe discrete stages of myogenesis as shown in Figure 2.12 (Goetsch et al., 2003; Shi and Garry, 2006). Figure 2.12A, 12B, and 12C show that a myonectotic molecule, cardiotoxin, induced muscle damage in adult mice. The injured muscle was recovered with days during regeneration. Similar to embryonic myogenesis, regenerative myogenesis occurred as followed: activation of satellite cells post-injury, proliferation of these myogenic cells, differentiation into myofibers with multinuclei, and finally maturation of myofibers for functions of muscle fibers (Goetsch et al., 2003; Shi and Garry, 2006). These molecular events of regenerating muscle are coordinated and complex. Using analyses of transcriptome or high-density oligo-nucleotide array, recent studies have revealed the molecular profile of gene expression that governs dissected stages of the muscle regenerative process as shown in Figure 2.12D (Goetsch et al., 2003; Porter et al., 2003;
As shown in Figure 2.12, if muscle stem cells, called satellite cells, located in basal lamina surrounding individual myofibers are activated after muscle injury or exercise (Schultz and McCormick, 1994; Zhao and Hoffman, 2004; Parise et al., 2006), embryonic MRF genes and paired box genes (Pax7 and Pax3) are re-expressed at proper stages for reforming myofibers (Schultz and McCormick, 1994; Buckingham, 2006; Shi and Garry, 2006). Collectively, understanding the muscle regenerative myogenesis and molecular physiology in the animals will provide an insight to those regenerative stages and processes in the avian species.

Figure 2. 12. Muscle repair by discrete stages of regeneration in mice (Shi and Garry, 2006; Goetsch et al., 2003). (A) Outlines of the distinct stages of muscle regeneration including activation of satellite cells, proliferation of the myogenic cells, differentiation into myofibers, and maturation of myofibers for normal function of muscles. (B) An intramuscular injection of cardiotoxin (a myonecrotic molecule), ~70%-90% of the muscle is damaged. (C) Restoration of the muscle fibers detected by Hematoxylin and eosin-staining (H&E) and tissue section. (D) Cardiotoxin-induced muscle injury and high-density oligonucleotide array analysis of pooled RNAs (n=3 animals).
2.3.4. Regeneration of tissues and DLK1 in the human and animals.

It has been reported that DLK1 was highly induced in embryonic tissues (liver, tongue, vertebrae, skeletal myobutes, chondroblasts, and pancreas) of the human and animals (Floridon et al., 2000; Yevtodiyenko and Schmidt, 2006). Recent regenerative studies, however, have demonstrated that a temporal expression of DLK1 is required for regeneration of ear, liver, and muscle tissues (Samulewicz et al., 2002; Jensen et al., 2004; Andersen et al., 2009). These studies have an objective to identify DLK1 as a marker of specific cell types during regeneration of tissues.

During ear wound healing in the mouse, DLK1 gene was highly induced at 12-14 days of the tissue regeneration and down-regulated until 20 days, suggesting that a temporal expression pattern of DLK1 gene may contribute to the regenerative capacity of the murine ear wound (Samulewicz et al., 2002). In addition, during liver regeneration of rats, DLK1 gene was re-expressed at post-injury day 9 and its protein was localized mostly in ductular oval cells and hepatocyte-like progeny, but not in bile duct cells, mature hepatocytes, and the endothelium of the hepatic arteries and veins during post-injury 5-9 days. This suggests that DLK1 plays a role, as a marker, in identifying liver specific stem cell types (Jensen et al., 2004). More recently, the spatial and temporal pattern of DLK1 re-expression was also found during muscle regeneration of rodents (Andersen et al., 2009). In this study, DLK1 was stained with mononucleated cells, but not myofibers. With days of muscle regeneration, DLK1 expression peaked at post-injury day 7 and then down-regulated at the levels of mRNA and protein when myogenic differentiation and fusion of mononuclear cells actively occurred. To characterize a DLK1 role in those stages of muscle regeneration, non-lesioned and post-lesioned
myogenic cell culture and double staining of DLK1 with one of the myogenic markers (Pax7, myogenin, desmin, and adult myosin heavy chain) were performed. Data showed that the majority of DLK1-positive cells were large, flat and non-myogenic, while a small population of DLK1-positive cells underwent myogenesis and formed into myotubes mostly containing 2-6 nuclei (Andersen et al., 2009). Nevertheless, the function of DLK1 during muscle regeneration is not completely identified.

2.3.5. Chicken as a model for muscle development & growth and muscle regeneration.

The chicken is an excellent model to study muscle development and regeneration because 1) the period of embryonic tissue development (17-19 days) in the chicken is relatively more shorter than that of mammals, 2) chickens have genetic disease models such as muscular dystrophy, which will contribute to understanding of the genetic and molecular mechanism of human muscle diseases, 3) the repair myogenic program of chicken muscle regeneration is similar to that of mammalian muscle regeneration, 4) the regenerative myogenesis can partially recapitulate embryonic myogenesis in chicken muscles, 5) handling of egg embryos and isolation of primary cells are easier than those of mammals, and 6) the results of chicken studies will contribute to agriculture and the chicken industry. This strongly suggests that the chicken can become an excellent model for muscle development and regeneration.

2.3.6. Comparison of muscularity of white leghorn layers with broilers

The effect of genetic selection on muscle growth and development has been
investigated in the chicken (Scheuermann et al., 2004; Aberle and Stewart, 1983). In chickens, broilers are genetically selected for meat production; whereas, leghorn layers are selected for egg production. Comparison of chicken genotypes demonstrated that the general parameters (body weight, breast yield, and pectoralis characteristics) at 7, 21, and 35 days of broiler lines were greater than those of layers (Scheuermann et al., 2004). Many investigators have reported that broilers have larger diameters of myofibers (Smith, 1963), a more rapid rate of myofiber radial hypertrophy (Aberle and Stewart, 1983), greater numbers of myofibers (Scheuermann et al., 2004), a faster proliferation rate of muscle cells (Moss, 1968; Blunn and Gregory, 1935), and hence, more muscle mass (Mizuno and Hikami, 1971) than layers. Therefore, broilers seem to have hypertrophy and hyperplasia of muscle cells. These differences lead to studying the reasons why they have different muscularity.

2.3.7. The low score normal (LSN) chickens

The LSN chickens have been actively studied in Dr. Velleman’s laboratory. They were detected in 1977 for the first time at the University of Connecticut. They were generated by an outcross of chickens with hereditary muscular dystrophy to White Leghorn chickens (Velleman et al., 2001). Genetically, they have inherited muscle weakness, showing phenotypes such as a decreased muscle function and a decreased ability of the birds to right themselves when placed in a supine position (Velleman et al., 1993). In addition, the LSN chickens have a 68% decrease in pectoralis muscle mass and a 60% reduction in body weight when compared to normal ones (Velleman et al., 1996). In Dr. Velleman’s lab, these distinct muscularity and muscle phenotypes between normal
and LSN chickens associated with, decorin, a transforming growth factor-β1, and extracellular matrix (ECM) have been studied during pectoralis major muscle growth and development (Velleman et al., 1998; Velleman et al. 2001; Li et al., 2009). How muscle regeneration in LSN compared to normal birds and its association with temporal gene expressions of myogenic regulatory factors and chicken DLK1 has not yet been studied.

2.3.8. The muscular dystrophic (MD) chickens

Muscular dystrophies in human and animals have been defined as inherited myogenic disorders that are characterized by progressive muscle wasting and weakness, leading to defects in muscle proteins and the death of muscle cells and tissues (Emery, 2002; Grounds et al., 2008). In the avian species, muscular dystrophic (MD) chickens with abnormal muscle (AM) have been found (Asmundson and Julian, 1956; Wagner and Peterson, 1970). The defect of the Am gene, a simple autosomal recessive gene, and its modification of expression seemed to destroy fast twitch alpha-white muscle fibers (Asmundson and Julian, 1956; Somes, 1990). The MD chickens with mutation of the Am gene are not lethal and have a normal lifespan. Recent genetic studies have verified the genetic characteristics of the AM locus using MD chickens (Lee et al., 2002; Yoshizawa et al., 2004). In the culture of Am/Am myoblasts, the cells were disorganized by 72 h postculture; in the histological analysis, lower proportion of glycolytic fibers (type IIb) and higher proportion of intermedeicate fibers (type IIa) were observed (Velleman et al., 1993). Unlike the human and mouse with muscular dystrophies, many things regarding the effect of an Am gene on the muscle characterizations of MD chickens is still unknown during muscle development, growth, and regeneration. Therefore, determining
differences in the pattern and level of myogenic regulatory factor and gDLK1 gene expressions during regenerative myogenesis may provide an insight for understanding muscular dystrophic phenotypes of chicken and shed light on the muscle disorders of humans.
2.4. Cell signaling pathways.

Signaling transduction is an essential process for responding to extracellular stimulus such as hormones, growth factors, and signal molecules to regulate cell proliferation, differentiation, apoptosis, and necrosis, resulting in regulating specific genes by suppression or stimulation (Lodish et al., 2003). To date, many types of signaling pathways have been identified to regulate gene expression, cell metabolism, and cell-cell interactions (Rosen et al., 1987; Lalli and Sassone-Corsi, 1994; Guo et al., 1995).

Figure 2.13. Overview of signaling pathways to regulate genes, resulting in cell proliferation or apoptosis (Lodish et al., 2003).
In general, a ligand molecule (a first messenger) recognizes its receptor on a cell surface. The surface receptor accepts the signal from the ligand and subsequently transfers the signal to associated proteins (a second messenger). Activation of the second messenger stimulates a target protein by phosphorylation or dephosphorylation. The target protein transmits the signal to a nucleus protein that can regulate a signal response gene. A target gene is expressed or suppressed by responding to the signaling originally from the extracellular stimuli (Figure 2.13).

Among many cell signaling pathways shown in Figure 2.13, the mitogen-activated protein kinase (MAPK) signaling proteins are composed of four distinct signaling pathways in skeletal muscle: 1) p38 MAPK; 2) c-Jun NH2-terminal kinases (JNK); 3) extracellular signal-regulated kinase (ERK) 1 and 2 (ERK1/2); and 4) ERK5 or big MAPK (Kramer and Goodyear, 2007). The MAPK family proteins are known to be evolutionary conserved in eukaryotes, suggesting the importance of this signaling pathway for the function of cell survival, adaptation, proliferation, and cell death through the control of gene expression (Chang and Karin, 2001).

Herein, MAPK/ERK signaling pathway associated with myoblast differentiation will be discussed among four distinct MAPK signaling pathways. In addition, it will be discussed how EGF domains of DLK1 are involved in the MAPK/ERK signal transduction during myogenesis.

2.4.1. MAPK/ERK signaling pathway in myogenesis

In general, MAPK/ERK signal pathway includes three critical steps: 1) ligand-receptor interaction to activate G proteins, 2) kinase cascade, and 3) regulation of
transcription and translation. Figure 2.14 shows and explains a summary of this MAPK/ERK signaling pathway. This MAPK/ERK signaling pathway has been known to be involved in cell proliferation and differentiation of various cell types (Chang and Karin, 2001; Pearson et al., 2001; Schramek, 2002). However, the role in cell proliferation or differentiation associated with MAPK/ERK signaling pathway still remains unclear.

Figure 2.14. The outline of MAPK/ERK signaling pathway (John Schmidt made this diagram; en.wikipedia.org/wiki/File:MAPKpathway). Binding of an epidermal growth factor (EGF), an extracellular ligand, to the EGF receptor (EGFR) activates the tyrosine kinase by phosphorylation. A docking protein, GRB2 containing SH2 domains, binds to an activated site of EGFR. In addition, GRB2 binds to SOS protein, a guanine nucleotide exchange factor, and activates it. Activation of SOS phosphorylates GDP-RAS into GTP-RAS. This phosphorylation of RAS activates RAF kinase. Subsequently, kinase cascade occurs to phosphorylate and activate RAF-MEK-MAPK kinases, involved in this signaling pathway to regulate the transcription of a target gene, such as c-myc or c-fos. The transcription of c-myc or c-fos regulates cell proliferation. RAF, MAPK kinase kinase (MAP3K); MEK, MAPK/ERK kinase 1 and MEK2 (MKK1 and MKK2); MAPK1, MAPK kinase ERK1 and ERK2
The MAPK/ERK signaling pathway associated with growth factors has also been studied on the differentiation of skeletal muscle cells (Olson et al., 1987; Bennett and Tonks, 1997; Tortorella et al., 2001), demonstrating that serum mitogens or growth factors positively act on this MAPK/ERK signal. The activation of the MAPK/ERK signaling pathway plays an important role in promotion of myoblast proliferation and inhibition of myoblast differentiation (Tortorella et al., 2001). Supportively, this negative function in myogenesis by MAPK/ERK signaling pathway was due to blocking the expression or function of myocyte differentiation factor (MyoD) and myocyte enhancer factor (MEF2) families (Winter and Arnold, 2000; Perry et al., 2001), which are essential for muscle cell differentiation. The retention of MEF2 and the suppression of MyoD were caused from tumorigenic expression of RAF, an upstream molecule of MAPK/ERK signaling pathway in 10T1/2 cells (Winter and Arnold, 2000; Perry et al., 2001), suggesting an inhibitory role of RAF in myogenesis. Interestingly, the suppression and retention of myogenic genes was accomplished by forced RAF signals, regardless of activation of RAF downstream signaling MEK1/2 (Dorman and Johnson, 1999 and 2000; Johnson et al., 2006), suggesting a sole inhibitory role of RAF in myogenesis.

However, treatment of a MAPK (MEK1) inhibitor (PD98059) enhanced the differentiation of avian myoblasts infected by retrovirus mediated expression of RAF (Dorman and Johnson, 1999), and myoblast fusion and terminal differentiation of C2C12 cells treated with basic fibroblast growth factor (bFGF), an activator of the MAPK/ERK signaling pathway (Tortorella et al., 2001). In addition, both insulin-like growth factor 1 (IGF1) and fibroblast growth factor 2 (FGF2) inhibited myogenesis through stimulation of the MEK signaling pathway; whereas, PD98059 restored the differentiation ability of
the 23A2 myoblasts in the presence of the two growth factors (Weyman and Wolfman, 1998). It was reported that sustained expression of MEK1 inhibited myogenesis (Perry et al., 2001). These suggest that the MEK signaling pathway mediated by IGF-1, FGF2, and bFGF may also play an inhibitory role in myogenesis.

Previous studies have shown that ERK1/2 activation, a downstream target of MEK1/2, negatively acts on myogenesis of skeletal muscles (Ramocki et al., 1997; Dorman and Johnson, 1999; Winter and Arnold, 2000; DeCahant et al., 2002; Dee et al., 2002). Nevertheless, an ERK1/2 knockdown study demonstrated that ERK2 phosphorylation and activation are essential for terminal differentiation in the mouse C2C12 cell line (Li and Johnson, 2006), supporting the observation that considerable activation of ERK2 is positively associated with myogenesis (Wang et al., 2004). This suggests that ERK2 activation is required for forming multinucleated myotubes during myogenesis, indicating a dual role of ERK2 for myogenesis.

Collectively, inducing RAF/MAPK/ERK signaling pathway by mitogens, growth factors, or genetic modification of the signals inhibits skeletal muscle cell differentiation, but this kinase cascade signaling involving myogenic differentiation still remains to be researched.

### 2.4.2. A role of DLK1 in MAPK/ERK cell signaling.

Because growth factors have been known as an essential extracellular signaling molecule that controls cell proliferation and differentiation via MAPK/ERK cell signaling (Florini et al., 1996; Lawlor and Rotwein, 2000; Miller et al., 2000; Ambrosio et al., 2009), understanding whether DLK1 containing EGF domains can control cell
proliferation and differentiation is of interest. Recent previous studies have suggested that DLK1 or a large EGF domain of DLK1 activates a MAPK/ERK signaling pathway during adipogenesis; consequently, inhibiting adipogenesis (Ruiz-Hidalgo et al., 2002; Kim et al., 2007; Nueda et al., 2007 and 2008; Sul, 2009).

Kim et al. (2007) showed that recombinant DLK1 containing 6-EGF domain fused with human Fc protein (hFc) activated the phosphorylation of MEK1 and ERK1/2 [Thr(202)/Tyr(204)], but not p38 MAPK or JNK in a time- and concentration-dependent manner in DLK1 null mouse embryo fibroblasts (MEF). In addition, in DLK1 MEF treatment of recombinant EGF-hFc of DLK1 inhibited adipogenesis. Moreover, PD98059, an inhibitor of MEK1, restored inhibition of adipogenesis by the recombinant 6-EGF-hFc protein. This suggests that 6-EGF can activate the MAPK/ERK signaling pathway, inhibiting adipogenesis (Kim et al., 2007; Sul, 2009).

On the other hand, Laborda’s group (Ruiz-Hidalgo et al., 2002; Nueda et al., 2007 and 2008) agreed with the inhibitory role of DLK1 protein in adipogenesis, but did not agree with the direct effect of EGF protein of DLK1 on MAPK/ERK signaling activation during adipogenesis. They suggested that 1) MAPK/ERK signaling activation is dependent on insulin-like growth factor 1 (IGF-1) or insulin; and 2) DLK1 expression controlled MAPK/ERK signaling pathway through insulin-like growth factor 1 (IGF-1) receptor, which correlates with differentiation.

In addition, they assumed that Notch-1, the structural homology of DLK1 with ligands for the Notch family of receptors (Laborda et al., 1993), is a good candidate for DLK1 receptor when considering its inhibitory role in adipogenesis (Garcés et al., 1997). However, due to the reason that DLK1 do not contain DSL (Delta Serrate Lin 12) domain
that is required for interaction with Notch, DLK1 has been predicted as not being a Notch ligand (Sul, 2009). Collectively, these controversial results of the DLK1 interaction with Notch-1 need to be further investigated. The effect of DLK1 action on the MAPK/ERK signaling pathway to regulate cell proliferation or differentiation is still unclear.

2. 5. Summary of Literature Review

Delta-like protein 1 (DLK1) has various transcripts by a mechanism of alternative splicing. Tissue-specific isoforms of DLK1 have been identified in mammalian species. Some major isoforms are developmentally down-regulated for animal tissue development. Regardless of various alternative splicing transcripts of DLK1, it is apparent that DLK1 is required for mammalian tissue development. The spliced isoforms of DLK1 are closely associated with cleaved protein products of DLK1 that can generate 3 or 6 epidermal growth factor (EGF)-like motifs. They are located in an extracellular domain of DLK1 protein which is cleaved by a disintegrin and metalloproteinase 17 (ADAM17/ TACE). Like the full length protein of DLK1, these motifs have been studied for regulating cell proliferation or differentiation in various cells, such as adipocytes, hematopoietic cells, chondrocytes, and adrenal gland and neuroendocrine cells. Genetic modification studies in transgenic or knockout mouse and murine adipocytes reported that overexpression of DLK1 inhibits adipocyte differentiation or adipose tissue development; whereas, absent expression of DLK1 promotes in vitro and in vivo adipose development. However, the tissue specific role of DLK1 has still remained to be identified in many other cells and tissues of animals.

DLK1 is also known as an imprinted gene that is paternally expressed on a
chromosome of animals. The animal genomic imprinting correlates with mammalian muscle genetic diseases. These are highly associated with an imprinted gene DLK1. In general, two general mechanisms of DLK1 regulation are explained for muscle genetic diseases by epigenetic modification through methylation in DLK1 locus and feedback function of a gene cluster from the maternal allele of the chromosomes. It has also been implicated in increasing muscle mass containing myofiber hypertrophy with centered nuclei in callipyge sheep, DLK1-transgenic mouse, and paternal uniparental disomy (pUPD) of human and mouse. This suggests that DLK1 may be involved in myogenic activity of muscle development in animals. In addition, the localization of DLK1 protein in membranes of embryonic or fetus myofibers in mammalian species further convince that it may have myogenic activity in a specific stage of myogenesis including myofiber formation or maturation.

Myogenic regulatory factors (MRFs) and paired box protein (Pax) 3 or 7 are important myogenic transcription factors that play a critical role in embryonic or regenerative myogenesis during muscle development. MRFs include myogenic differentiation 1 (MyoD), myogenic determination factor 5 (Myf5), myogenin (Myog), and myogenic regulatory factor 4 (MRF4/ Myf6). These sequential expressions of MRFs are associated with early or late stages of myogenesis where myoblasts are migrated, lined, and fused together, and hence formed into multinucleated myofibers. MyoD and Myf5 are induced at early stages of myogenesis, suggesting early myogenic markers. Myogenin and MRF4 are induced at late stages of myogenesis, suggesting late myogenic markers. Pax7 expression is indicative as determining activation of satellite cells, or lineage marker. Association of MRF gene expressions with DLK1 gene expression
during muscle development and regeneration allows determining a specific stage of
myogenesis.

Muscle regeneration is an excellent method to recapitulate embryonic myogenesis. During cardiotoxin, a muscle injury molecule-induced muscle regeneration in mammals, MRFs and Pax7 are re-expressed and involved in muscle regenerative myogenesis. Unlike embryonic myogenesis where the expression of myogenic early and late markers is coordinated regardless of distinctive sequential expressions, regenerative myogenesis has an advantage to observe discrete stages of myogenesis. In addition, DLK1 has been shown to contribute to the regenerative capacity of ear, liver, and muscle tissues. Therefore, it is of interest whether DLK1, which highly induced at fetal stages of myogenesis in animals, can re-induce at a specific stage of regenerative myogenesis in their muscles.

Chicken may be an excellent model to study muscle development and regeneration because the period of chicken embryonic development is shorter than mammals; chicken has different genetic selection on muscle growth and development of layers and broilers; chicken also has muscle genetic disease model such as muscular dystrophy; the repair program of chicken muscle regeneration is also able to recapitulate embryonic myogenesis, and handling of egg embryos is easier and cheaper.

To date, there is no study of DLK1 on muscles of birds from embryonic to adult development and regeneration. There is no 1) nucleotide sequence information of DLK1 in birds; 2) no sequence comparison of DLK1 between bird and mammal; 3) no alternative splicing information of DLK1 in birds; 4) no information about the regulation of DLK1 gene during muscle development and regeneration and muscle cell
differentiation; 5) no distinctive comparison of DLK1 gene expression between white leghorn layers, selected for egg production, and broilers, selected for meat production; 6) no comparison of DLK1 gene expression during muscle regeneration between control and Low Score Normal (LSN) and Muscular dystrophy (MD) chickens that have muscle wasting and weakness. These investigations about chicken DLK1 can provide new insight of its expression role in muscles during embryonic and regenerative myogenesis in poultry.
Chapter 3: Cloning and Expression of Delta-like protein 1 mRNA during Development of Adipose and Muscle Tissues in Chickens.

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3.1 Abstract

Delta-like protein 1 (DLK1) is involved in adipose and muscle development as shown by the reduction of fat mass in DLK1 transgenic mice and in muscle hypertrophy of callipyge sheep. However, no study on DLK1 has been investigated in avian species. Cloning and sequencing of a full length of chicken DLK1 (gDLK1) cDNA revealed that gDLK1 contains a total of 1,161 base pairs, encoding 386 amino acids. The similarity of gDLK1 nucleotide and protein sequences was over 50% compared to other mammalian species. In addition, chickens only express one full length of gDLK1 in various tissues at different ages without the alternative splicing variants of DLK1 found in mammalian species. This suggests that the full length form of gDLK1 may be sufficient for normal development in the chicken. In adipose tissue, the gDLK1 gene was highly expressed in preadipocytes as compared to adipocytes (P < 0.05); whereas, expression levels of
adipogenic marker genes such as stearoyl-Coenzyme A desaturase 1 (SCD-1) and fatty acid binding protein 4 (FABP4) were higher in mature adipocytes than in preadipocytes ($P < 0.05$ and $P < 0.01$, respectively). Expression of gDLK1 in adipose tissue tends to decrease with age. The expression of gDLK1 gene in pectoralis major muscle was significantly higher in 13- and 17-d old embryos ($P < 0.05$), decreased in 1- and 5-d old chicks ($P < 0.05$), and further decreased in 11- and 33-day old chickens ($P < 0.05$). This expression pattern of gDLK1 was very similar to the expression patterns of myogenin and Pax7 genes, suggesting a close association with myogenic activities. In conclusion, the developmental regulation of gDLK1 expression might play an important role in the early stages of adipose and muscle tissue development.

### 3.2 Introduction

Delta-like protein 1 (DLK1) has been known as Pref-1, SCP-1, FA-1, Zog, and pG2. DLK1 is a transmembrane glycoprotein containing six epidermal growth factor (EGF) repeats homologous to the Notch/Delta/Serrate family. The DLK1 gene has been widely studied in embryo development (Georgiades et al., 2000), obesity (Moon et al., 2002; Lee et al., 2003), human genetic disease (Berends et al., 1999), skeletal stem cells (Abdallah et al., 2004), and ear wound repair (Samulewicz et al., 2002). The DLK1 is an imprinted gene that is expressed from the paternal allele in mammals (Lin et al., 2007; Sutton and Shaffer, 2000). Recent studies using DLK1 transgenic mice models have provided direct evidence that DLK1 is a promoting factor of muscle development (Davis et al., 2004) and an inhibitory factor of adipose development (Lee et al., 2003). These characteristics of DLK1 may be useful for producing maximal meat yield from animals.
by increasing muscle mass and decreasing fat content.

The callipyge phenotype in sheep shows an inherited muscular hypertrophy with larger longissimus muscle area and leg scores, resulting in a 42 to 50% increase in the muscles compared to those of normal sheep (Cockett et al., 1993; Jackson et al., 1997). The gene responsible for this increased muscle mass in sheep was recently identified as DLK1 with the phenotype inherited from the paternal allele (Takeda et al., 2006). Recent studies on transgenic mice overexpressing the DLK1 gene confirm the pro-myogenic function of DLK1 (Davis et al., 2004). Although the DLK1 gene expression is associated with muscle growth and development, the DLK1 gene has not been reported in avian species.

The DLK1 gene has been extensively studied in the areas of adipocyte development and obesity. For example, previous studies demonstrated that DLK1 gene expression is decreased during adipocyte differentiation in vivo and in vitro (Deiuliis et al., 2006; Smas and Sul, 1993). Our previous in vivo studies clearly indicate that overexpression of the DLK1 gene in adipose tissue of transgenic mice inhibited adipocyte development (Lee et al., 2003) and that the knockout of the DLK1 gene induced the obesity phenotype in mice (Moon et al., 2002). An increasing number of studies show that the hormonal and genetic modulation of adiposity successfully used DLK1 as a marker of preadipocyte and adiposity indicator. However, the DLK1 gene has never been studied in adipose development in poultry.

It has been reported that DLK1 has several isoforms generated by alternative splicing in the tissues of various species (Smas et al., 1994 and 1997; Vuocolo et al., 2003; Deiuliis et al., 2006). Therefore, the first aim of this study was to clone and sequence
chicken DLK1 gene (\textit{gDLK1}), compare its nucleotide and deduced protein sequences with mammalian species, and investigate the possible alternative splicing of \textit{gDLK1} transcript. A second objective was to investigate developmental regulation of DLK1 gene in chickens and assess how specific developmental patterns of \textit{gDLK1} expression correlate with the specific stages of chicken muscle and adipose development. Our current studies on chicken DLK1 were initiated to enhance our basic understanding of DLK1 gene in avian muscle and adipose development.

3.3 Materials and Methods

3.3.1 Experimental animals

Animal care and procedures were approved by the OSU Institutional Animal Care and Use Committee. A total of 40 chicken eggs (White leghorn) were incubated and sampled at various developmental times [embryonic day (E) 13, 17 and posthatch day (P) 1, 5, 11 and 33]. Hatched chicks were fed a standard diet \textit{ad libitum} throughout the growth period. At each developmental time, pectoralis major muscle and fat tissues were collected from four or five chickens per group and kept at -80\(^\circ\)C for total RNA isolation, regular or quantitative real-time PCR (qRT-PCR), and western blotting. To investigate alternative splicing variants of \textit{gDLK1}, chicken pectoralis muscle, heart, fat, liver, lung, and intestine tissues at E17 were snap frozen in liquid nitrogen and kept at -80\(^\circ\)C for total RNA isolation, reverse transcriptase (RT)-PCR, and qRT-PCR.

3.3.2 Cloning of chicken DLK1

Chicken DLK1 was cloned using a set of forward and reverse primers designed
according to two predicted DLK1 sequences: The Institute for Genome Research (TIGR) database sequence (TC214622) and NCBI database sequence (XM_421369.1). Briefly, chicken DLK1 gene was amplified by using two primer sets: gDLK1-F1 (5’-CCA GAG GCC CCA ACA TGA G-3’) and gDLK1-R1 (5’-ACC TGC ACC AAT ATC TGT GCA CG-3’) as shown in Figure 3.1. Chicken cDNA from embryonic muscle tissue total RNA was used as a template for PCR. The putative gDLK1 cDNA was ligated to the PCR 2.1 vector after gel extraction using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) according to the instruction of manufacturer. The TOP 10 chemical competent cells (Invitrogen) were transformed with the PCR 2.1 plasmid including the putative gDLK1 insert. Transformants were grown on a kanamycin agar plate containing X-gal. Plasmids isolated from positive colonies were sequenced by The Ohio State University Sequencing Core Facility using an Applied Biosystems 3730 DNA Analyzer.

3.3.3 Chicken Stromal-Vascular and Adipocyte cell fractionation

Chicken stromal-vascular and adipocyte cell fractionation was performed by following the cell fractionation procedure of Deiuliis et al. (2006, 2008). Briefly, adipose tissue (3 to 5 g) was isolated from 20-day-old chickens (n = 4), minced, and incubated with 3.2 mg/ml collagenase II (Sigma-Aldrich, St. Louis, MO) for 1 hour in a shaking water bath (180 rpm, 37 ºC) to separate adipose tissue into stromal-vascular (SV) and adipocyte fractions. The suspension was passed through a 100-µm nylon cell strainer (BD falcon, Franklin Lakes, NJ) to remove undigested tissue. The filtrate was centrifuged at 200 × g for 5 min. The top layer (adipocyte fraction) and the pellet (SVF) were collected for total RNA isolation.
3.3.4 RNA Isolation, Regular PCR, and quantitative Real-Time (qRT) PCR

Chicken pectoralis muscle, heart, fat, liver, lung, and intestine tissues were snap frozen in liquid nitrogen and homogenized using a TissueMiser homogenizer (Fisher Scientific, Pittsburgh, PA). Total RNA from the tissue was isolated using Trizol (Invitrogen) following the manufacturer’s instructions, and RNA quality was assessed by electrophoresis. Reverse transcription was performed using 1 μg of total RNA and M-MLV reverse transcriptase (Moloney murine leukemia virus RT, Invitrogen). Reverse transcription conditions for each cDNA amplification were 65 ºC for 5 min, 37 ºC for 52 min, and 70 ºC for 15 min. To examine whether chickens have alternative splicing isoforms of gDLK1, the regular PCR was performed with gDLK1-F1 and gDLK1-R1 primer set to amplify the full length of gDLK1 for various tissues and ages. Regular reverse transcription-PCR conditions were 95 ºC for 2 min and 37 cycles of 94 ºC for 15 sec, 60 ºC for 1 min, and 72 ºC for 1 min. The PCR products were separated in 1% agarose gel. In addition, quantitative Real Time-PCR (qRT-PCR) using gDLK-F2 and gDLK1-R3 primer set was performed to measure the relative levels of gDLK1 expression in muscle and adipose tissues. The glyeraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as a housekeeping gene. The gene expression was quantified by SYBR green real-time PCR as described previously (Deiuliis et al., 2008, 2006). The PCR was performed using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), and SYBR green was used as the detection dye. Primer sequences used for the qRT-PCR are shown in Table 3.1. Primers (gDLK1-F2 and gDLK1/R3; Figure 1A) to quantify total gDLK1 gene expression were designed to align exon 4 and 5 of chicken DLK1 gene,
respectively. Therefore, the primers that span genomic intron (2.1 kb) between exon 4 and 5 could avoid amplification of contaminated genomic DNA during the PCR reactions. Conditions for the qRT-PCR were 95 ºC for 10 min and 40 cycles of 94 ºC for 15 sec, 60 ºC for 40 sec, 72 ºC for 30 sec, and 82 ºC for 32 sec. Real-time PCR was performed in duplicate in 25 µl reactions on an ABI 7300 Real-Time PCR Instrument (Applied Biosystems). The relative level of target gene expression, as determined by ABI software, was calculated using the comparative 2−ΔΔCt method for relative quantification (Livak and Schmittgen, 2001).

3.3.5 Bioinformatics, sequence analysis, and statistical analysis

Applied Biosystems Sequence Scanner v1.0 was used to analyze the chromatogram. Homology analysis was performed using BLAST at NCBI as previously described (Deiuliis et al., 2006). Sequence alignment and comparison in addition to protein translation were done using the ClustalX and GeneDoc™ software (Deiuliis et al., 2008, 2006). Results are presented as mean ± SEM. Comparison of two means was accomplished by a Student’s t-test at P < 0.05 and 0.01. Comparisons among gene expression data were performed using one way analysis of variance (ANOVA) followed by the Tukey’s test at P < 0.05. Statistical analysis was performed using Minitab software (version 15.0).

3.4 Results

3.4.1 Chicken DLK1 (gDLK1) gene cloning and sequence comparisons

The gDLK1 cDNA was cloned and sequenced (Genbank #: EU_288039), which is
1,161 bp and encodes 386 amino acids (Figure 3.1, 3.2, and 3.3). The multiple nucleotide sequence comparison between species (using ClustalX and GeneDoc software programs) showed that gDLK1 has 55, 55, 56, 56, and 55% similarity to that of the mouse, rat, human, pig, and cattle, respectively (Figure 2). In addition, the alternative splicing sites such as acceptors or donors of B, C, C2, D, D2 forms of gDLK1 gene at the fifth exon were not conserved across mammals (Figure 2). Putative protein sequence comparison also showed that the amino acid sequence of gDLK1 has 48% (65%; similar amino acid group), 49% (65%), 51% (65%), 51% (65%), and 50% (65%) similarity to that of the mouse, rat, human, pig, and cattle, respectively (Figure 3.3). Interestingly, the gDLK1 protein sequence contains an 7-cysteine repeat (C11 to C17 from N-terminus), which is not contained in mammalian species (Figure 3.3). The first two N-linked glycosylation sites (black arrow) across species also were present in the chicken; however, the third located in the juxtamembrane region of the protein was substituted with glutamine (Figure 3.3). Furthermore, alternative splicing donor or acceptor sites (black diamond arrow) for DLK1-B and C2 forms among others were not conserved in those of gDLK1 amino acid sequence (Figure 3.3).

3.4.2 Identification of alternative splicing in tissue distribution and in vivo fat and muscle developmental expression of gDLK1

Reverse transcription-PCR was performed for tissue distribution of a full length of gDLK1 mRNA using total RNA isolated from the pectoralis major muscle, heart, fat, liver, lung, and intestine collected at E 17. As shown in Figure 3.4A, only a single PCR product was detected in these tissues of egg-type chickens. Next, alternative splicing
transcripts of gDLK1 were investigated in fat and muscle tissue from E13 to P33. There were no alternative splicing variants of the gDLK1 gene during chicken adipose and muscle development (Figure 3.4B and 3.4C). Therefore, it was confirmed that chickens express only the A form of gDLK1 mRNA in multiple tissues and ages in adipose and muscle tissues.

### 3.4.3 The relative gDLK1 gene expression in the stromal-vascular and adipocyte fractions, and during chicken adipose development

It is of interest to examine the gene expression of gDLK1 with other adipogenic marker genes such as stearoyl-Coenzyme A desaturase 1 (SCD-1) and fatty acid binding protein 4 (FABP4) in two types of fractionated cells, SV and adipose cells. The SV cells contain mostly preadipocytes, whereas adipose cells contain mostly mature adipocytes. The levels of FABP4 and SCD-1 mRNA expression were significantly higher in mature adipocytes than those levels in preadipocytes ($P < 0.01$ and $P < 0.05$, respectively) as shown in Figure 3.5A. However, the level of gDLK1 mRNA expression was significantly higher in preadipocytes than in mature adipocytes ($P < 0.05$). The in vivo temporal expression of gDLK1 during adipose tissue development was further investigated. The gDLK1 mRNA expression tended to be low at E17 and tended to increase at P1 as shown in Figure 3.5B. Thereafter, it seemed gradually down-regulated until P33 during adipose development, although there was no statistical significance in differences ($P = 0.18$).
3.4.4 The relative temporal expression of gDLK1 gene during chicken muscle development

The gene expression of gDLK1 with other myogenic marker genes such as Pax7 and myogenin during chicken muscle development were shown in Figure 3.6. The mRNA expression of gDLK1 was gradually down-regulated during muscle development. First, it was significantly expressed at E13 and E17 ($P < 0.05$) and thereafter, four-fold decreased at P1 and P5 ($P < 0.05$) until P11 and P33 ($P < 0.05$). The gene expression of Pax7 was significantly higher at E13 and E17 than at other time points, 34-fold decreased after post hatch until P33 ($P < 0.05$). Myogenin mRNA was highly expressed at E13 and significantly decreased until P1 ($P < 0.05$), and slightly increased at P5 and down-regulated up to P33 ($P < 0.05$).

3.5 Discussion

Improving muscle growth and reducing amount of fat in avian species is essential in maximizing poultry production. Understanding the role of factors and their interplay in muscle and adipose development will lead to strategies to ultimately improve muscle growth and decrease fat deposits. The pro-myogenic and anti-adipogenic functions of DLK1 have been known in mammalian species (Davis et al., 2004; Lee et al., 2003). Here, we report for the first time cloning, sequencing, alternative splicing, and temporal expression of gDLK1 during muscle and adipose development in the chicken.

The DLK1 gene has several spliced transcripts generated by alternative splicing in the fifth exon across various species. A total of six different isoforms of DLK1 such as DLK1-A, -B, -C, -C2, -D, and -D2 have been identified in mice (Smas et al., 1997, 1994).
Pigs, cattle, and sheep have mainly the DLK1-C2 isoform. Humans express only the A form of DLK1. Moreover, the role of DLK1 isoforms has been studied in cell culture as well as in animals (Smas et al., 1997, 1994; Davis et al., 2004). Overexpression of A and B forms of DLK1 inhibits adipocyte differentiation (Lee et al., 2003; Mei et al., 2002). Callipyge sheep primarily express the C2 form with minor expression of the A form (Davis et al., 2004). Overexpression of the C2 form of mouse DLK1 in the muscle of transgenic mice appears to recapitulate the callipyge phenotype. Our previous studies with pig DLK1 showed that the C2 form was the most abundant isoform, whereas the B form was present to a lesser extent (Deiuliis et al., 2006). Similar to humans, chickens express only the A form, which is a full length form of chicken DLK1. In addition, no detectable amplification of other splicing transcripts was shown in various tissues including adipose and muscle tissues at different ages of chickens (Figure 3.4). An absence of alternative splicing of gDLK1 transcripts can be possibly explained by a comparative point of view in their sequences across the species. Our comparative analysis of the multiple nucleotide sequences of DLK1 genes (Figure 2) revealed that the alternative splicing donor or acceptor sites of B, C, C2, D and D2 of DLK1 shown in mammalian species were not conserved at the fifth axon of chicken DLK1 nucleotide sequences. In addition, the obligatory adenosine residue [CT(A/G)A(C/T)] of a lariat structure or a branch point conserved in mammalian species (Figure 2), which is required for steps of alternative splicing, was not found in chickens. Therefore, a lack of conserved sequences for the formation of a branch point as well as splicing donor and acceptor sites may result in chickens expressing only the A form of DLK1. From the evolutionary point of view, it appears that chickens have evolved to have only the A form
of DLK1, which is sufficient for normal tissue and organ development.

Glycosylation affects the structure and function of many proteins in eukaryotes. The glycosylation of DLK1 protein has been reported in mammalian species. As shown in Figure 3.3, the full length of DLK1 proteins of mice, rats, humans, and cattle have three conserved N-linked glycosylation sites at the 105th, 139th and 301st amino acid residues. However, like the pig, chicken DLK1 protein does not have the third N-linked glycosylation site of DLK1 amino acid sequences. The splicing of DLK1 mRNA into the C2 form generates a major isoform in pigs, cattle, and sheep, resulting in deletion of amino acids from 237 to 312, including the third glycosylation site at 301. The deleted amino acid sequences of DLK1 are most variable across species, conceivably being evolved under less evolutionary pressure to keep a glycosylation site in this residue of gDLK1 protein. However, future studies on the biological function of DLK1 glycosylation in various cells and tissues of many species need to be performed.

Adipose tissue contains many types of cells including mainly adipogenic cells at various stages of adipocyte development. Fractionation of individual cells based on the densities of cells containing various amounts of lipids could separate two major populations of cells; the SV fraction contains mostly preadipocytes, and the adipocyte fraction contains adipocytes filled with lipids. Comparing the expression of target genes in two different fractions of cells enables us to determine which fraction of cells dominantly expresses the target gene and to understand developmental regulation of gene expression in vivo. Two well-known adipogenic marker genes, SCD-1 and FABP4, were predominantly expressed in the adipocyte fraction (Cohen et al., 2002; Lee et al., 2003), indicating successful fractionation of cells in chicken adipose tissue. In addition, our data
showed that DLK1 was highly expressed in the SV fraction of cells in chicken adipose tissue. In general, DLK1 expression was found in many cell types at undifferentiated stages (Carlsson et al., 1997; Costaglioli et al., 2001; Tanimizu et al., 2004; Abdallah et al., 2007), although it is absent at the terminal differentiation stage. Like in mouse and human, DLK1 can be used as a marker gene for preadipocytes in chickens.

The function of DLK1 in cellular development has been mostly studied in adipocyte development. Our previous studies clearly indicate that overexpression of the DLK1 gene in adipose tissue of transgenic mice inhibited adipocyte development, and knockout of the DLK1 gene induced the obesity phenotype in mice (Lee et al., 2003; Moon et al., 2002). In chickens, it has been reported that the rapid growth of adipose tissue and its enlargement after the hatching period depends on adipocyte differentiation and fat storing (Leclercq, 1984). Considering DLK1’s anti-adipogenic activities, the down-regulation of gDLK1 gene during adipose tissue development indicates that DLK1 is negatively correlated with adipose tissue development in the chicken.

The importance of DLK1 for the fetal and neonatal muscle development has been demonstrated by the high levels of the DLK1 expression during fetal development and myogenesis in human and mouse genetic diseases, affecting muscle development (Berends et al., 1999; Georgiades et al., 2000; Schmidt et al., 2000; Yevtodiyenko and Schmidt, 2006). Like in normal sheep (Fleming-Waddell et al., 2007; White et al., 2008), our current study showed that gDLK1 gene expression in the muscle was the highest in the fetal stages, whereas its expression was decreased during the posthatch period. Therefore, these suggest that high levels of DLK1 expression are generally associated with active myogenesis that occurs during the fetal muscle
The processes of embryonic or fetal myogenesis have been characterized with expression of myogenic marker genes such as myogenin and Pax7 (Hasty et al., 1993; Zammit et al., 2006). Myogenin is one of the transcription regulatory factors that play an important role in myoblast differentiation and myofiber formation (Nabeshima et al., 1993; Yablonka-Reuveni and Paterson, 2001). Along with Pax7 and myogenin, gDLK1 gene expression level was investigated during chicken muscle development. The high expression of myogenin gene in the embryonic muscle may indicate the active processes of chicken myoblast differentiation and myotube formation. During the posthatch period, there was a sharp decline in the expression of the myogenin gene in 1-day-old chicks followed by a rebound of myogenin gene expression in 5-day-old chicks in our study. A similar pattern of myogenin protein levels was reported by Halevy et al. (2004) who showed low levels of myogenin protein at hatch and the increased expression of myogenin in 3-day-old chicks. The expression pattern of myogenin suggests that there is a transient suppression of myogenin expression at hatching, due to the possible associated stress of posthatch starvation. It has been reported that posthatch starvation resulted in decreases in myofiber size in avian species (Halevy et al., 2000; Moore et al., 2005).

Pax7 has been known as a marker gene required for the specification of satellite cells that are important for postnatal muscle growth and muscle regeneration (Seale et al., 2000; Halevy et al., 2004). The high expression of Pax7 gene may be indicative as a proliferating marker of satellite cells at fetal stages (Halevy et al., 2004). The levels of Pax7 gene expression during embryonic or fetal skeletal muscle development were seen in normal and callipyge sheep (White et al., 2008), chickens (Otto et al., 2006), and mice
(Relaix et al., 2005). Our current study also shows that the Pax7 gene was highly expressed in fetal muscle and dramatically down-regulated after hatch. This indicates that satellite cells may be actively proliferating during embryonic stages and inactivated after the hatching period. Alternatively, the down-regulation of Pax7 gene expression may represent the loss of mononucleated cells as they fuse into myofibers. Similar to the expression patterns of myogenin and Pax7 during muscle development, the expression levels of gDLK1 gene were very high in embryonic stages, declined during the early posthatch period, and further decreased with increasing age. Taken together, the abundance of gDLK1 mRNA is correlated with the expression levels of myogenin and Pax7 genes and closely associated with myogenic activities.

Here, we report for the first time that chickens express only the A form of gDLK1 (gDLK1-A), which is 1,161 bp, encoding 386 amino acids. The expression of gDLK1-A in various tissues at different ages of chickens suggests that the gDLK1-A is sufficient for normal development of the chicken. The predominant expression of gDLK1 in preadipocytes indicates that gDLK1 gene can function as an early marker gene of chicken adipose development. The down-regulation of gDLK1 during adipose development may reflect a negative association with fat cell development and fat deposition. In addition, high levels of gDLK1 gene expression correlate with the high levels of myogenin and Pax7 gene expression in embryonic muscle. However, the precise mechanism how DLK1 affects specific stages of muscle development remains to be investigated. In addition, it will be interesting to discover if the differences in expression pattern and level of DLK1 gene are associated with the rates of muscle growth in avian species.
3.6 Acknowledgements

This work was supported by the JAAP Poultry Endowment Fund (K. Lee and J. D. Latshaw) and partially supported by the grant (J. Shin, No.C00137) from the Korean Science and Engineering Foundation. We are grateful to Dr. Sandra G. Velleman (the Department of Animal Sciences, The Ohio State University) for the critical review of this manuscript.
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¹gDLK1 = chicken delta-like protein 1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; FABP4 = fatty acid binding protein 4; SCD-1 = stearoyl-Coenzyme A desaturase 1; Pax7 = Paired box gene 7; F = forward; R = reverse.
Figure 3.1. The putative nucleotide sequence of chicken delta-like protein 1 (gDLK1) complementary DNA cloned from embryonic muscle tissue at d 14 with a length of 1,161 bp. The boldfaced nucleotides indicate the start codon and the stop codon of gDLK1. The arrows indicate the primer sets for the cloning and quantitative real-time PCR (qRT-PCR). The primers (gDLK1-F1/R1) were designed for cloning the full length of gDLK1, whereas the primers (gDLK1-F2/R3) were designed to perform qRT-PCR for the gDLK1 mRNA amplification.
Figure 3. 2. Alternative splicing variants of delta like protein (gDLK1)-A between the chicken and selected mammalian species. The nucleotide alignments of gDLK1 with mammals. The known 5’ (arrow) and 3’(diamond) splice sites of DLK1 B, D, C2, D, and D2 forms in the mouse (GenBank #. BC052159), rat (GenBank #. NM053744), human (GenBank #. BC007741), pig (GenBank #. NM001048187), and cow (GenBank #. AF181466) are illustrated. Black gradient shading indicates identical nucleotides, and gray shading indicates highly conserved nucleotide sequence stretches.
Figure 3.2
Figure 3. Putative amino acid sequence comparison of chicken delta-like protein 1 (gDLK1; A-form) among species. The arrows indicate the deduced N-linked glycosylation sites in the protein. Black shading indicates identical amino acids and gray shading indicates highly conserved amino acid sequence stretches. Chicken DLK1-A lacks the third N-linked glycosylation site at amino acid 301 which is highly conserved in the human, rat, mouse, and cow. Instead, the putative third N-linked glycosylation site is at amino acid 259, unique to the chicken.
Figure 3.3
Figure 3.4. The expression of a full length of chicken delta-like protein 1 (gDLK1) in chickens without alternative splicing variants. (A) Total RNA was isolated from muscle (M), heart (H), fat (F), liver (Li), lung (Lu), and intestine (Int) from E17 embryos. (B and C) Total RNA was isolated from adipose and muscle tissues at embryonic day (E) 17, and post-hatch day (P) 1, 5, 11 and 33. PCR for gDLK1 using gDLK1-F1 and gDLK1-R1 primer set is shown in 1% agarose gel. The first lane is a DNA standard ladder. An arrow indicates 1,161 base pairs of full-length of gDLK1.
Figure 3.5. The chicken delta-like protein 1 (gDLK1) gene expression in preadipocytes and adipocytes fractionated from chicken adipose tissues, and during adipose tissue development.

(A) Expression levels of gDLK1, SCD-1, and FABP4 genes in adipose tissue were measured by quantitative real-time PCR (n=4). The bar represents mean ± SEM. Significant difference is indicated by *, ** between preadipocytes and adipocytes at $P < 0.05$ and $P < 0.01$ by using the student t-test. SV = stromal-vascular fraction; FC = fat cell fraction. (B) Expression levels of gDLK1 gene in adipose tissue at indicated ages of chickens were measured by quantitative real-time PCR (n=4 to 5 at each time point). The bar represents mean ± SEM. NS: not significant ($P > 0.05$). GAPDH = glyceraldehyde-3-phosphate dehydrogenase; FABP4 = fatty acid binding protein 4; SCD-1 = stearoyl-Coenzyme A desaturase 1; E = embryonic day; P = posthatch day.
Figure 3.6. The temporal gene expression of chicken delta-like protein 1 (gDLK1) during muscle development.
Expression levels of gDLK1, Pax7, and myogenin gene in muscle tissue at indicated ages were measured by quantitative real-time PCR (n=4 at each time point). The bar represents mean ± SEM. The letters (a-c) represent a significant difference among groups at various time points by using one-way ANOVA at $P < 0.05$. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Pax7 = Paired box gene 7; E = embryonic day; P = posthatch day.
Chapter 4: The Ontogeny of Chicken Delta-Like Protein 1 mRNA Expression during Muscle Development and Regeneration: Comparison of Broiler and Leghorn Chickens.


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4.1 Abstract

Delta-like protein 1 (DLK1) has been implicated in the muscle hypertrophy observed in DLK1 transgenic mice, callipyge sheep, mouse paternal uniparental disomy (pUPD) 12 and human pUPD14 syndromes. The current study was aimed to determine gDLK1 mRNA expression during primary muscle cell differentiation and during muscle regeneration after cold injury and to compare gDLK1 mRNA expression during skeletal muscle development in layers and broilers. In chicken primary muscle cell culture, gDLK1 mRNA expression was significantly increased from 12 h to 48 h ($P \leq 0.05$) when the nascent myotubes were actively formed at days 2-3. Myogenin, a late myogenic marker gene, mRNA expression peaked at 36 to 48 h. MyoD and Pax7, early myogenic
marker genes, mRNA expressions gradually decreased during myogenic differentiation. During muscle regeneration, the expression of MyoD and Pax7 peaked at day 2 ($P \leq 0.05$), and myogenin mRNA expression peaked at day 4 ($P \leq 0.05$). The induction of gDLK1 gene appeared between days 7-10 post-injury ($P \leq 0.05$) when myotubes were actively formed as also demonstrated in histological sections. The expression of gDLK1 was slowly down-regulated to the control levels at day 14 when the damaged muscle appeared nearly healed. These data suggest that gDLK1 may be involved in the late myogenic stages of primary muscle cell differentiation and muscle regeneration. The gDLK1 mRNA in the muscle tissues was very abundant at embryonic ages but decreased after hatching in both broiler and layer chickens. Compared to layers, broiler muscle at embryonic day 13 had a 3-fold greater expression of DLK1 ($P \leq 0.01$). In addition, the gDLK1 mRNA expression at days 1, 11, and 33 post-hatch was significantly higher in broilers than layers ($P \leq 0.05$). Therefore, the relatively greater expression of the gDLK1 gene in muscles of broilers compared to layers suggests that gDLK1 may serve as a new selection marker for high muscle growth in chickens. These findings may provide new insight into chicken muscle development and regeneration.

4.2 Introduction

The stages of embryonic myogenesis are determination of myogenic precursor cells, proliferation and migration of myoblasts, fusion of myoblasts into myotubes, and terminal differentiation into functional myofibers. In addition, muscle regeneration from muscle stem cells or satellite cells after muscle injury or exercise partially recapitulates the process of embryonic myogenesis (Schultz and McCormick, 1994; Zhao and
Hoffman, 2004; Parise et al., 2006). These developmental processes, accompanied by morphological and biochemical changes of muscle cells, are governed by a complex orchestration of signals that are mediated by myogenic regulatory transcription factors (MRFs) (Schultz and McCormick, 1994; Buckingham, 2006), the extracellular matrix (ECM) (Velleman, 1999), and growth factors (Tatsumi et al., 1998; McFarland et al., 1993; McFarland, 1999). The MRFs include Myf5, MyoD, myogenin, and MRF4 (Schultz and McCormick, 1994; Buckingham, 2006). These genes have been used as myogenic markers for indicating different stages of myogenesis. Numerous studies have revealed the expression and involvement of new factors, their functions, and their signaling at specific stages of muscle development (Dogra et al., 2007; Ochi et al., 2008; Li and Johnson, 2006). One of these factors is delta-like protein 1 (DLK1).

DLK1 has been referred to as Pref-1, SCP-1, FA-1, Zog, and pG2. DLK1 is a glycosylated protein homologous to the Notch/Delta/Serrate family. DLK1 has been studied in various areas such as embryonic development (Georgiades et al., 2000), obesity (Moon et al., 2002; Lee et al., 2003), human genetic disease (Berends et al., 1999), skeletal stem cells (Abdallah et al., 2004), ear wound healing (Samulewicz et al., 2002), and muscle development (Davis et al., 2004). DLK1 is an imprinted gene paternally expressed in mammalian species and located in syntenic chromosomes 14, 12 and 18 in mice, humans, and sheep, respectively (Berends et al., 1999; Georgiades et al., 2000; Takeda et al., 2006). Dysregulation of DLK1 gene expression in human and mouse genetic diseases leads to developmental abnormalities including either muscle hypotonia in the case of the absence of DLK1 expression, or muscle hypertrophy in the case of abnormally high levels of DLK1 expression (Berends et al., 1999; Georgiades et al., 2000;
Davis et al., 2004). In addition, callipyge sheep expressing high levels of DLK1 gene expression in postnatal ages are characterized by muscle hypertrophy (Cockett et al., 1996, Takeda et al., 2006). Although the DLK1 gene is involved in muscle development, little is known about the developmental and physiological regulation of chicken DLK1 (gDLK1) during muscle cell differentiation, muscle development, and regeneration.

DLK1 mRNA is abundantly expressed in the fetal muscle of mammalian species as well as chickens (Floridon et al. 2000, White et al., 2008; Shin et al., 2008), suggesting that DLK1 may be actively involved in fetal muscle development. Previously, we cloned chicken gDLK1 cDNA, compared its nucleotide and protein sequences to those of mammals, and showed the developmental regulation of gDLK1 mRNA during chicken (White leghorn) muscle development (Shin et al., 2008). The current aims of this study were: 1) to examine whether the gDLK1 mRNA expression is induced at specific stages of myogenesis during chicken primary muscle cell differentiation, 2) to further examine gDLK1 mRNA expression during muscle regeneration, and 3) to investigate if the difference in muscularity between broiler- and leghorn-type chickens correlates with different levels of gDLK1 mRNA expression during development.

4.3 Materials and Methods

4.3.1 Experimental animals

Animal care procedures were approved by the OSU Agricultural Care and Use Committee. A total of forty layer (White leghorns) and forty broiler (Ross 708) eggs were incubated. Five muscle tissues of layers and five of broilers were collected at embryonic day (E) 13 and 17. Thereafter, five muscle samples of layers (weighing
average (~) 39g at post-hatch day (P) 1, ~54g at P5, ~99g at P11, and ~302g at P33) and five of broilers (weighing ~48g at P1, ~100g at P5, ~243g at P11, and ~1,508g at P33) were collected after chicks were euthanized with CO2. Chickens were fed a standard diet *ad libitum* throughout the growth period. At each ontogenic time-point, pectoralis major muscle tissues from leghorn layers and broilers were kept in -80ºC for isolating total RNAs and performing quantitative real-time PCR (**qRT-PCR**).

### 4.3.2 The injury of chicken pectoralis major muscle

A total of forty-two layer adult chickens weighing ~473g (White leghorns, 40-day-old) were used to study muscle regeneration because our previous study showed that very low expression of myogenic marker genes (myogenin, and Pax7) and gDLK1 was shown at day 33 post-hatching (Shin et al., 2008), indicating that activities of satellite cells will be low at day 40. Therefore, it was hypothesized that muscle damage induces gDLK1 mRNA during muscle regeneration. The chickens were anesthetized after inhalation of isoflurane, and their skins were incised for exposure of the left breast muscle surface. The tip of a metal rod with a flat 5 mm² surface area was dipped in liquid nitrogen for 1-2 min, and then held gently against the left breast muscle surface (2-3 cm) for 4-5 sec. The injured chickens were kept warm during recovery of anesthetization. Samples were collected at various days (D0, D2, D4, D7, D10, and D14) after cold injury for histological analysis and measurement of gene expression levels by qRT-PCR. The undamaged right side of the muscle was used as a control.
4.3.3 Chicken primary muscle cell culture and differentiation

White leghorn eggs were provided by The Ohio State University Poultry Center. Chicken primary muscle cells were isolated from pectoralis major muscles in 13-day old embryos. The cells were cultured for primary cell muscle differentiation as previously reported (ref). Briefly, the muscle tissues were finely minced and dissociated with 0.05% trypsin-EDTA for 20 min at 37 °C (Invitrogen, Carlsbad, CA). The cells were sedimented at 1,500 x g for 5 min at room temperature and subsequently seeded onto 0.01% calf skin collagen-coated plates in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin sulfate (100 μg/mL) (Invitrogen, Carlsbad, CA). After 2 days, differentiation was induced by incubation with differentiation media (DMEM with 2% horse serum) for 3 days in primary muscle cells. The cells were collected at 0, 12, 24, 36, 48, and 72 hours for total RNA isolation, qRT-PCR, and cell morphological images.

4.3.4 Immunostaining of myosin heavy chain (MyHC) and staining of nuclei with 4', 6-diamidino-2-phenylindole (DAPI)

Fluorescent immunostaining of MyHC and nucleus staining were performed in chicken primary muscle cells from day 0 to day 3 after differentiation as previously described (Li et al., 2008). Briefly, the cells were rinsed with phosphate buffered saline (PBS), fixed in 10% buffered formalin, and rinsed with PBS again. After the cell fixation, the primary antibody [pan sarcomeric MyHC (NA4), 1:500; The Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA] was incubated with PBS in the fixed muscle cells overnight at 4°C. After several rinses in PBS, the cells were incubated with
rhodamine conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch, West Grove, PA), for 1 hour at room temperature. In addition, the cells were nuclear-stained with 300 nM of DAPI (Invitrogen, Carlsbad, CA) in PBS. The cells were rinsed 5 times with PBS and subsequently imaged using an inverted fluorescent microscope at 100 X magnifications (Olympus IX50, Olympus, Melville, NY).

4.3.5 RNA Isolation and quantitative Real-Time PCR (qRT-PCR)

Chicken pectoralis muscle tissue at selected ages was snap frozen in liquid nitrogen and homogenized using a Tissuemiser homogenizer (Fisher Scientific, Pittsburgh, PA). Briefly, total RNA isolation and qRT-PCR were performed as previously described (Shin et al., 2008). Total RNA from the tissue was isolated using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions, and RNA quality was assessed by electrophoresis (1% agarose gel). Reverse transcription (RT) was performed using 1 μg of total RNA and moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription condition for cDNA amplification was 65 °C for 5 min, 37 °C for 52 min, and 70 °C for 15 min. The qRT-PCR using gDLK-F2 and gDLK1-R3 primer set was performed to measure the relative levels of gDLK1 expression in muscle tissues. The glyeraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as a housekeeping gene as previously described (Shin et al., 2008; Lowe and Always, 1999). The quantitative real time-PCR was performed using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), and SYBR green was used as the detection dye. Primer sequences used for the qRT-PCR are shown in Table 4.1. Primers (gDLK1-F2 and gDLK1/R3) to quantify total gDLK1 gene
expression were designed to align with the sequences in exon 4 and 5 of the chicken DLK1 gene, respectively. Therefore, the primers that span the genomic intron (2.1 kb) between exon 4 and 5 could avoid amplification of contaminated genomic DNA during the PCR reactions. Conditions for qRT-PCR were 95 °C for 10 min and 40 cycles of 94 °C for 15 sec, 60 °C for 40 sec, 72 °C for 30 sec, and 82 °C for 32 sec. Quantitative real-time PCR was performed in duplicate on 25 μl reactions using an ABI 7300 Real-Time PCR Instrument (Applied Biosystems). The relative level of target gene expression, as determined by ABI software, was calculated using the comparative $2^{-\Delta\Delta Ct}$ method for relative quantification (Livak and Schmittgen, 2001).

4.3.6 Histological analysis

The skin was immediately removed from the breast region after euthanizing the birds and a sample of the pectoralis major muscle, approximately 0.5 × 3.0 cm, was obtained by carefully dissecting muscle fibers following the orientation in a manner to prevent contraction (Velleman et al, 2003). Injury samples were taken from the area (right side of P. major) of muscle injury of the chicken breast muscle while control samples were taken from the uninjured left side of P. major muscle. The tissue samples were dehydrated, cleared, embedded in paraffin, sectioned at 5 μm, and mounted on slides as previously reported (Velleman et al, 2003). Prior to staining with hematoxylin and eosin (H&E), the tissue sections were incubated and rehydrated (Velleman et al., 2002). The stained sections were viewed for muscle morphological characteristics with an Olympus XI 70 microscope and digitally recorded with an Olympus Magna Fire digital camera (Melville, NY). Four sections from each bird were placed on a slide. Five
fields of each section were viewed.

4.3.6 Statistics

Results are presented as mean ± SEM. Comparisons between leghorn and broiler chickens were performed using one way analysis of variance (ANOVA) followed by the Tukey’s test at \( P \leq 0.05 \). Pairwise analysis was performed using Student’s t-test at a significant level set at \( P \leq 0.05 \). All statistical analyses were performed using Minitab software (version 15.0).

4.4 Results

4.4.1 Induction of gDLK1 mRNA expression during chicken primary muscle cell differentiation

The chicken primary muscle cells were cultured to examine the gene expression of gDLK1 along with the expression of myogenic marker genes and the morphological changes of primary muscle cells during differentiation (Figure 4.1 and 4.2). MyHC, a marker for nascent myotubes was not detected at D0 but started to show as early as D1. Its staining increased until day 3 after differentiation. The staining of nuclei with DAPI showed that myotubes exhibit multiple nuclei. These observations demonstrate that primary myoblasts were quickly fused into myotubes at D1 and myotubes became longer and thicker during D2-3 (Figure 4.1).

The ontogeny expressions of myogenic marker and gDLK1 genes are shown in Figure 4.2 (A-D). After induction of myogenesis on the chicken primary muscle cells by treatment with differentiation media, MyoD mRNA, an early marker of myogenesis, was
gradually down-regulated from 0 to 72 h ($P \leq 0.05$). Similarly, before the induction of myogenesis the mRNA expression level of Pax7, another early marker gene or lineage, was also high at 0 hr, significantly decreased to 12 h ($P \leq 0.05$), and remained at low levels to 72 h. On the other hand, myogenin, a late marker of myogenesis, mRNA expression was low at the early time points (12 and 24 h). However, when the early myogenic markers were down-regulated at 36 to 48 h, the levels of myogenin were increased by 10-20 folds, compared to those at 12 and 24 h ($P \leq 0.05$). The level of myogenin mRNA expression then decreased to a level similar to that at 0 h ($P \leq 0.05$). Similar to the pattern of myogenin mRNA expression, the high expression level of gDLK1 mRNA at 0 hr was significantly decreased at 12 h ($P \leq 0.05$). The mRNA expression of gDLK1 was gradually up-regulated from 12 hr to 48 h (around 2.5 fold induction, $P \leq 0.05$) when the myotubes are actively formed and enlarged as shown in Figure 4.1.

### 4.4.2 The temporal expression of gDLK1 mRNA during chicken muscle regeneration

Along with changes in the morphology of muscle fibers after injury, the expressions of gDLK1 gene and myogenic marker genes (MyoD, myogenin, and Pax7) were measured to investigate the coordination of gDLK1 expression with developmental stages of muscle regeneration. Histological analysis at D0 showed that 40-day-old layer chickens had well-organized myofibers. At D5 after muscle damage, the injured muscle contained unorganized small fibers with centered nuclei. In addition, an increased cell number caused by infiltration and proliferation of cells was shown in the damaged area at
D5 (Figure 4.3), indicating the early processes of muscle regeneration. Through D10 to D14 after muscle injury, the muscle fibers returned to a normal shape as seen in the control tissue at D0, indicating that the injured muscle was almost recovered (Figure 4.3).

As shown in Figure 4.4A, Pax7 mRNA was highly induced at D2 and D4 by 7- to 10-fold after the injury ($P \leq 0.05$). The high expression level of Pax7 gene was significantly decreased from D2 to D7 ($P \leq 0.05$). There was no difference in the expression level of Pax7 from D7 to D14 during the muscle recovery period. In addition, the MyoD mRNA was significantly and highly induced at D2 by 5-fold after muscle injury ($P \leq 0.05$). Its gene expression level was decreased from D2 to D4, and significantly down-regulated to D7 when compared to the level of MyoD mRNA expression at D2 ($P \leq 0.05$). Thereafter, the gene expression level of MyoD from D7 up to D14 was maintained at the D0 level (Figure 4.4B). Unlike early expression of Pax7 and MyoD with peaks at D2, relatively late marker, myogenin mRNA expression peaked at D4 with a 5,000-fold higher level when compared to the level at D0 ($P \leq 0.05$). Its mRNA expression was significantly decreased from D4 to D10 ($P \leq 0.05$), but tended to be remained at higher levels over a thousand fold at D7 and D10, then returned to control levels afterwards (Figure 4.4C).

The mRNA expression of gDLK1 in the uninjured left pectoralis major muscle (control) consistently remained at low levels from D0 to D14 (Figure 4.4D). In the injured right pectoralis major muscle, the mRNA expression of gDLK1 was not significantly changed from D0 to D4. However, it was highly induced from D4 to D7 ($P \leq 0.05$). At D7, gDLK1 gene expression in the damaged tissue was about 8-fold higher than the control (Figure 4.4B, $P \leq 0.05$). The gDLK1 mRNA expression level was
gradually down-regulated from D7 to D14 ($P \leq 0.05$), but the level was still highly maintained when compared to the control level.

4.4.3 Comparison of the mRNA expression levels of myogenic regulatory factors (MRFs) and gDLK1 in the pectoralis major muscles of leghorns with those levels of broilers

The comparison of the gene expression patterns or levels of MRFs and gDLK1 between broilers and layers during embryonic skeletal muscle development and growth after hatching was investigated (Figure 4.5). As shown in Figure 4.5A, both layers and broilers expressed similar levels of Pax7 mRNAs at embryonic muscle developmental stages (E13 and E17). In layers, the mRNA expression level of Pax7 was decreased 29-fold from E17 to P1 ($P \leq 0.05$). In contrast its expression level in broilers was decreased 7.7 fold from E17 to P1 ($P \leq 0.05$). This resulted in a significantly different level of Pax7 mRNA expression between them at P1 ($P \leq 0.05$). At P5, however, the expression of Pax7 was maintained from P1 in layers while its expression level in broilers tended to decrease. There were no significant differences of the Pax7 gene expression level between the two selected chick lines from P11 to P33.

A declining pattern of MyoD gene expressions in both types of chickens was shown from E13 to P33 (Figure 4.5B). In layers, MyoD gene expression decreased sharply from E13 to P1 by 5-fold ($P \leq 0.05$) and maintained from P1 to P5 until P33. In broilers, however, its sharp decline (25-fold) was found from P1 to P5 ($P \leq 0.05$). This resulted in significantly different levels in MyoD mRNA expressions between the two chickens at P1 (4-fold high in broilers, $P \leq 0.001$) and P5 (3-fold high in layers, $P \leq 0.01$),
respectively. In addition, a decreased level of MyoD mRNA expression in layers was found from P5 to P11, resulting in a 2-fold lower expression level than broilers ($P \leq 0.05$).

The level and pattern of ontogenic gDLK1 mRNA expression in the pectoralis major muscle in both types of chickens is shown in Figure 4.5C. Gene expression of the layer gDLK1 at E13 and E17 declined significantly to P5 ($P \leq 0.05$), and remained at that level. Similarly, broiler gDLK1 gene expression was the highest at E13, significantly down-regulated to E17 ($P \leq 0.05$), and remained unchanged until P1. Subsequently, the gene expression of broiler gDLK1 was significantly down-regulated from P1 to P5 ($P \leq 0.05$), and the expression level of broiler gDLK1 gene remained unchanged from P5 until P33. The gene expression levels of gDLK1 at various sampling times ranged from E13 to P33 when compared between broilers and layers. There were significantly higher expression levels of gDLK1 mRNA in broilers than layers at time points, E13 (2.5-fold, $P \leq 0.05$), P1 (2.5-fold, $P \leq 0.01$), P11 (2-fold, $P \leq 0.005$), and P33 (3-fold, $P \leq 0.01$). The exceptions were at E17 and P5.

### 4.5 Discussion

Myogenic transcription regulatory factors (MRFs) that function in processing myogenesis and their expressions has been used as indicators of muscle development as well as regeneration (Shi and Garry, 2006; Buckingham, 2006). In the current study, a chicken primary muscle cell culture system was used to determine the temporal expression of gDLK1 during muscle development. The gradual reduction of expression of early myogenic marker genes (Pax7 and MyoD) during the differentiation period, and induction of a late marker gene (myogenin) at the time of active myotube formation
(Nabeshima et al., 1993; Dedieu et al., 2002) indicates that primary chicken muscle cells can serve as an excellent in vitro system for evaluating stages of muscle development. In addition, immunostaining using MyHC and DAPI staining also confirmed that chicken primary myoblasts successfully underwent myogenesis. The gene expression of gDLK1 (Figure 4.2) was induced at the late stages of muscle development when the myotubes were actively forming and elongating (Figure 1), suggesting possible involvement of gDLK1 in these developmental processes.

Muscle regeneration has been used as an excellent in vivo model for investigating muscle growth and developmental biology. Its advantages include: 1) re-establishment of the activation of quiescent satellite cells (Halevy et al., 2004), 2) recapitulation of the developmental processes during embryonic myogenesis (Parker et al., 2003) and, 3) prediction of myogenic functions of genes at the specific stages of muscle development (Jørgensen et al., 2008). Sequential induction of marker genes during the developmental process of muscle regeneration has been demonstrated in mice (Goetsch et al., 2003; Garry et al., 2000). Based on morphological characteristics of regenerating chicken muscles, the recovery period of chicken muscle is between 2-3 wk which is similar to the mouse. In this study the sequential induction of marker genes was clearly shown in regenerating chicken muscles: induction of early myogenic gene (Pax7 and MyoD) expression at D2-4 post-injury; the late myogenic gene (myogenin) expression at D4-10 post-injury; and the gDLK1 gene expression at D7-14 post-injury.

The expression patterns of Pax7 and MyoD suggest the potential importance of their roles in the early stages of muscle regeneration. Similarly, the sequential expression of these genes was also found during the differentiation of primary chicken muscle cells.
This indicates that \textit{in vitro} myogenic gene expression patterns are recapitulated during regenerative myogenesis. Importantly, the late induction of gDLK1 during \textit{in vitro} myogenesis and also muscle regeneration suggests that gDLK1 gene expression is highly associated with the late stages of muscle development including myotube formation. In support of this, our unpublished data and previous reports showed that DLK1 protein was exclusively found in the developing myotubes in the fetus of humans and mice (Floridon et al., 2000; Yevtodiyenko and Schmidt, 2006). Our data also suggest that the temporal expression of gDLK1 may be involved in tissue regeneration and differentiation of stem and /or satellite cells.

The effect of genetic selection on muscle growth and development has been widely investigated in the avian species of chickens (Scheuermann et al., 2004; Aberle and Stewart, 1983), turkeys (Velleman et al., 2003), and quail (Ye et al., 1999; Campion et al., 1982). In chickens, broilers are genetically selected for meat production; whereas, leghorn layers are selected for egg production. Many investigators have reported that broilers have larger diameters of myofibers (Smith, 1963), a more rapid rate of myofiber radial hypertrophy (Aberle and Stewart, 1983), greater numbers of myofibers (Scheuermann et al., 2004), a faster proliferation rate of muscle cells (Moss, 1968; Blunn and Gregory, 1935), and hence, more muscle mass (Mizuno and Hikami, 1971) than layers. In the current study, we investigated whether the temporal expression of myogenic marker genes and the gDLK1 gene during avian muscle development and growth can account for the distinct different muscle growth characteristics between the two lines of chickens. Relatively high levels of gDLK1 mRNA were expressed in developmental muscles of broilers compared to those of layers (Figure 4.5). The greater
expression of gDLK1 in broilers than layers may affect muscle hypertrophy in broilers.

The promyogenic activities of DLK1 were demonstrated by muscle hypertrophy in callipyge sheep (White et al., 2008), and transgenic mice ectopically overexpressing DLK1 (Davis et al., 2004). In addition, a paternally imprinted DLK1 gene has been reported to be involved in genetic diseases associated with muscle development. Absence of the DLK1 gene expression in the human genetic disease (maternal UPD14 chromosome) causing hypotonic muscles, suggests that DLK1 plays an important role in the muscular development of humans (Berends et al., 1999). Similarly, absence of DLK1 gene expression in the mouse having maternal UPD12 chromosome caused muscle hypotonia (Georgiades et al., 2000). In contrast, double dose expression of the DLK1 gene in the mouse paternal UPD12 chromosome exhibited myofiber hypertrophy (Georgiades et al., 2000). The greater expression of gDLK1 in broilers could positively affect muscle growth in broilers, implicating it as a new selection marker for large muscling chickens. Thus, the DLK1 gene may be an important factor for controlling muscle growth and development in the chicken as well as mammals.

Expression of myogenic marker genes was also compared between broilers and layers during muscle development. The high levels of Pax7, and MyoD mRNA expressions at the embryonic stages suggest that active myogenesis occurs during embryonic development in both leghorns and broilers (Figure 4.5). Pax7 is required for the specification of satellite cells (Seale et al., 2000) and serves as a early marker for proliferation of satellite cells (Halevy et al., 2004), providing new mononuclei for growing myofibers (Moss and Leblond, 1971). Therefore, the relatively higher mRNA expression level of Pax7 in the muscles of broilers at P1 compared to layers may indicate
that broilers have a greater population of reserve satellite cells than layers. In addition, MyoD is required for myoblast differentiation by controlling the cell cycle exit (Zhang et al., 1999). This supports our conclusion that the relatively higher levels of MyoD in broilers from embryonic to post-hatching days than layers contribute to the extended differentiation of muscle cells in broilers. Taken together, relatively high expression levels of MyoD and Pax7 after hatching supports the conclusion that broilers have a larger population of reserved satellite cells that undergo differentiation for a relatively longer period of time to increase muscle mass.

We report here the temporal expression of gDLK1 mRNA during in vitro muscle cell differentiation and in vivo muscle regeneration in chickens. In addition, the greater expression of gDLK1 gene along with the higher levels of expression of the myogenic genes (MyoD and Pax7) in broilers seems to contribute to higher myogenic activities and greater muscle mass compared to leghorns. Furthermore, because of the apparent critical role of DLK1 to muscle hypertrophy our results suggest that gDLK1 can be a new candidate marker for selection of high muscularity in chickens. The mRNA expression of gDLK1 followed by the peak expressions of myogenic markers (MyoD, Pax7, and myogenin) was clearly shown in injured muscle tissue and primary muscle cell differentiation, indicating that gDLK1 may be essential for a specific stage of muscle development. However, the mechanism of gDLK1 role in skeletal muscle development remains to be further investigated.
4.6 Acknowledgements

This work was supported by the Edna Jaap Poultry Endowment Fund to K. Lee, and J. D. Latshaw and partially supported by a Korean Science and Engineering Foundation grant to J. Shin, No.C00137. We are grateful to Michelle Milligan (The Ohio State University) for editing this manuscript.
Table 4.1. Primer sequences for quantitative real-time polymerase chain reaction.

<table>
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<th>Primer</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>GenBank accession number</th>
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<tr>
<td>gDLK1-F2</td>
<td>5'- TGT GTG CCC AGG GAT TTA CAG GA-3’</td>
<td>233</td>
<td>EU_288039</td>
</tr>
<tr>
<td>gDLK1-R3</td>
<td>5'-ACC TGC ACC AAT ATC TGT GCA CG-3’</td>
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<tr>
<td>gGAPDH-F1</td>
<td>5’-CTC TGT TGT TGA CCT GAC CTG-3’</td>
<td>262</td>
<td>NM_204305</td>
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<tr>
<td>gMyoD-F</td>
<td>5’-AGC TCT CGC AGG AGA AAC AGC TA -3’</td>
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<td>NM_204214</td>
</tr>
<tr>
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<tr>
<td>gMyogenin-F</td>
<td>5’-TGC TCA GCA GCC TCA ACC AGC A-3’</td>
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<td>D90157</td>
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<td>gMyogenin-R</td>
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<td></td>
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<tr>
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<td>5’-TTG CCA CCA TCC ACC ATG CA-3’</td>
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<td>NM_205065</td>
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<tr>
<td>gPax7-R</td>
<td>5’-CAC CTG GAG CAC TGC ATC TT-3’</td>
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</table>

1gDLK1 = chicken delta-like protein 1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; MyoD = Myogenic differentiation 1; Myogenin = Myogenic factor 4; Pax7 = Paired box gene 7; F = forward; R = reverse. (Shin et al., 2008)
Figure 4.1. Differentiation of chicken primary muscle cells.
The microscopic images of primary muscle cells at D0-D3 indicate the morphological changes during cell differentiation. The muscle cells isolated from pectoralis major muscle tissue of a 13-day embryo were cultured in differentiation medium. The differentiated muscle cells at each sampling time were analyzed by the immunostaining MyHC (Red) and staining nuclei with DAPI (Blue). A scale of white bar = 50 μm.
Figure 4.2. The mRNA expressions of myogenic markers and gDLK1 during the differentiation of chicken primary muscle cells. (A-D) The mRNA expressions of MyoD (A), Pax7 (B), myogenin (C), and gDLK1 (D) were analyzed by quantitative RT-PCR (n = 4). GAPDH was used as a housekeeping gene. The bar represents mean ± SEM. The letters (a, b) represent a significant difference among groups at various time points by using one-way ANOVA at $P \leq 0.05$. 
Figure 4.3. Histology of skeletal muscles in leghorn chickens during muscle regeneration.
The histological tissue sections for undamaged pectoralis muscle (left) at day 0 and damaged pectoralis muscle (right) 5, 10, or 14 days after muscle injury were stained with H&E. A scale of white bar = 50 μm.
Figure 4.4. The gene expression of gDLK1 during muscle regeneration in leghorn chickens.
(A-C) Expression levels of Pax7 (A), MyoD (B), and myogenin (C) mRNAs in injured muscle tissue during various time points (day 0, 2, 4, 7, 10, and 14) were measured by qRT-PCR (n=7). (D) Comparison of gDLK1 gene expression between control and injured muscle tissue during various time points (n=7). GAPDH was used as a housekeeping gene. The bar represents mean ± SEM. The letters (a, b) represent a significant difference among groups at various time points by using one-way ANOVA at p ≤ 0.05. Significant difference is indicated by *, **, or *** between control and injured groups at p ≤ 0.05, p ≤ 0.01, p ≤ 0.005, respectively, by using the student t-test.
Figure 4.5. The gene expression of gDLK1 and other myogenic regulatory factors during muscle development in layers (White leghorn) and broilers.

(A-C) Relative amounts of Pax7 (A), MyoD (B), and gDLK1 (C) mRNAs in the pectoralis major muscle were measured by quantitative real-time PCR (n = 4). GAPDH was used as a housekeeping gene. The bar represents mean ± SEM. The letters (a, b) represent a significant difference among groups at various time points by using one-way ANOVA at p ≤ 0.05. Significant difference is indicated by *, **, or *** between leghorn and broiler chickens at p ≤ 0.05, p ≤ 0.01, p ≤ 0.005, respectively, by using the student t-test.
Chapter 5: Impaired muscle regeneration and its coordinated myogenic gene expression in Low Score Normal (LSN) and Muscular Dystrophy (MD) chickens.

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5.1 Abstract

Muscle regeneration recapitulates embryonic skeletal muscle development that is associated with the coordinated gene expression of myogenic regulatory factors and chicken delta-like protein 1 (gDLK1). Muscular dystrophy (MD) and low score normal (LSN) chickens have a genetic muscle weakness, losing pectoralis major (PM) muscle function. Therefore, the objectives for the current study were to compare: 1) the morphological changes and 2) the expression levels and patterns of myogenic regulatory genes (Pax7, MyoD, myogenin, and MRF4) and the gDLK1 gene during muscle regeneration of control, LSN, and MD chickens. To induce muscle regeneration in the three types of chickens, a liquid nitrogen chilled-rod was used to damage their left pectoralis major muscles at 6wks of age. Thereafter, undamaged and damaged tissue samples from each group during set time points (d 0, 2, 4, 7, 10, 14, and 20) were
collected for both histological analysis and quantitative real-time PCR to study the expression of myogenic marker genes. Histological data showed the delayed and impaired myofiber formation in LSN and MD chickens. A significantly high basal level of MyoD, Pax7, myogenin, and gDLK1 mRNA expression ($P < 0.05$) in LSN chickens suggests that muscle degeneration may have already progressed. LSN chickens had a delayed pattern of myogenin mRNA expression and maintained expression of Pax7 and MyoD mRNA from D4 to D7 ($P < 0.05$); whereas, MD chickens had a significantly high peak expression of myogenin mRNA, a low peak expression of MRF4 mRNA, and a high peak expression of gDLK1 mRNA ($P < 0.05$). Although both LSN and MD chickens have delayed muscle regeneration, distinctive expression of stage-specific myogenic markers suggests that the regenerative mechanisms for their impaired muscles might be different. Understanding morphological and molecular events during muscle regeneration of muscular dystrophic chickens will further aid to identify muscle characteristic genes responsible for muscle developmental disorders.

**Keywords:** chicken delta-like protein 1 (gDLK1), myogenic regulatory factor (MRF), muscular dystrophic (MD), low score normal (LSN), chicken, muscle regeneration.
5.2 Introduction

Skeletal muscle is regenerated after injury or exercise in humans and animals (Schultz and McCormick, 1994; Parise et al., 2006). The studies of muscle regeneration in animals have largely been performed to recapitulate and understand the developmental process of embryonic myogenesis (Goetsch et al., 2003; Parker et al., 2003; Turk et al., 2005). The multiple cellular processes of regenerative myogenesis are accomplished by activation and proliferation of muscle stem cells or satellite cells after the injury, differentiation of these cells into myofibers with multi nuclei, and finally terminal differentiation and maturation of myofiber for the function of muscle (Halevy et al., 2004; Buckingham, 2006; Shi and Garry, 2006). The processes are controlled by a complex of myogenic transcription regulatory factors (MRFs), the extracellular matrix (ECM), and growth factors (McFarland, 1999; Velleman, 1999; Martin, 2003; Buckingham, 2006).

The MRFs, a family of basic helix-loop-helix (bHLH) transcription factors, play essential roles in the developmental processes of muscle development and regeneration (Pownall et al., 2002; Martin, 2003). Their family members consist of myogenic differentiation 1 (MyoD), myogenic determination factor 5 (Myf-5), myogenic factor 4 (Myogenin), and myogenic factor 6 (MRF4/Myf6). Previous studies indicate that MyoD plays a role in proliferation of myoblasts, cell cycle exit, and initial differentiation of myoblasts (Rudnicki et al., 1992 and 1993), suggesting that MyoD expression indicates the early stages of myogenesis. Moreover, it has been reported that myogenin promotes fusion of myoblasts and the formation of myofiber with multi-nuclei (Hasty et al., 1993); MRF4 are involved in terminal differentiation of myofibers (Hinterberger et al., 1991; Dedieu et al., 2002), suggesting that myogenin and MRF4 expression may be
important for the late stages of myogenesis. In addition, paired-box gene 7 (Pax7) has previously been used as a myogenic lineage marker to determine activation of muscle satellite cells during muscle regeneration (Seale et al., 2000; Halevy et al., 2004). These gene expressions have been used and proven as myogenic markers for indicating early (MyoD and Pax7) and late (myogenin) developmental stages of embryonic and regenerative myogenesis in avian species (Shin et al., 2008 and 2009). Therefore, the correlation of the gene expression of MRFs and Pax7 with muscle morphological changes during muscle regeneration can be used to understand which stages of muscle regeneration are affected in muscular dystrophic models.

The myogenic function of delta-like protein (DLK1) has been implicated for muscle hypertrophy in callipyge sheep, transgenic mouse ectopically over-expressing DLK1, and genetic diseases in the mouse and human (Cockett et al., 1996; Georgiades et al., 2000; Davis et al., 2004). DLK1 was highly induced during embryonic or fetal muscle development of animals (White et al., 2008; Andersen et al., 2009). Recently, the expression pattern of DLK1 at the late developmental stages has also been studied in muscle regeneration of rodents (Anderson et al., 2009) as well as ear wound healing of the mouse (Samulewicz et al., 2002), suggesting a role in tissue regeneration. In our previous studies, chicken DLK1 (gDLK1) was cloned, and its regulation has been studied for the first time in poultry (Shin et al., 2008 and 2009). The high induction of gDLK1 mRNA at the embryonic stage during muscle development and the temporal expression of gDLK1 mRNA during the regeneration have been identified. The peak expression of gDLK1 mRNA was shown in the late stages of regenerative myogenesis, which was also confirmed in primary chicken muscle cell culture. However, the regulation and
comparison of gDLK1 gene expression has never been studied in chickens with muscular dystrophy.

Muscular dystrophies in human and animals have been defined as inherited myogenic disorders that are characterized by progressive muscle wasting and weakness, leading to defects in muscle proteins and the death of muscle cells and tissues (Allamand and Campbell, 2000; Emery, 2002; Grounds et al., 2008). To understand the muscle physiology, pathology, and regeneration of human Muscular Dystrophies, mice with muscular dystrophy have generally been used and studied (Fargas et al., 2002; Collins and Morgan, 2003; Marotta et al., 2007). However, avian muscular dystrophic models, low score normal (LSN) and muscular dystrophy (MD) chickens, have never been used for studying myogenic gene expression and its associated muscle morphology during muscle regeneration.

Low Score Normal and Muscular dystrophy chickens have been characterized during muscle development and growth (Asmundson and Julian, 1956; Wagner and Peterson, 1970; Velleman et al., 1993; Velleman et al., 1996; Velleman et al., 2001; Li et al., 2009). LSN chickens were generated by an outcross of chickens with hereditary muscular dystrophy to White Leghorn chickens (Velleman et al., 1993). Previous studies reported that LSN and MD chickens have inherited muscle weakness, showing a decreased muscle function (Velleman et al., 1993; Velleman et al., 1996). The LSN chickens have severe muscle phenotypes such as a 68% decrease in pectoralis muscle mass and a 60% reduction in body weight when compared to normal chickens (Velleman et al., 1996). Unlike humans and mice with muscular dystrophies, the molecular muscle characteristics of chickens with muscular dystrophy are still unclear during muscle
development, growth, and regeneration.

Therefore, the objectives of the current study were to compare the morphological characteristics, and the expression level and pattern of myogenic regulatory genes (MyoD, Pax7, myogenin, and MRF4) and gDLK1 gene during muscle regeneration among control, LSN, and MD chickens. In addition, the association of morphological changes of damaged muscles with the gene regulation of MRFs and DLK1 during regenerative myogenesis was investigated to understand involvement of MRFs and DLK1 in specific stages of regenerative myogenesis in muscular dystrophic chickens.

5.3 Material and Methods

5.3.1 Experimental birds and the injury of the pectoralis major muscles

Animal care procedures were approved by the OSU Agricultural Care and Use Committee. The Low Score Normal (LSN) chickens have been actively studied since it was detected in 1977 for the first time at the University of Connecticut (L. J. Pierro and J. S. Haines, Department of Animal Genetics, University of Connecticut, Storrs, CT 06268). The muscular dystrophic (MD) chickens had been maintained at the University of Connecticut for many years prior to being transferred to the Ohio State University in 1997 (L. J. Perro and J. S. Haines, Department of Animal Genetics, University of Connecticut, Storrs, personal communication referred to Velleman and Nestor, 2001). A total of one hundred fifty chickens [specific pathogen free (SPF), LSN, and MD chickens] at 40-days-old were used to study muscle regeneration. The chickens were anesthetized after inhalation of isoflurane, and their skins were incised for exposure of the right pectoralis major (PM) breast muscle surface. The tip of a metal rod with a flat 5 mm²
surface area was dipped in liquid nitrogen for 1-2 min, and then held gently against the right breast muscle surface (3-5 cm) for 5 sec. The injured chickens were kept warm during recovery from anesthetization. Samples were collected at various days (D0, D2, D4, D7, D10, D14, and D20) after cold injury for histological analysis and measurement of gene expression levels by qRT-PCR. The undamaged left side of the muscle was used as a control for the damaged right side of the muscle.

5.3.2 Total RNA Isolation and qRT-PCR

Chicken pectoralis muscle tissues at selected ages in each group were snap frozen in liquid nitrogen and homogenized using a Tissuemiser homogenizer (Fisher Scientific, Pittsburgh, PA). Total RNA isolation and qRT-PCR were performed as previously described (Shin et al., 2008). Briefly, total RNA from the tissue was isolated using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions, and RNA quality was assessed by electrophoresis (1% agarose gel). Reverse transcription (RT) was performed using 1 μg of total RNA and moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription condition for cDNA amplification was 65 ºC for 5 min, 37 ºC for 52 min, and 70 ºC for 15 min. Primer sequences used for the qRT-PCR are shown in Table 5.1. The qRT-PCR using gDLK-F2 and gDLK1-R3 primer set was performed to measure the relative levels of gDLK1 expression in muscle tissues. The glyeraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as a housekeeping gene as previously described (Shin et al., 2008 and 2009). Primers (gDLK1-F2 and gDLK1/R3) to quantify total gDLK1 gene expression were designed to align with the sequences in exon 4 and 5 of the chicken DLK1 gene,
respectively. Therefore, the primers that span the genomic intron (2.1 kb) between exon 4 and 5 could avoid amplification of contaminated genomic DNA during the PCR reactions. The quantitative real time-PCR was performed using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), and SYBR green was used as the detection dye. Conditions for qRT-PCR were 95 ºC for 10 min and 40 cycles of 94 ºC for 15 sec, 60 ºC for 40 sec, 72 ºC for 30 sec, and 82 ºC for 32 sec. Quantitative real-time PCR was performed in duplicate on 25 µl reactions using an ABI 7300 Real-Time PCR Instrument (Applied Biosystems). The relative level of target gene expression, as determined by ABI software, was calculated using the comparative \(2^{-\Delta\Delta Ct}\) method for relative quantification (Livak and Schmittgen, 2001).

### 5.3.3 Histological analysis

The skin was immediately removed from the breast region after euthanizing the birds and a sample of the pectoralis major muscle, approximately 0.5 × 3.0 cm, was obtained by carefully dissecting damaged muscle tissues following the orientation of the muscle fibers in a manner to prevent contraction (Velleman et al, 2003). Injured samples were taken from the area (right side of PM) of muscle injury of the chicken breast muscle while control samples were taken from the uninjured left side of PM muscle. The tissue samples were dehydrated, cleared, embedded in paraffin, sectioned at 5 µm, and mounted on slides as previously reported (Velleman et al, 2003). Prior to staining with hematoxylin and eosin (H&E), the tissue sections were incubated and rehydrated (Velleman et al., 2002). The stained sections were viewed for muscle morphological characteristics with an Olympus XI 70 microscope and digitally recorded with an
Olympus Magna Fire digital camera (Melville, NY). Four sections from each bird were placed on a slide. Five fields of each section were viewed.

5.3.4 Statistics

Results are presented as mean ± SEM. Comparisons among control, LSN, and MD chickens were performed using one way analysis of variance (ANOVA) followed by the Duncan’s test at $P < 0.05$. All statistical analyses were performed using SPSS software (Version 15.0; SPSS Inc., Chicago, IL).

5.4 Results

5.4.1 Histological analysis of control, LSN, and MD type chickens during muscle regeneration

Hematoxylin and eosin histology for muscle tissues of control, LSN, and MD chickens were performed to compare the morphological changes of their muscles during regeneration (Figure 5.1). As shown in Figure 5.1A, at D0, control (SPF) chickens had relatively well-organized myofibers with mostly peripheral nuclei and homogeneous size. LSN and MD chickens seemed to have larger myofibers with more centered nuclei at D0 than control. At D2 post-injury, less myofibers, damaged border lines (endomysium and perimysium) of myofibers, and many new nuclei were shown around the scar area in damaged muscles of control, LSN, and MD chickens. New and small myofibers with central multi-nuclei were observed in the damaged area of control, LSN, and MD chickens from D4 to D7 (Figure 5.1B). At D10, the border lines of the myofiber perimysium and endomysium began to reappear in control chicken; whereas, those were
not observed in LSN and MD chickens. From D10 to D20 of muscle regeneration in the control chicken, the damaged muscle tissue was nearly healed with well-organized myofibers with both central and peripheral nuclei (Figure 5.1C). Unlike control muscle at D10, the myofibers were not completely formed yet, indicating the delayed formation of myofibers in LSN and MD chicken. In addition, the organization and morphological shapes of myofibers were not similar to D0 of LSN and MD chicken muscles at D20 after the injury. Considerably large myofibers with centered nuclei, un-organized myofibers, and myofibers with variable size were regenerated in muscles of LSN and MD chicken at D20 (Figure 5.1C).

### 5.4.2 Comparison of the level and pattern in the expressions of early or lineage marker genes (MyoD and Pax7) during muscle regeneration among control, LSN, and MD chickens

To investigate whether the distinct levels or patterns of MyoD and Pax7 gene expressions are associated with the delayed or impaired myogenic regeneration of LSN and MD chickens, the Pax7 and MyoD gene expressions were compared (Figure 5.2 and 5.3). The levels of Pax7 expression were compared at D0 before muscle injury, D2 for the peak stage, and D20 after muscle injury (Figure 5.2A). The basal level of Pax7 mRNA expression of LSN chicken at D0 was 2-fold higher than those of control and MD chickens \((P < 0.05)\); the level of Pax7 expression of MD chicken at D20 post-injury was 2-fold higher than control and LSN chickens \((P < 0.05)\). There were no differences among the peak gene expression of Pax7 at D2. Figure 5.2B shows a similar pattern of the temporal mRNA expression of Pax7 during muscle regeneration in all three types of
chickens. From D2 to D20 after muscle injury of the three lines of chickens, the peak mRNA expressions of Pax7 were down-regulated \((P < 0.05)\) except that the Pax7 expression at D4 of only LSN chickens highly remained at D7 post-injury.

As shown in Figure 5.3A, the basal level of MyoD mRNA expression of LSN chicken at D0 was 2-fold higher than those of control and MD chickens \((P <0.05)\). There were no different levels in MyoD peak expression at D2 and D20 among all three lines of chickens. Figure 5.3B shows a temporal expression of MyoD mRNA during muscle regeneration in all three types of chickens. At D2 after their muscle injuries, the MyoD mRNA expression levels of all chickens peaked \((P < 0.05)\). Thereafter, the peak induction of MyoD mRNA in control and MD chickens was gradually down-regulated to D20 \((P < 0.05)\). However, the relatively high level of MyoD gene expression at D4 was maintained to D7 in LSN chicken and then down-regulated to D20 \((P < 0.05)\).

5.4.3 Comparison of the level and pattern of the expressions of late marker genes (myogenin and MRF4) during muscle regeneration among control, LSN, and MD chickens

The comparison of myogenin and MRF4 mRNA expression during muscle regeneration in all three types of chickens was performed to determine if the distinct differences of the level or pattern could be associated with delayed muscle regeneration of chickens with muscular dystrophy (Figure 5.4 and 5.5). As shown in Figure 5.4A, LSN chicken had a 2-fold higher basal level of myogenin mRNA expression at D0 than control and MD chickens \((P <0.05)\); MD chickens had about 2-fold higher expression of myogenin mRNA at post-injury D4 than control and LSN chickens \((P <0.05)\). In
addition, MD chicken had 2-fold higher expression of myogenin mRNA than LSN chickens at D20 post injury ($P<0.05$). Figure 5.4B shows a temporal mRNA expression of myogenin during muscle regeneration in all three types of chickens. Control and MD chickens showed the peak induction of myogenin mRNA at post-injury D4 ($P<0.05$) and considerably down-regulated up to D7 ($P<0.05$). Unlike the myogenin expression patterns of control and MD chickens, the LSN chickens had the peak mRNA expression of myogenin at D7 ($P<0.05$). Thereafter, the myogenin expression of LSN chickens was considerably decreased to D10 post-injury ($P<0.05$).

As shown in Figure 5.6, MD chicken had 3-fold lower basal level of MRF4 mRNA expression at D0 than control chicken ($P<0.05$). In addition, at D4 the peak expression level of MRF4 mRNA in MD chickens was considerably lower than that of control and LSN chickens ($P<0.05$). However, MD chickens showed 2-fold higher expression of MRF4 mRNA than that of control and LSN chickens at D20 ($P<0.05$). In Figure 5.5B, MRF4 mRNA peaked at D4 after the injury in all three types of chickens ($P<0.05$); the peak expression of MRF4 gradually decreased up to D20 in all three groups ($P<0.05$).

### 5.4.4 Comparison of the level and pattern of the expression of gDLK1 mRNA during chicken muscle regeneration among control, LSN, and MD chickens

To investigate whether the distinct difference in the level and pattern of gDLK1 gene expression during muscle regeneration among control, LSN, and MD chickens could be associated with morphological changes in damaged muscles, the temporal gene expression of gDLK1 was examined and compared (Figure 5.6). As shown in Figure
5.6A, LSN chickens had a 2.5-fold higher basal level of gDLK1 mRNA expression at D0 before muscle injury than control and MD chickens ($P < 0.05$). At D7 post-injury, MD chickens had about 1.5- or 2-fold higher level of gDLK1 mRNA expression than control and LSN chickens ($P < 0.05$). There was no difference in the gDLK1 expression among three types of chickens at D20. Figure 5.6B shows a temporal expression of gDLK1 during muscle regeneration in all three types of chickens. At D7, the gDLK1 gene expression peaked in all three lines of chickens ($P < 0.05$). Thereafter, the peak expression of gDLK1 mRNA was significantly decreased from D7 to D20 ($P < 0.05$).

5.5 Discussion

In our previous research, a temporal expression of early or late myogenic marker genes (MRFs and gDLK1) was used for identifying multiple stages of regenerative myogenesis in chickens (Shin et al., 2009). In the present study, the similar temporal expression patterns, as shown in our previous work, were confirmed again by these myogenic stage-dependent orders: MyoD and Pax7 mRNA expressions at the early stages (d 2), myogenin and MRF4 mRNA expressions at the late stages (d 4-7), and gDLK1 mRNA expression at the late stage (d 7) during muscle regeneration in all three types of chickens (Figure 5.2-5.6). Similarly, these regulated patterns and expression orders of MRFs and DLK1 with specific myogenic stages were also shown in the muscle regenerative study of the mouse and rat (Andersen et al., 2009), supporting our results. These indicate that the regenerative expression patterns and orders of those myogenic marker genes seem to be important regardless of muscle phenotypes or species. In addition, this suggests that our muscle regeneration may be an excellent model to study
for recapitulating the in vivo muscle developmental process.

Our histological analysis revealed that LSN and MD chickens had a considerable impairment in the formation of myofibers after injury as shown by: 1) irregular shapes of myofibers, 2) abnormal endomysium and perimysium, 3) partial myofiber hypertrophy with centered nuclei at D20 post-injury, and 4) macrophage infiltration (Figure 1). Similar histological observations were also shown in muscular dystrophic animals during muscle regeneration (Nonaka, 1991; Zhao et al., 2006; Cohn et al., 2007; Cerri et al., 2008). Furthermore, real-time PCR data analysis showed the abnormal levels or delayed patterns in MRF and gDLK1 gene expression during muscle regeneration of LSN and MD chickens (Figure 5.2-5.6). These data suggest that LSN and MD chickens may be excellent experimental chicken models to facilitate the histological and molecular biology of muscle regeneration mechanisms.

In the muscle dystrophic (mdx) mouse, the cycles of degeneration and regeneration occur in skeletal muscles with age (Yablonka-Reuveni and Anderson, 2006). In the present study, LSN chickens, at the basal level (post-hatch d 40) before muscle injury, showed significantly 2-fold higher gene expressions of MyoD, Pax7, myogenin, and gDLK1 than control and MD chickens, suggesting that activation, proliferation, and incorporation of satellite cells into myofibers might be active at this age in LSN chickens. In addition, the histological section of LSN muscles showed myofibers with some centered nuclei at this age. Collectively, these results suggest that muscle degeneration and regeneration of LSN chicken may have already progressed at 40 days old. This is consistent with the data regarding muscle protein synthesis and degradation (Velleman, 1997). Interestingly, Velleman et al. (1993 and 1996) reported that LSN chicken at
around this age had normal myofibers in size distribution, but seemed to have a considerably decreased muscle mass, suggesting less number of myofibers in muscles of LSN chicken. Thus, this less number of myofibers may result from degeneration and regeneration of LSN birds that were earlier than normal birds (Velleman, 1997).

Compared to control chickens during muscle regeneration, LSN chicken had the distinctive characteristics of the molecular phenotypes, including 1) the abnormally extended expression of Pax7 and MyoD mRNA from D4 to D7, 2) the delayed pattern of myogenin expression (peak at D7), 3) no distinctive different pattern of MRF4 gene expression, and 4) delayed myofiber formation at D7 during muscle regeneration. The prolonged mRNA expressions of Pax7, MyoD, and myogenin at D7 suggest that activities of early regenerative myogenesis including activation, proliferation, and fusion of satellite cells may be maintained for a relatively longer period of time. This may be supported by morphological data at D7, showing many un-incorporated nuclei and relatively less small fibers in LSN chicken. Taken together, reduced myofiber number, impairment of myofiber formation and lagging early stages of regenerative myogenesis may be associated with sustained expression of Pax7, MyoD, and myogenin in LSN chicken.

On the other hand, there were no distinct differences in the levels and patterns of Pax7, MyoD, and myogenin expression between control and MD chickens. However, MD chickens had a 3-fold lower basal level of MRF4 mRNA expression at D0 before muscle injury than that of control chickens. In addition, when compared to morphological and myogenic gene expression data of control chicken, MD chicken had 1) the 4-fold low expression of MRF4 mRNA at D0 (basal) and D4 (peak), 2) about 10-fold
low temporal expression of MRF4 during muscle regeneration, 3) about 3.5-fold high peak expression of myogenin mRNA at D4, 4) the 1.5-fold high expression of gDLK1 mRNA at D7, and 5) delayed and impaired myofiber formation at D7 to D20. Because MRF4 is responsible for terminal differentiation of the cells and maturation of myofibers (Hinterberger et al., 1991; Dedieu et al., 2002), a low temporal expression of MRF4 associated with delayed or impaired muscles of MD chicken at D7 to D20 may reflect a defect of myofiber maturation during regenerative myogenesis. Interestingly, it was reported that adult dystrophic birds failed to repress the neonatal isoform of myosin heavy chain (MyHC) that disappeared in normal adult birds (Bandman, 1985; Velleman et al., 1993), suggesting a defect in muscle maturation. Taken together, these muscle defects of MD chickens might result in impaired ability to right themselves when repeatedly placed on their backs (exhaustion score test) at 2-3 month of age (Velleman et al., 2001). The muscle disorder characteristics of MD chickens, however, still remain to be investigated.

A conscious effort has been made to find a certain role of DLK1 in muscle development and regeneration of mammals and bird (Georgiades et al., 2000; Floridon et al., 2000; White et al., 2008; Shin et al., 2008 and 2009; Andersen et al., 2009). There were the similar patterns of gDLK1 mRNA expression with peak at D7 during muscle regeneration in all three lines of chicken (Figure 5.6). In addition, the temporal gene expression of gDLK1, shown in the previous and current studies, seems to be required for repairing damaged muscles in all three types of chickens regardless of the status of muscular dystrophies. However, concomitant with the high expression of Pax7, MyoD, and myogenin mRNA, 2-fold higher level of gDLK1 mRNA expression in LSN chickens
at 40 days old (D0) than control chicken may indicate that LSN PM muscles have a high myogenic activity at that age. The 1.5-fold higher level of gDLK1 mRNA expression in MD chicken at D7 than control chicken may also indicate that gDLK1 expression is associated with the process of myofiber immaturation, resulting in myofiber hypertrophy in MD muscles at D20.

Questions arise about the role of gDLK1 expression at the late stages of regenerative myogenesis in normal, LSN, and MD chickens. Three possible roles of gDLK1 during muscle regeneration are hypothesized here. First, gDLK1 expression during myofiber formation may play a role in maintenance of satellite cells to aid self-renewal like the role of Pax7. Otherwise, it might be important for helping the fusion of satellite cells into myofibers. The first hypothetical role of gDLK1 was assumed from the following results: 1) a peak expression of DLK1 protein or gene was shown at late stage of regenerative myogenesis and primary chicken muscle cells, when myofibers are formed (Shin et al., 2009; Andersen et al., 2009); 2) DLK1 protein was localized in myofibers during embryonic and fetal muscle development in mammals (Georgiades et al., 2000; Floridon et al., 2000; White et al., 2008) and postnatal muscle regeneration after the injury (Andersen et al., 2009); 3) DLK1 sought to act as a regulator of cell fate determination (Laborda, 2000).

Secondly, it may play a role in the prevention of fibroblast differentiation into adipocytes in the scar area of injured muscles. Overexpression of DLK1 in transgenic mice inhibited adipogenesis while knockout of DLK1 in mice promote adipogenesis (Moon et al., 2002; Lee et al., 2003). In addition, the inhibitory role of DLK1 has been identified in differentiation of 3T3-L1 preadipocytes (Smas et al., 1993). Therefore,
DLK1 expression at the late stage during the formation of myofibers may prevent the adipogenesis of other stem cells (hematopoietic cells).

Finally, it may play a role in myofiber hypertrophy – increased muscle size and myofibers with centered nuclei in chickens. DLK1 has been implicated in muscular hypertrophy in callipyge sheep, DLK1-transgenic mice, and muscle genetic disorders of the human and mouse (paternal uniparental disomy 14 and pUPD12) (Cockett et al., 1996; Georgiades et al., 2000; Davis et al., 2004). In addition, DLK1 is considered as a candidate gene for high muscularity in chickens (Shin et al., 2009). All these results may support the third hypothetic role of gDLK1 expression at the late stages during regenerative myogenesis. In this regard, MD myofiber hypertrophy may be further associated with high expression of gDLK1 gene at the late stage of regenerative myogenesis, which was not associated with LSN myofiber hypertrophy.

In summary, the correlation of the gene regulation of MRFs and gDLK1 with the morphological process of myofiber formation was investigated during the muscle regeneration in chickens with muscular dystrophies (LSN and MD chickens). In the analysis of histology, delayed formation of myofibers, irregular shapes of myofibers, and myofiber hypertrophy with centered nuclei were shown in LSN and MD chickens at 20 days of muscle regeneration compared to control chickens, suggesting a delayed and impaired process of regenerative myogenesis in LSN and MD muscles. Associated with histological evidences, the higher expression of Pax7 and MyoD mRNA at D7 and delayed expression of myogenin mRNA during muscle regeneration in LSN chickens may be represented in LSN molecular muscle phenotypes. Whereas, the MD molecular muscle phenotypes in MD chickens include the low peak expression of MRF4, and a high
peak gene expression of myogenin at D4 and gDLK1 at D7 during muscle regeneration. Therefore, the association and comparison of histological and gene expression data in LSN and MD chickens provide an insight for a muscle dys-function caused from muscular dystrophy and muscle wasting. Here, although the genetic and molecular mechanisms undergoing these muscle disorders of chickens are still unclear, understanding morphological and molecular events during muscle regeneration of muscular dystrophic chickens will further aid to identify muscle specific genes responsible for muscle developmental disorders.

5.6 Acknowledgements

We are grateful to Michelle Milligan (The Ohio State University) for editing this manuscript.
Table 5.1. Primer sequences for quantitative real-time PCR.

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1 gDLK1 = chicken delta-like protein 1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; MyoD = Myogenic differentiation 1; Myogenin = Myogenic factor 4; Pax7 = Paired box gene 7; MRF4 = myogenic factor 6 (herculin; Myf6); F = forward; R = reverse. (Shin et al., 2008 and 2009)
Figure 5. Impaired and delayed muscle regeneration in LSN and MD chickens. Hematoxylin and eosin (H&E) staining of regenerative pectoralis major muscles of control, LSN, and MD chickens after the cold injury. The histological tissue sections were indicated for undamaged pectoralis muscle (left) at day 0 and damaged pectoralis muscle (right) 2, 4, 7, 10, or 20 days after muscle injury. N, nucleus; en, endomysia; p, perimysium; ep, epimysium. A black bar scale = 50 μm. The arrow indicates myofibers with central nuclei.
Figure 5. 1
Figure 5.2. Comparison of the mRNA expression level and pattern of Pax7 during muscle regeneration of control, LSN, and MD chickens.
(A) Quantitative real-time-PCR was performed to examine a significant difference of the gene expression level of Pax7 at D0, post-injury D2, and post-injury D20 among three types of chickens (n = 6–7, respectively). (B) Comparison of the pattern in the mRNA expression of Pax7 during muscle regeneration of three types of chickens (n = 5–7). GAPDH was used as a housekeeping gene. The bar represents mean ± SEM. The letters (a, b) represent a significant difference among groups at various time points by using one-way ANOVA at $P < 0.05$. 
Figure 5.3. Comparison of the mRNA expression level and pattern of MyoD during muscle regeneration of control, LSN, and MD chickens.

(A) Quantitative real time-PCR was performed to examine a significant difference of the gene expression level of MyoD at D0, post-injury D2, and post-injury D20 among three types of chickens (n = 6–7, respectively). (B) Comparison of the pattern in the mRNA expression of MyoD during muscle regeneration of three types of chickens (n = 5–7). GAPDH was used as a house keeping gene. The bar represents mean ± SEM. The letters (a, b) represent a significant difference among groups at various time points by using one-way ANOVA at P < 0.05.
Figure 5.4. Comparison of the mRNA expression level and pattern of myogenin during muscle regeneration of control, LSN, and MD chickens.

(A) Quantitative real time-PCR was performed to examine a significant difference of the gene expression level of myogenin at D0, post-injury D2, and post-injury D20 among three types of chickens (n = 6–7, respectively). (B) Comparison of the pattern in the mRNA expression of myogenin during muscle regeneration of three types of chickens (n = 5–7). GAPDH was used as a housekeeping gene. The bar represents mean ± SEM. The letters (a, b) represent a significant difference among groups at various time points by using one-way ANOVA at $P < 0.05$. 
Figure 5. Comparison of the mRNA expression level and pattern of MRF4 during muscle regeneration of control, LSN, and MD chickens. (A) Quantitative real-time PCR was performed to examine a significant difference of the gene expression level of MRF4 at D0, post-injury D2, and post-injury D20 among three types of chickens (n = 6–7, respectively). (B) Comparison of the pattern in the mRNA expression of MRF4 during muscle regeneration of three types of chickens (n = 5–7). GAPDH was used as a housekeeping gene. The bar represents mean ± SEM. The letters (a, b) represent a significant difference among groups at various time points by using one-way ANOVA at $P < 0.05$. 
Figure 5.6. The mRNA expression level and pattern of gDLK1 during muscle regeneration of control, LSN, and MD chickens.

The mRNA expression level and pattern of gDLK1 during muscle regeneration of control, LSN, and MD chickens. (A) Quantitative real-time PCR was performed to examine a significant difference of the gene expression level of gDLK1 at D0, post-injury D2, and post-injury D20 among three types of chickens (n = 6-7, respectively). (B) Comparison of the pattern in the mRNA expression of gDLK1 during muscle regeneration of three types of chickens (n = 5-7). GAPDH was used as a housekeeping gene. The bar represents mean ± SEM. The letters (a, b) represent a significant difference among groups at various time points by using one-way ANOVA at P < 0.05.
Chapter 6: Epilogue

To date, delta-like protein 1 (DLK1), a paternally expressed gene, has been studied in callipyge sheep, and paternal uniparental disomy (UPD) syndromes of humans and mice, implicating a role in the muscle hypertrophy of mammals. However, there has been no information about the correlation of DLK1 expression with muscle hypertrophy during development in poultry. Therefore, the ultimate goal of this dissertation was to determine the role of DLK1 expression in skeletal muscle development and regeneration in the avian species. In particular, the effort has been made to understand correlation of muscle fiber formation with chicken DLK1 (gDLK1) expression in different types of chickens, White leghorn layers vs. Broilers, and chickens with or without muscular dystrophies, low score normal (LSN) and muscular dystrophy (MD). This was done to prove our four hypotheses as follows: 1) chicken delta-like protein 1 (gDLK1) gene expression is associated with early embryonic muscle developmental stages during embryogenesis; 2) the high expression of gDLK1 mRNA correlates with a certain specific stage of primary cell and regenerative myogenesis in chickens; 3) the high expression of gDLK1 mRNA is implicated for muscle hypertrophy of chickens that have high muscularity; 4) muscle weakness chicken models express a distinct expression pattern and level of gDLK1 mRNA associated with morphological changes in muscles during regeneration.

In the first study, cloning and sequencing of chicken DLK1 (gDLK1) was done to
verify its mRNA expression in adipose and muscle tissues during the embryonic and post-natal development of chickens. In addition, the nucleotide and protein sequence comparisons of gDLK1 with that of mammals and PCR data were performed to investigate the alternative splicing of gDLK1 during tissue development. The first study demonstrated that 1) gDLK1 has a total 1,161 bp, putatively encoding 386 amino acids; 2) only the full length form (A form) of gDLK1 mRNA was expressed in various tissues and developmentally down-regulated in adipose and muscle tissues; and 3) gDLK1 mRNA was induced considerably at the embryonic stages of muscles. This first study provided initial information of the gDLK1 gene and its expression to begin understanding the role of gDLK1 during chicken muscle development.

In the second study, a comparison of the mRNA expressions of myogenic regulatory factors and gDLK1 between two different types of chickens, broilers (high muscularity) and layers (low muscularity), was examined. In addition, the gDLK1 gene expression during primary muscle cell differentiation and muscle regeneration after the cold injury to muscles were investigated. This study found that 1) the peak expression of gDLK1 mRNA was associated with the late stage of myogenesis, which was also confirmed in the study of muscle regeneration; 2) broilers with high muscle mass and increase body weight had a relatively higher expression of gDLK1 mRNA than layers within a developmental age. This comparative study provided insight on muscle hypertrophy and contributed toward understanding the role of gDLK1 in the development of chicken muscle tissue.

In the third study, a comparison of regenerative myogenesis after cold injury to the muscles of control and muscular dystrophic chickens, including LSN and MD, was
evaluated. In addition, the patterns and levels of myogenic regulatory factor (MRF) and gDLK1 mRNA expression were compared in three lines of chickens. Finally, the association of MRF and gDLK1 expression with muscle morphological changes during the regeneration of three chickens was discussed. The third study showed that, 1) LSN and MD chickens had impaired myofiber with irregular size and shape at 20 days after the muscle injury; 2) LSN chickens had a significantly delayed expression of myogenin mRNA and maintained expression of Pax7 and MyoD mRNA at post-injury day 7; 3) MD chickens had a significantly higher level of myogenin gene expression and a lower level of MRF4 gene expression at post-injury day 4 than control and LSN chickens; and 4) gDLK1 mRNA was highly induced, without any difference in pattern and level, at the late stages (post-injury day 7) of regenerative myogenesis of all three chickens.

Several conclusions can be made from the studies outlined in this dissertation. First, the overall conclusion from these studies suggests that DLK1 has an important role in myofiber formation during both embryonic and regenerative myogenesis. Data from the first study shows that the full length of gDLK1 mRNA expression was associated with myogenic activity during myogenesis. In particular, the high expression of the gDLK1 gene correlates with embryonic stages of myogenesis. In fact, data from the second study strongly support that the time-dependent expression of gDLK1 is associated with that of MRFs, suggesting a role in myogenesis at the late stages during differentiation of primary myoblasts and regenerative myogenesis. Broilers displaying greater muscle mass than layers expressed higher levels of gDLK1 mRNA associated with the higher expression of myogenic marker genes, suggesting that gDLK1 may be a selective marker for high muscle breeds in chickens. It may be associated with boosting
muscularity in chicken. When myotubes are newly formed during muscle regeneration, the gDLK1 gene expression indicates the high association of gDLK1 with myofibers. Moreover, data from the third study strongly supports the second finding that the peak expression of gDLK1 mRNA correlates with late myogenic marker gene expression during muscle regeneration in chickens, suggesting a role in myofiber formation. Therefore, three gDLK1 studies for proving our hypotheses proved that the gDLK1 expression was associated with embryonic stages of muscle development, strongly correlated with late stages of in vivo regenerative myogenesis and in vitro primary myoblast differentiation, and correlated with increased muscle mass only in a case of broiler and layer. However, there was no distinctive mRNA expression pattern of gDLK1 among control, LSN, and MD chickens during muscle regeneration. Although the function and mechanism of gDLK1 were not identified, the effort of cloning and sequencing gDLK1 cDLK1 has been made to compare gDLK1 gene and protein sequences with those of other mammals, as well as identifying alternative splicing and expression of gDLK1 during muscle development and regeneration.

The studies outlined in this dissertation provide important insight for the myogenic activity during myogenesis in poultry. These studies are important for four main reasons: 1) We verified that the chicken is an excellent model to study muscle development, growth, and regeneration; 2) Chickens with muscular dystrophies are represented as a model of human muscular dystrophies to identify the certain mechanisms undergoing muscle wasting and weakness; 3) The system of primary myoblast culture can be used to further confirm the in vivo molecular and cellular mechanisms and signaling studies during myogenesis; and 4) By using these chicken models, we can
continue to elucidate a role of chicken DLK1 in myogenesis, its associated myogenic program, and the involvement of myogenic signaling pathway during muscle development and regeneration.

However, some of general questions still arise and need to be answered in further research. First, it is wondered why gDLK1 does not have alternative splicing during transcription. Second, it is wondered whether the chicken muscle has a cleaved enzyme such as a tumor necrosis factor-α converting enzyme (TACE/ADAM17) discovered in mammals. Then, does it cleave gDLK1 protein, producing the soluble isoforms? Third, it is wondered why gDLK1 expression involves late stages of muscle regeneration. Fourth, it is wondered how gDLK1 acts on myofiber hypertrophy during muscle development, growth, and regeneration. Fifth, it is wondered what mechanism and which signaling pathway of gDLK1 are involved in muscle development. Finally, it is wondered whether gDLK1 can play a role in self-renewal of satellite cells like a role of Pax7. Answers to these questions may provide better insight and strengthen our knowledge for understanding an unknown role and mechanism of gDLK1 in muscles during the development, growth, and regeneration.
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Shin, J., S. Lim, J. D. Latshaw, and K. Lee. 2008. Cloning and expression of delta-like protein 1 messenger ribonucleic acid during development of adipose and muscle in


Appendix A: (Technical note) A Gene Delivery System in the Embryonic Cells of Avian Species Using a Human Adenoviral Vector

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ABSTRACT

Adenovirus (Ad) has been used in vivo and in vitro as a vector to carry a foreign gene for efficient gene delivery into various cell types and tissues of animals. The aim of the current study was to evaluate the adenovirus delivery system in the primary avian cells. Primary cells isolated from the embryonic pectoralis major muscles of the chicken and quail were cultured and incubated with human recombinant Ad serotype 5 (Ad5) containing sequences encoding either the green fluorescence protein (GFP) gene alone, as a tracking marker, or both GFP and murine 3-hydroxyisobutyryl-CoA hydrolase (mHIBCH) as a target gene. The fluorescent GFP images showed the successful delivery of a target gene using Ad5 in the primary avian cultured cells. In addition, immunostaining of the myosin heavy chain (MyHC) in these cells indicates that a large population of the cells is myogenic. Colocalization of GFP-positive cells with MyHC staining was mostly found in MyHC negative cells, indicating successful delivery of Ad5
into a large population of mononucleated cells. Furthermore, the current fluorescence study detected the dual expression of GFP and mHIBCH protein in GFP-positive cells. Finally, western blot analysis confirmed that the adenovirus-mediated expression of mHIBCH protein was specific in primary cultures of avian myogenic cells and that the mHIBCH protein expression was continued for 15 days (d) after infection in chicken primary cells. These data demonstrate that Ad5 is a feasible tool to express foreign genes in primary cultured cells of avian species, providing a new approach to study the function of genes of interest in muscle development and metabolism.

**Key words:** human recombinant adenovirus, gene delivery, avian primary cells
INTRODUCTION

Modification of gene expression and function has emerged as a promising approach to understand gene functions that may find an application in basic and applied animal research. Adenoviral (Ad) vectors have been developed as a method of the gene delivery system and are currently used in many human cell types and other animal species for gene therapy (Brunori et al., 2001; Jogler et al., 2006). Advantages of Ad vectors include relative ease of DNA manipulation, infection of dividing and non-dividing cells, higher levels of gene expression, rare incidence of integration into the chromosomes of host cells, the ability of the Ad vectors to accommodate larger gene inserts, and their extended duration of adenoviral-mediated gene expression (Young et al., 2006; Kochanek et al., 2001; Lee et al., 1995).

Among the 50 unique Ad serotypes, the human Ad serotype 5 (Ad5), used in the current study, has been the most extensively studied and is being studied for the treatment of cancer and genetic diseases in humans (Crystal et al., 1994; Addison et al., 1995). Bergelson et al. (1997) showed that Ad5 successfully enters into the host cells through the attachment of the Ad fiber knob protein to the Coxackie and Adenovirus Receptor (CAR) of host cells, a critical step for the infection of Ad5 in different species of animals. Several studies, including ours, have demonstrated that Ad5 efficiently delivers genes of interest into cells or tissues in the mammalian species (Jacobs et al., 1994; Lee et al., 1999; Fan et al., 2002; Quesada et al., 2007). However, the feasibility of the human Ad vector system has not been evaluated in primary cultured cells isolated from embryonic muscles of the chicken and quail. Therefore, the current studies tested whether Ad5 is an effective tool to express a foreign gene in the primary cultured muscle cells of avian
species.

MATERIALS AND METHODS

Avian primary cell cultures

Animals were sampled under guidelines of Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Ag Guide). Chicken and quail eggs were provided by The Ohio State University, OARDC Poultry Research Center, Wooster, Ohio. Embryonic pectoralis major muscle tissues of the chicken and quail were dissected from 11- and 10-d-old embryos, respectively. The isolation and culture of primary cells from pectoralis major muscles was performed as previously described (Shin et al., 2009). All primary avian cells were cultured 3–4 d for the studies of Ad5 transduction and dual expression of Ad5 containing a target gene or cultured 15 d for a duration study of Ad5 transduction. After the periods of time indicated in the Figure 1 and 2, the cells were collected and lysed for western blot, or fixed in 10% buffered formalin for immunohistochemical analysis.

Cloning of HIBCH gene, adenovirus construction, and transduction

The entire coding sequence for the mouse HIBCH (Genbank #, NM_146108) cDNA was amplified using a set of primers: mHIBCH-F (5’-TAG GCC ATG GGG CAG CCA TA-3’), and the reverse primer: mHIBCH-R (5’-GAT GTG TTG ACC TTT GCT CCA-3’). Cloning of mHIBCH by PCR, bacterial transformation, and the sequencing of cloned mHIBCH were performed as described previously in the method (Shin et al., 2008). The positive clones were sequenced to confirm the sequence fidelity of mHIBCH
gene by the Ohio State University Sequencing Core Facility using an Applied Biosystems 3730 DNA Analyzer.

To generate recombinant Ad5 containing the mHIBCH cDNA, a commercially available pAd-Track-CMV adenoviral vector (American Type Culture Collection, Manassas, VA) was used. Homologous recombination, amplification, and infection of adenoviruses were performed as previously described (He et al., 1998; Li et al., 2009). An Ad5 without mHIBCH was used as a control vector for the Ad5 containing both GFP and mHIBCH genes. The titer of infectious virus particles was assessed using the cytopathic effect (CPE) assay as previously described (Lee et al., 1999). The CPE value for Ad5 containing GFP (control) was measured as $1.6 \times 10^{11}$ pfu/mL; whereas, the CPE value for Ad5 containing GFP plus mHIBCH was $1.6 \times 10^{10}$ pfu/mL. Primary muscle cells ($1.02 \times 10^6$ cells/mL) obtained from chicken and quail embryos were infected with Ad5 containing GFP [multiplicity of infection (MOI): 7,000] for studying the feasibility of Ad5. For other studies on the dual expression of GFP and HIBCH protein, Ad5 containing GFP plus mHIBCH (MOI: 700) was used. The chicken primary muscle cells were incubated overnight with either Ad5 containing GFP alone (MOI: 1,000) or Ad5 containing GFP plus HIBCH (MOI: 1,000) and cultured for additional 15 d to determine duration of exogenous mHIBCH protein expression.

**Immunohistochemistry**

Cell fixation, washing, and immunostaining of primary and secondary antibodies were followed as previously described (Shin et al., 2009; Li et al., 2009). The primary antibodies were a pan sarcomeric MyHC [(NA4), 1:500; The Developmental Studies
Hybridoma Bank (DSHB), Iowa City, IA] and the anti-HIBCH rabbit serum (1:500, a generous gift from John W. Hawes at Miami University, Oxford, OH). A rhodamine conjugated goat anti-rabbit IgG (H+L) (1:200, Jackson ImmunoResearch, West Grove, PA) was used as a secondary antibody. An inverted fluorescent microscope was used for photo images at 100 X or 200 X magnifications (Olympus IX50, Olympus, Melville, NY).

**Western blot**

Cell lysis and Western blot analysis was performed as described previously (Li et al., 2009). A rabbit anti-HIBCH (1:15,000) and β-actin (1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used as primary antibodies. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit and donkey anti-goat antibodies at 1:3,000 (Cell Signaling Technology, Danvers, MA) were used as secondary antibodies. The proteins were detected with an enhanced chemiluminescence system (ECL plus, Amersham Biosciences, Piscataway, NJ) and visualized on Hyperfilm (Amersham).

**RESULTS AND DISCUSSION**

For the evaluation of the gene delivery using the human Ad5 system in these avian species, primary avian cells isolated from embryonic muscles of the chicken and quail were cultured and infected with Ad5 containing the GFP gene only. As shown in Figure A.1, the expression of the GFP protein in the primary cells was detected under a fluorescent microscope, showing a high possibility for Ad5 gene delivery in avian cells.
In addition, all primary cells seemed to undergo myogenesis spontaneously when considering the morphological evidence such as alignment or arrangement of primary cells and GFP-positive cells. To confirm whether these primary cultured cells are able to be formed into myotubes, immunostaining of myosin heavy chain (MyHC) protein, a well-known marker of muscle cell differentiation (Li and Johnson, 2006), was performed. In addition, the co-localization of MyHC protein with GFP expression was assessed to determine whether GFP-positive cells can be found in myotubes. The immunostaining of MyHC protein in these primary cells clearly showed the red immunofluorescence staining on myotubes, indicating that a large population of the cultured cells of the chicken and quail are primary myogenic cells. This also suggests that the delivery and expression of a target gene can be generated in the primary cultured cells of avian species by using this Ad5 vector system.

Nevertheless, a considerable population of GFP-positive cells was not overlapped with MyHC positive cells. These results suggest three possibilities: 1) existence of non-myogenic cells, such as fibroblasts that could be transduced by Ad5, 2) different activities of cytomegalovirus (CMV) promoter to drive GFP expression of Ad5 among cells at different stages of muscle development such as less CMV activities of Ad5 in myotubes, and 3) difficulty of myotube formation from GFP-positive myogenic cells. However, the fiber shaped-GFP cells and a small population of GFP-positive cells in the MyHC positive myotubes (Figure A.1) suggest that the GFP-positive cells may be myogenic cells that can undergo myogenesis.

To confirm further whether Ad5 preferentially transduces mononucleated cells over myotubes, adenoviral transduction was performed 3 d after initiation of
differentiation when there are two populations of cells, multinucleated myotubes and mono-nucleated cells. As a result, predominant expression of GFP was found in mono-nucleated cells (data not shown). This strongly suggests that the Ad5 was efficiently and preferentially transduced into mononucleated cells rather than cells associated with myotubes. In addition, this indicates that less expression of GFP in myotubes may not be due to different CMV promoter activities at the different stages of muscle cell differentiation.

In fact, successful transduction of genes of interest into target cells by Ad5 is dependent on the entry of the virus via the Coxackie and Adenovirus Receptor (CAR) on the membrane of host cells. Because CAR expression was barely detectable in murine mature myofibers and muscles, overexpression of CAR overcomes the difficulty of Ad5 transduction into muscle cells and muscles (Kimura et al., 2001) and mature skeletal muscle (Nalbantoglu et al., 2001). This emphasizes the importance of CAR expression in muscle cells. Although avian CAR expressions were not studied, the successful transduction of primary cultured avian myogenic cells by our recombinant Ad5 system suggests that the mononucleated cell membranes of the chicken and quail could contain compatible receptors that are permissive for human Ad5 entry, but the membranes of multinucleated myotubes could not.

The AdTrack-CMV shuttle vector used in the current study contains two expression cassettes, accommodating both a target gene (mHIBCH cDNA) and the GFP gene, a convenient tracer of infected cells. To test the dual expression system of the recombinant Ad5, primary avian cells transduced with the Ad5 containing both GFP and mHIBCH genes were stained with rhodamine-labeled antibodies specific to mHIBCH
proteins. In this experiment, the low titer of Ad5 was used to avoid too much transduction, so both non-infected and infected cells in the same images could be shown. The fluorescent images presented in Figure A.2A showed that only the GFP positive cells were stained with red fluorescence specific to mHIBCH protein, indicating that all avian primary myogenic cells infected with the Ad5 expressed both genes. Dual expression cassettes confirmed that Ad5 containing target genes successfully infected the target cells, and that GFP is a reliable tracer for cells expressing a target gene.

To further confirm the protein expression of mHIBCH from Ad5 in primary cultured cells of the chicken and quail, Ad5 with or without the mHIBCH gene was transduced, and western blot analysis was performed to detect the target protein mHIBCH. As shown in Figure 2B, protein bands corresponding to the expected molecular weight of mHIBCH (36 kDa) were detected in cells transduced with the Ad5 containing the mHIBCH gene. However, cells transduced with control Ad5 containing only the GFP gene did not show the presence of the mHIBCH protein. These data clearly demonstrate that both primary avian cultured cells from chickens and quails exhibited efficient Ad5-mediated mHIBCH gene transfer and expression.

In addition, we also examined the duration of the adenovirus-mediated expression of HIBCH protein in chicken primary myogenic cell culture (Figure A.2C). Exogenous mHIBCH protein expression was detected 2 d after transduction, increased to the maximum level at 7 d, and then was maintained for up to 15 d after transduction. Over the entire 15 d, the mHIBCH protein expression was not detected in the control cells infected with the control virus containing GFP. This indicates that mHIBCH antibodies did not detect endogenous avian HIBCH protein, but detected exogenous mHIBCH
protein derived from Ad5 containing the mHIBCH gene. Taken together, the data show that the duration of Ad vector-mediated gene expression in chicken myogenic cells can be sustained for at least 2 wk. The persistent expression of a target protein allows for the study of the long term effect of a target gene in embryonic muscle development and physiology.

In conclusion, the present study showed that human Ad5 has promise for transferring a foreign gene to primary myogenic cells of avian species, but the efficiency of its transduction into myotubes of all avian species was low during myogenesis. This system can be applied to future studies in elucidating the function of genes expressed in primary myogenic cells of avian species. In addition, studying and determining the function of genes of interest by using Ad5 will give new insight into avian muscle development and metabolism.

ACKNOWLEDGMENTS

This work was partially supported by an Ohio Agricultural Research and Development Center (K. Lee), and the HK Biotech Company (K. Lee; Jinju, South Korea). We are grateful to John W. Hawes (Miami University, Oxford, Ohio 45056) for providing HIBCH antibody and Michelle E. Milligan (The Ohio State University) for formatting and revising the manuscript.
LITERATURE CITED


FIGURE A. 1. Adenovirus-mediated green fluorescent protein (GFP) expression and myosin heavy chain (MyHC) protein expression in the primary cultured cells of the avian species, chicken and quail. (Bright field+GFP) The cells were isolated from embryonic muscles, infected overnight by 7,000 MOI of Ad5 containing GFP alone, and cultured for 3 d. The cells were fixed and imaged at 3 d after adenoviral transduction. (GFP, MyHC, and Merge) The primary cells were isolated from 11- and 10-d embryos and cultured with normal media (DMEM + 10% FBS) for 3-4 d. The green color indicates successful transfecion of Ad5 containing the GFP gene. The red fluorescence indicates the protein expression of MyHC in the primary avian myogenic cells. The white arrows indicate GFP positive/ MyHC positive myotubes. A scale of the white bar = 50 μm.
FIGURE A. 2. Dual expression of GFP and mHIBCH proteins and its duration in the primary cultured avian myogenic cells transfected with Ad5 containing GFP and mHIBCH genes.  (A) 2 d after adenoviral transduction, the fixed cells were immunostained with rhodamine conjugated rabbit anti-mHIBCH (Red color) to confirm the expression of mHIBCH protein. The green color indicates the expression of GFP. A scale of the white bar = 50 μm. (B) Western blot analysis was used to detect mHIBCH protein expression in the primary myogenic cells from the chicken and quail. The mHIBCH protein was expressed by Ad5 in the cells. The number 1 indicates that cells were infected with Ad5 containing only GFP; while number 2 indicates that cells were infected with Ad5 containing GFP plus HIBCH gene. (C) The duration of the protein expressions of control and mHIBCH transgene in primary cultured chicken myogenic cells. Cells were infected at 0 d with Ad5 containing the mHIBCH gene. Samples were collected at 1, 2, 4, 7, 10, and 15 d post transduction. β-Actin was used as a reference of protein amounts.
Appendix. B: Comparative analysis of fatty acid binding protein 4 promoters; Conservation of PPAR binding sites

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Abstract

The objective of this research was to further characterize the promoter regions of the bovine and porcine fatty acid binding protein 4 (FABP4) genes relative to that of other mammals. The DNA sequences of FABP4 promoters for the human, mouse, cattle, pig, and dog were obtained from the genomic database of the National Center for Biotechnology Information (NCBI) and also from sequencing of the bovine and porcine genomic DNA clones obtained by 5’ racing of genomic DNA. Sequence alignments of these FABP4 promoters using BLAST of the NCBI revealed 3 highly conserved promoter regions across the species. Two computational bioinformatics databases and literature identified the conserved transcription factor binding sites for C/EBPs, AP-1, and boxes of CAAT and TATA in the first conserved proximal promoter region, a direct repeat (DR) 1-type peroxisome proliferator-activated receptor (PPAR) responsive element (PPRE) in the second distal conserved region, and another PPRE element in the third distal conserved

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promoter region of FABP4 in all 5 mammals. Five new short interspersed repetitive elements (SINE) in the bovine FABP4 promoter and 2 new SINEs in the porcine were found, but these SINEs did not disrupt the 3 conserved regions, suggesting that important regulatory elements are maintained regardless of evolutionary pressure. In conclusion, the conserved cis-acting elements, especially 2 key adipocyte transcription factors such as C/EBP and PPARs, may contribute greatly to adipogenic regulation and adipose tissue-specific expression of FABP4 in these mammals. This helps to further characterize and decipher important cis-acting elements that are important for adipocyte development in adipose and muscle tissue.

**Key words:** adipose-specific, comparative analysis, fatty acid binding protein 4 (FABP4), promoter, peroxisome proliferator activated receptor (PPAR), short interspersed repetitive element (SINE)
INTRODUCTION

Fatty acid binding protein 4 (FABP4) belongs to a family of 9 currently identified as cytoplasmic fatty acid binding proteins. The role of FABP4 in fatty acid trafficking has been identified in animal fat tissues by showing involvement of FABP4 in efflux and influx of fatty acids in the adipocyte in response to anabolic and catabolic conditions, respectively (Bernlohr et al., 1984; Armstrong et al., 1990; Shen et al., 1999; Hertzel et al., 2006; Vural et al., 2008). Recent studies on the linkage analysis of fatness traits for FABP4 loci in pigs and cattle have suggested an association of animal marbling with FABP4, suggesting the FABP4 gene as a strong candidate gene for marbling among adipose-specific genes (Estellé et al., 2006; Wibowo et al., 2008). Understanding the regulatory role of FABP4 in lipid metabolism and regulation of FABP4 gene expression in domestic animals may contribute to improving product efficiency and quality such as fattening or marbling.

Tissue specific promoters generally contain cis-acting elements, where tissue specific transcription factors bind and regulate the tissue-specific expression of genes. There has previously been interest in finding fat-specific regulatory regions in both human and mouse FABP4 promoter (Ross et al., 1990; Rival et al., 2004). The 5.4 kb of FABP4 5’-flanking promoter is essential and sufficient to direct the adipose-specific expression of transgenes in mice (Ross et al., 1990; Graves et al., 1992) and to regulate differentiation-dependent gene expression in vitro (Christy et al., 1989; Tontonoz et al., 1994b). Furthermore, PPARγ2, a member of the PPAR family at 5.4 kb upstream of the promoter, has an important role in regulation of the differentiation- or adipose tissue-specific expression of the FABP4 gene (Graves et al., 1991, 1992; Tontonoz et al., 1994b).
However, the bovine and porcine FABP promoters have not yet been studied. Therefore, the aims of the current study were to clone, sequence, and align the FABP4 promoter region among mammals. In addition, the major conserved regions were identified, and the transcription binding protein elements in these regions were further related to regulation of FABP4 expression in these animals.

**MATERIALS AND METHODS**

**Genomic DNA Isolation**

Animal care and use procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. Porcine (Landrace; n = 1) and bovine (Angus; n = 1) genomic DNA was isolated from blood buffy coats. The white blood cell layers were harvested and digested with buffer containing 0.1 M MgCl₂, 0.02 M EDTA, 0.5% SDS, 0.01 M Tris, pH 8.0, and 0.1 mg/mL protease K at 55°C overnight. The digested cells were centrifuged at 12,000 rpm for 10 min. DNA from the supernatant was purified by double phenol/chloroform extractions and ethanol precipitation. The genomic DNA was dissolved in TE buffer. The amount of genomic DNA was quantified by spectrophotometric analysis and the quality of genomic DNA was checked for high molecular weight using 0.7% agarose gel electrophoresis.

**Genomic DNA Libraries**

To obtain the unknown sequences of the pig and bovine FABP4 promoter by 5’ PCR racing, a Universal GenomeWalker kit (Clontech Laboratory Inc., Mountain View,
CA) was used with methods previously described (Siebert, 1995). To generate pig and bovine genomic DNA libraries containing a known sequence of adapter, 2.5 µg of the genomic DNA was digested with 1 of 4 blunt-end restriction enzymes including *Dra*I, *EcoRV*, *Pvu*II, and *Sul*I. After purification of digested genomic DNA, each digested genomic DNA was ligated with 48 bp of adapter to provide sequence for the forward primer of PCR amplification of the gene of interest.

**PCR Amplification, Cloning, and Sequencing**

Five nano-grams of each genomic DNA library containing adapter were used as templates for PCR amplification of the pig and bovine FABP4 promoters. The sense adapter primer 1 and 2 was provided from the commercial kit (Clontech Laboratory Inc.) and the gene specific antisense primers for porcine and bovine FABP4 promoters are listed in Table B.1. The conditions for long distance PCR in an MJ Research Thermocycler were as follows: denaturation for 15 sec at 94°C, and annealing and elongation for 5 min at 68°C with 40 cycles. The primary PCR products from each library were separated in 0.8% agarose gels to visualize the bands. To confirm amplification of the primary PCR products from the specific genomic DNA, 1 µL of primary PCR products was used as the template for a nested PCR reaction using the adaptor primer 2 and gene specific primer 2. The conditions for the nested PCR were as follows: denaturation for 15 sec at 94°C, and annealing and elongation for 3 min at 66°C with 30 cycles. The resulting secondary PCR products were separated on 0.8% agarose gels. The specific bands with the expected sizes were excised from the gel and the DNA fragments in the gels were purified using a gel extraction kit (Qiagen, Valencia, CA).
The purified PCR products were cloned into a pCR 2.1 vector using a TOPO-TA cloning kit (Invitrogen). For the long PCR product cloned into the pCR 2.1 vector, the PCR products were subcloned into a pBluescript vector (Invitrogen) and sequenced at the Sequencing Core Facility in the Comprehensive Cancer Center at The Ohio State University.

**Bioinformatics and Sequence Analysis**

Bioinformatics and sequence analysis of mammal FABP4 promoters were performed as previously described (Deiuliis et al., 2008; Shin et al., 2008; Lee et al., 2009). The sequence scanner (v 1.0) was used to generate the chromatogram of cloned bovine and porcine FABP4 sequences [-1,189 ~ -4,519 bp (FJ884068), and -1 ~ -4,748 bp (FJ884069), individually] by using Applied Biosystems genetic analyzer instruments. In addition, long distal promoter sequences of FABP4 in the mouse (-1 ~ -5,545; NT 078380), human (-1 ~ -9,600; NT 008183), dog (-1 ~ -13,494; NW 876284), cattle (-1 ~ -1,188 and -4,520 ~ -10,730; NW 001493222), and pig (-4,749 ~ -9,582; NW 001886208) were obtained from the 5’- upstream region of individual mammal FABP4 shown in the Genome database at NCBI. Homology analysis was performed using align 2 sequences in a basic local alignment search tool (BLAST) at NCBI (Tatusova et al., 1999). The parameters for the BLASTN program were described as follows: reward for a match (1); penalty for a mismatch (-2); penalties for open gap (5) and extension gap (2); gap x dropoff 50 expect 10.0 ward size 11 filter. Next, alignment and comparison of the promoter sequences among the 5 mammals were carried out using the ClustalX (V 2.0) and GeneDoc™ software (V 2.7). To predict putative elements of transcription binding
factors on FABP4 promoters, 2 databases from TFSEARCH software (Searching
Transcription Factor Binding Sites, V 1.3;
http://www.cbrc.jp/research/db/TFSEARCH.html) and from TRANSFAC (v6.0) of
Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess)
were used.

**Western Blot**

Western blot analysis was performed as described previously (Li et al., 2009). Briefly, tissue protein samples (white adipose, heart, muscle, spleen, lung, liver, and kidney) were isolated from the mouse (FVB; n = 4; 70-day-old), cattle (Angus; n = 4; 450-600 kg), and pig (Landrace; n = 4; 100-120 kg). Frozen tissue samples were lysed in 60 mM Tris-HCl (pH 6.8), 1% SDS, and 1:100 dilution of proteinase inhibitor cocktail (Sigma, St. Louis, MO). The protein lysates were centrifuged at 12,000 × g for 3 min. Equal amounts of each protein lysate were mixed with Laemmli buffer (Bio-Rad, Hercules, CA), and boiled for 2 min at 95°C before a discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used. After the electrophoresis, proteins were transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ), and the membranes were incubated in blocking buffer [150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.05% Tween 20 (TBST) with 5% non-fat dry milk]. The membranes were probed with a goat antibody against FABP4 (R&D systems, Minneapolis, MN) and β-actin (Cell Signaling Technology, Danvers, MA) at 1:2,000, and with a mouse antibody against α-tubulin at 1:3,000 [The Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA] in TBST with 5% non-fat dry milk overnight at
4°C. Membranes were washed 6 times with TBST and incubated for 1 h with horseradish peroxidase (HRP)-conjugated donkey anti-goat at 1:3,000 (Bio-Rad, Hercules, CA), and horseradish peroxidase (HRP)-conjugated goat anti-mouse at 1:5,000 (Cell Signaling Technology, Danvers, MA). After washing 5 times in TBST, the proteins were detected with an enhanced chemiluminescence system (ECL plus, GE Healthcare Life Sciences, Piscataway, NJ) and visualized on Hyperfilm (GE Healthcare Life Sciences, Piscataway, NJ).

**Total RNA Isolation and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)**

Adipose tissue, heart, muscle, spleen, lung, liver, and kidney of the mouse (FVB; n = 4), cattle (Angus; n = 4), and pig (Landrace; n = 4) were harvested after slaughter of animals at the Ohio State University’s Meat Science Laboratory located in the Department of Animal Sciences Building, Columbus, OH. Tissues were immediately snap frozen in liquid nitrogen and homogenized using a Tissuemiser homogenizer (Fisher Scientific, Pittsburgh, PA). Total RNA isolation and qRT-PCR were performed as previously described (Shin et al., 2008). Total RNA from the tissue was isolated using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions, and RNA quality was assessed by electrophoresis (1% agarose gel). Reverse transcription (RT)-PCR was performed using 1 μg of total RNA and moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription conditions for cDNA amplification were 65°C for 5 min, 37°C for 52 min, and 70°C for 15 min. Primer sequences used for the qRT-PCR are shown in Table B.1. The qRT-PCR using
murine, porcine, and bovine PPARγ-F and PPARγ-R primer sets was performed to measure the relative levels of PPARγ gene expression in various tissues. The murine, porcine, and bovine cyclophilin (CYC) genes served as housekeeping genes. All primers for qRT-PCR that span the genomic introns for qRT-PCR could avoid amplification of contaminated genomic DNA during the PCR reactions. The quantitative real time-PCR was performed using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), and SYBR green was used as the detection dye. Conditions for qRT-PCR were 95°C for 10 min and 40 cycles of 94°C for 15 sec, 60°C for 40 sec, 72°C for 30 sec, and 82°C for 32 sec. Quantitative real-time PCR was performed in duplicate on 25 μL reactions using an ABI 7300 Real-Time PCR Instrument (Applied Biosystems). The relative level of target gene expression, as determined by ABI software, was calculated using the comparative $2^{-\Delta \Delta Ct}$ method for relative quantification (Livak and Schmittgen, 2001).

Statistics

Results are presented as mean ± SEM. Comparisons of PPARγ mRNA expression among various tissues of the mouse, cattle, and pig were performed using one-way analysis of variance (ANOVA) followed by the Tukey’s test at $P \leq 0.05$. All statistical analyses were performed using Minitab software (version 15.0).

RESULTS AND DISCUSSION

In general, the lipids in adipocytes originate from either dietary fat or de novo synthesis of fatty acids in adipose and liver depending on the species. The hydrophobic fatty acids inside of adipocytes are chaperoned by FABP4 (Coe and Bernlohr, 1998).
Adipose tissue-specific gene expression of FABP4 was demonstrated in mammalian species (Lee et al., 2003; Stejskal et al., 2006; Li et al., 2007; Soliman et al., 2007). However, the tissue-specific expression of porcine and bovine FABP4 at the protein level has not been reported. In the current study, Western blot analysis of FABP4 in adipose, heart, muscle, spleen, lung, liver, and kidney, showed the predominant expression of FABP4 protein in the adipose tissue of mice, cattle, and pigs (Figure B.1). These results led us to perform comparative analysis of FABP4 promoters to identify conserved regulatory elements in the promoters regulating expression of FABP4 in these species. Generally, the highly conserved nucleotide sequences in promoter regions are more likely to be functionally important to regulate transcription of a gene in a similar manner in different species (Dieterich et al., 2005).

The expression of FABP4 gene is extremely low in preadipocytes, but dramatically induced in adipocytes during differentiation in the mouse and pig (Chmurzyńska, 2006; Li et al., 2007, 2009; Deiuliis et al., 2008), which is referred to as “adipocyte-specific”. In addition, FABP4 mRNA is exclusively expressed in adipose tissues of most mammalian species including the human, rat, mouse, and pig (Bernlohr et al., 1985; Stejskal et al., 2006; Li et al., 2007), which is referred to as “adipose tissue-specific”. For these reasons, the FABP4 gene has been widely used as an adipose and adipocyte marker gene in studies on obesity and adipocyte development in the human, mouse, and food animals (Chavey et al., 2006; Li et al., 2007 and 2009).

For the present comparative analysis study, the FABP4 promoter sequences for human, mouse, cattle, pig, and dog available in the NCBI were downloaded. However, the relatively large sequences for bovine and porcine FABP4 promoters comparable to
the -5.4 kb of mouse FABP4 promoter were not available in the NCBI genome database. Therefore, 5’ racing of genomic DNA for bovine and porcine was performed and several clones were obtained, covering the nucleotide sequences (total 3,331 bp located in -4,519 bp to -1,189 bp) of bovine 5’-flanking FABP4 promoter and sequences (total 4,748 bp located in -4,748 bp to -1 bp) of porcine FABP4 promoter (Figure B.2A). These sequences were reported in Genbank [bovine (FJ884068) and porcine (FJ884069)]. Recently, the NCBI updated and included the sequences for bovine and porcine FABP4 promoters. The promoter sequences that we obtained by the 5’ racing are almost identical to the sequences reported in the NCBI (Figure B.2). In addition, our promoter sequences could fill the gap of 100 bp of missing nucleotide sequences (NNNN…..NNNN) in porcine FABP4 promoter regions at -830 to -731 and 355bp of missing sequences of bovine FABP4 promoter regions at -1,730 to -1,376 that were reported in GenBank [bovine (FJ884068) and porcine (FJ884069)]. As shown in Figure B.3C, the sequences of FABP4 promoters for 5 mammalian species were available to align them using a basic local alignment search tool (BLAST) at NCBI.

To perform alignment of 5 FABP4 promoter sequences and identify highly homologous regions of the FABP4 promoter among mammalian species, mouse sequences of FABP4 promoters were chosen to compare with those of the human, bovine, pig, and dog, individually, because the mouse FABP4 promoter was previously studied. In addition, all possible cross comparisons with all 5 species were made to locate evolutionarily conserved regions of the promoters. As shown in Figure B.2B, nucleotide alignment of all FABP4 promoters revealed a total of 5 conserved promoter regions. Among them, the 2nd region was specifically conserved between mouse and cattle, and
the 4th region was conserved among mouse, pig, and dog. However, the 1st, 3rd, and 5th regions were conserved in all species, indicating that these conserved regions may contain important cis-regulatory elements regulating FABP4 gene expression in animals.

To find the important regulatory elements among the 5 species, 2 computational bioinformatics databases (TFSEARCH and TRANSFAC software) were used. Studies relative to FABP4 and its promoter were also widely reviewed. As shown in Figure B.3, 5 major transcription factor binding elements, CCAAT box/enhancer-binding protein (C/EBP), activation protein 1 (AP1), fat specific element 1 (FSE1), CAAT box, and TATA box, were found in the 1st conserved region of the FABP4 promoters. CCAAT box/enhancer-binding protein α gene expression is positively associated with adipocyte cell differentiation in mice (Christy et al., 1989), cattle (Ohsaki et al., 2007), pigs (Lee et al., 1998), and humans (Chiche et al., 2009). C/EBPs are master transcription factors that not only play a regulatory role in differentiation of preadipocytes into adipocytes (Birkenmeier et al., 1989), but also induce adipogenic genes, including FABP4 (MacDougald and Lane, 1995). Mouse models with knockout of the C/EBPα gene and overexpression of the dominant negative form of C/EBPα in adipose tissue showed a complete loss of fat accumulation in adipose tissue, further supporting the importance of C/EBPα in adipose development (Wang et al., 1995; Moitra et al., 1998).

In humans, a single nucleotide polymorphism (SNP) was found and reported at -89 (T to C) of the FABP4 promoter region (Tuncman et al., 2006), resulting in reduced binding activity of C/EBPα to the element of the promoter, and, consequently, reduced transcription activity from the FABP4 promoter. Interestingly, this C/EBPα binding site (CCAAAGTTGAGAAATT), located between 140 and 155 bp of the 1st conserved
region, is 100% homologous in all 5 species, further supporting the importance of conservation of the C/EBPα binding site for the regulation of FABP4 expression. Indeed, a mutation in the C/EBP binding site of the murine FABP promoter region decreased CAT reporter gene expression during preadipocyte differentiation (Herrera et al., 1989). However, the proximal FABP4 promoter (-168 bp to 21 bp) containing the C/EBP binding site showed ubiquitous expression of the CAT reporter in transgenic mice (Ross et al., 1990), indicating that the C/EBP element is not sufficient for adipose-specific expression of FABP4. In support of this, a search of the NCBI’s GEO Profiles database for the human and mouse (http://www.ncbi.nlm.nih.gov/geo/ GEO accession #: GDS181 for human and GDS182 for mouse) revealed that C/EBPα is expressed in many tissues including adipose tissue, liver, intestine, lung, and muscle (Su et al., 2002). Taken together, conservation of the C/EBP element in FABP4 promoters has an important role in the up-regulation of FABP4 expression during adipocyte differentiation, but contributes less to adipose tissue-specific expression of FABP4.

The putative cis-acting elements in the 3rd conserved distal promoter region were two peroxisome proliferator-activated receptor binding elements (PPRE), C/EBP binding element, NF-CLE0a, HSF, and POU1F1a (Figure B.4). Among them, it is worthy to focus on and discuss the conserved PPRE sites for predicting a role of FABP4 promoters for the pig, cattle, and dog, because 1) PPRE is known as an important cis-acting element of the mouse and human FABP4 for its tissue specific expression, and 2) PPARγ promotes expression of adipogenic genes that are involved in lipid metabolism in their adipose tissues. A meta-analysis of PPAR response element (PPRE) sites (Lemay and Hwang, 2006; Heinäniemi et al., 2007) showed a consensus sequence
(AGGTCA\textsubscript{AGGTCA}) of DR1 (direct repeat 1)-type PPRE. In the present study, the consensus sequence of the DR1-type PPRE was highly conserved in all 5 mammalian species. Compared to the mouse sequences, 83% homology was shown in the human sequences, 92% homology was shown in cattle; and 100% homology was shown in the pig and dog. Furthermore, many other adipogenic genes, such as perilipin (Shimizu et al., 2006), fatty acid transporter proteins (FATPs) (Frohnert et al., 1999), human peroxisomal fatty acyl coenzyme A oxidase (ACOX1) (Varanasi et al., 1996), lipoprotein lipase (Schoonjans et al., 1996), phosphoenolpyruvate carboxykinase (Tontonoz et al., 1995), acyl-CoA synthetase (Schoonjans et al., 1995), and malic enzyme (Castelein et al., 1994), contain the consensus sequence of DR1-type PPRE. This strongly suggests that the consensus PPRE may be one of the key elements for adipose tissue-specific expression of FABP4 in the pig, cattle, and dog.

Analysis of the 5\textsuperscript{th} distal conserved promoter region of FABP4 revealed several conserved \textit{cis}-regulatory elements including ARE6, ARE7, TCF-2a, ARE2, GATA-1, and ARE4 (Figure B.5). Among them, ARE7, a PPAR binding site, is an important \textit{cis}-regulatory element for adipose tissue-specific expression of murine FABP4 (Graves et al., 1992; Tontonoz et al., 1994a and b). Here, conservation of the ARE7 (TGA\textsc{ACT}CTG\textsc{ATCC}) site indicates that the PPRE sites may contribute to the adipose tissue-specific regulation of FABP4 in the human, pig, cattle, and dog.

To investigate the relation of PPAR\textgamma expression with predominant expression of FABP4 in fat tissue, qRT-PCR for PPAR\textgamma was performed in various tissues of mice, cattle, and pigs (Figure B.6). As expected, the gene expression of PPAR\textgamma was significantly predominant in fat tissue of the mouse (P<0.05) when compared with the level of PPAR\textgamma.
gene expression in other tissues. Likewise, the porcine and bovine PPARγ mRNAs were abundantly expressed in white adipose tissues \((P < 0.05)\). This suggests that porcine and bovine PPARγ may contribute to adipose tissue-specific expression of FABP4 in pigs and cattle. The positive association of FABP4 expression with PPARγ expression during adipocyte differentiation in pigs (Ding et al., 1999; Samulin et al., 2008) and cattle (Soliman et al., 2007) and induction of FABP4 expression by treatments with PPARγ agonists (Hausman et al., 2008) further supported the involvement of PPARγ in regulation of FABP4 expression.

In humans, mice, pigs and cattle, PPARγ is produced in 2 isoforms, γ1 and γ2, by alternative promoter usage and alternative splicing (Tontonoz et al., 1994a, b; Elbrecht et al., 1996; Sundvold et al., 1997; Houseknecht et al., 1998). Quantitative RT-PCR analysis for total PPARγ mRNA expression showed considerably higher levels of PPARγ expression in the spleen and lung in pigs and cattle, which is in agreement with the previous findings that the gene expression of PPARγ is abundant in the adipose tissue, spleen, and lung tissues of pigs and cattle (Sundvold et al., 1997; Houseknecht et al., 1998). However, PPARγ2 is selectively expressed in adipose tissue, but very low in the spleen and lung in which PPARγ1 is a dominant form in pigs and cattle (Sundvold et al., 1997; Houseknecht et al., 1998). Studies on the functional role of the PPARγ isoforms revealed that knockdown of both PPARγ1 and γ2 expression in 3T3-L1 preadipocytes completely inhibited adipogenesis and FABP4 expression (Ren et al., 2002), suggesting an important role of PPARγ in induction of FABP4 expression. In addition, exogenously expressed PPARγ2 in these cells restored adipogenesis, but not by PPARγ1, indicating an
adipogenic capacity of PPARγ2. Furthermore, the capacity of transcription activation of PPARγ1 is much lower than that of PPARγ2 (Werman et al., 1997). Taken together, the adipose-tissue specific protein expression of FABP4 in pigs and cattle (Figure B.1), even though total PPARγ mRNA expression was relatively high in the spleen and lung (Figures B.5A and B. 5B), may be largely due to the predominant expression of PPARγ2 in adipose tissue. Other possible explanations include 1) different modulation of chromatin structures to expose the promoter region of FABP4, 2) the cooperative regulation of the FABP4 promoter by factors binding to PPARγ such as a co-activator (PGC-1α) (Hondares et al., 2006) and a repression factor (COUP-TF1) (Brodie et al., 1996; Tsai and Tsai, 1997; Brandebourg and Hu, 2005), and 3) different levels of PPAR ligands among tissues.

Previous studies using transgenic mice, expressing the CAT reporter gene under the control of different sizes of mouse FABP4 promoter, found that -5.4 kb of the FABP promoter, including ARE6 and ARE7 at the 5th conserved region, was sufficient for adipose tissue-specific expression of the FABP4 gene. In support of this, adipose tissue-specific expression of target genes in numerous transgenic mice models was achieved using -5.4 kb of mouse FABP promoter (Lee et al., 2003; Takasawa et al., 2008; Wang et al., 2008). This suggests that murine ARE6 and ARE7 could be the main cis-acting elements to regulate the predominant adipose tissue-specific expression of FABP4. In addition, the PPRE site at the 3rd conserved region in human increased activity of luciferase reporter gene by treatments of PPAR agonists such as rosiglitazone (Wurch et al., 2002; Rival et al., 2004). This indicates that the consensus DR1 type-PPRE of the human FABP4 promoter that is located in the 3rd conserved distal area (-5.2~-5.1 kb) may
play an additional role in adipose tissue-specific expression of FABP4. The positive associated expression of FABP4 with PPAR\(\gamma\) during adipocyte differentiation in the pig (Ding et al., 1999; Li et al., 2007; Samulin et al., 2008) and cattle (Soliman et al., 2007; Taniguchi et al., 2008) has been identified. In this regard, 2 conserved PPAR binding sites, the ARE6 and ARE7 elements at the 5\(^{th}\) conserved region and DR1-type PPRE at the 3\(^{rd}\) conserved region, may be important to regulate the FABP4 gene in these species, although it has not been determined which site of the PPREs is required for adipose tissue specific expression of porcine and bovine FABP4.

Alignment of the bovine and porcine FABP4 promoters with the FABP4 promoters of other species revealed not only the conserved homologous area, but also a diverse area including possible insertions of sequences in the promoter area. As shown in Figure B.2C, a BLAST search of these sequences against the bovine genome database at the NCBI revealed that 5 different regions, located at -2,724 to -1,666 bp (total 1,058bp), -4,578 to -4056 bp (521 bp), -5,807 to -5,388 bp (420 bp), -6,196 to -5,908 bp (378 bp), and -7,929 to -7,725 bp (205 bp), are repetitively found throughout the entire bovine genome. The 1\(^{st}\) insertion (1,058 bp, located at -2,724 to -1,666 bp of the bovine FABP4 promoter region) was shown to distribute 3,308 blast hits on the query sequence in the entire bovine genome. Likewise, the 2\(^{nd}\) insertion was shown to distribute 14,335 blast hits; the 3\(^{rd}\) was 14,838 blast hits; the 4\(^{th}\) was 14,968 blast hits; finally, the 5\(^{th}\) was 11,876 blast hits. In the porcine FABP4 promoter, 2 new repetitive sequences were found at -1,542 to -1,314 bp (285 bp with 2,243 blast hits) and -4,678 to -3,437 bp (1,242 bp with 12,563 blast hits) (Figure 2C). In general, a considerable portion of animal genomes contains long interspersed repetitive elements (LINEs) (Girardot et al., 2006) and short
interspersed repetitive elements (SINEs) (DeCerbo and Carmichael, 2005). Because SINEs should have at least $10^4$ to $10^5$ copies per genome (Sheikh et al., 2002), the 7 regions of repeated DNA sequences in bovine and pig FABP4 promoters can be considered as unreported new SINEs. The insertion of these SINEs resulted in relatively long promoters as shown by the location of the conserved areas at the far upstream region of the bovine and porcine FABP4 promoters, compared to the mouse FABP4 promoter. Like Alu sequences in the human, these repetitive sequences may stabilize genome sequences or inversely allow recombination of genomes for evolutionary diversity. In addition, a BLAST search of these inserted sequences against genome databases of other species could not find homologous sequences, indicating that these sequences are unique to the bovine and porcine genomes. However, insertions of these SINEs did not disrupt the conserved homologous sequences in the FABP4 promoters, suggesting conservation of important regulatory cis- elements for the regulation of FABP4 expression across the animal species examined.

In the current study, we cloned porcine and bovine sequences of FABP4 5’-flanking promoter regions, finding missing sequences shown in the genome database of BLAST at NCBI. The comparative analysis of FABP4 promoters of all 5 mammals examined revealed 3 highly conserved promoter regions (1st proximal, 3rd distal, and 5th distal). Each conserved promoter region contains differentiation-regulatory elements, hormone-regulatory elements, and fat-specific regulatory elements. Among them, a conserved PPAR binding element such as DR1 type PPRE in the 3rd distal promoter region of FABP4 that may govern fat-specific expression of FABP4 was unexpectedly found across the 5 mammals. In addition, ARE7, a necessary PPAR binding element for
fat-specific expression of FABP4 in the mouse, was also highly conserved in the 5th distal
promoter region of FABP4 among the 5 species. This suggests that PPAR binding sites
may also play an important role in adipose-specific expression in these species. This
study provides a new bioinformatics approach for comparative analysis of promoters and
to identify putative conserved transcription factor binding sites.

ACKNOWLEDGMENTS

This work was supported by an Ohio Agricultural Research and Development
Center grant to K. Lee. We are grateful to Michelle Milligan (The Ohio State University)
for editing this manuscript.
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TABLE LEGEND

**TABLE B. 1.** Primer sequences for quantitative real-time PCR and racing of 5'-flanking sequences of bovine and porcine fatty acid binding promoter 4 (FABP4).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
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<tr>
<td>mCYC-F</td>
<td>5'-GGT GGA GAGCACCAA GAC AGA-3’</td>
<td>Li et al., 2009</td>
</tr>
<tr>
<td>mCYC-R</td>
<td>5'-GCC GGA GTC GAC AAT GAT G-3’</td>
<td></td>
</tr>
<tr>
<td>mPPARγ-F</td>
<td>5'-CCG AAG AAC CAT CCG ATT GAA-3’</td>
<td></td>
</tr>
<tr>
<td>mPPARγ-R</td>
<td>5'-GCC CAA ACC TGA TGG CAT T-3’</td>
<td></td>
</tr>
<tr>
<td>bCYC-F</td>
<td>5'-GTG GTC ATC GGT CTC TTT GG-3’</td>
<td></td>
</tr>
<tr>
<td>bCYC-R</td>
<td>5'-CAC CGT AGA TGC TCT TAC CTC-3’</td>
<td></td>
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<tr>
<td>bPPARγ-F</td>
<td>5'-CTG TGA AGT TCA ACG CAC TGG A-3’</td>
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<tr>
<td>bPPARγ-R</td>
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<tr>
<td>pCYC-F</td>
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<td>Li et al., 2007</td>
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<tr>
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</tr>
<tr>
<td>pPPARγ-F</td>
<td>5'-TAG ATG ACA GCG ACC TGG CGA-3’</td>
<td>Li et al., 2007</td>
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<tr>
<td>pPPARγ-R</td>
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<td>AP-F2</td>
<td>5'-ACT ATA GGG CAC GCG TGG T-3’</td>
<td>Clontech.</td>
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</table>

1m = mouse; b = bovine; p = porcine; r = racing; AP = commercial adaptor primer; CYC = cyclophilin; PPAR = peroxisome proliferator-activated receptor; F = forward; R = reverse.
Figure B. 1. Western blot analysis represents tissue distribution of fatty acid binding protein 4 (FABP4) protein expressions for mouse, cattle, and pig. β-Actin was used as reference for FABP4 protein in the mouse, whereas α-tublin was used as reference for FABP4 protein expressions of the cattle and pig.
Cloning of porcine and bovine nucleotides of FABP4 promoters, alignment and schematic comparison of the sequence homology of mouse FABP4 promoter with other mammal FABP4 promoter regions. (A) Cloning of porcine and bovine nucleotides of FABP4 promoters by 5’ racing of individual genomic DNA. Restriction enzymes are DraI, EcoRV, DraI, and StuI. The arrows indicate the 5’ racing direction for cloning of bovine and porcine FABP4 promoters in 5’ flanking regions. Individual primer for cloning of the bovine and porcine promoter regions was shown in Table 1. (B) Murine FABP4 promoter sequence (-1 ~ 5,545) was aligned with that of the human (-1 ~ 9,592), cow (-1 ~ 10,609), pig (-1 ~ 9,582), and dog (-1 ~ 13,494). The small boxes indicate conserved regions of FABP4 promoter among species. (C) Schematic representation of highly conserved FABP4 promoter regions between the mouse and human, cow, dog, and pig, individually. Seven numbered boxes indicate putative short interspersed nucleotide elements (SINEs) in the promoter regions of the cattle and pig.
Figure B. 2
Figure B. 3. Sequences and muti-alignment of the 1st conserved proximal FABP4 promoters of 5 mammals, and putative or reported transcription binding sites on their FABP4 promoters. The abbreviation under dot empty boxes indicates myocyte-specific enhancer-binding factor DNA binding site, MEF-2; CCATT/enhancer-binding protein alpha, beta, or delta, C/EBPa, C/EBPb, or C/EBPδ; activation protein-1 (AP-1); fat specific enhancer 1 (FSE1); CAAT box; TATA box. The black arrow under ATG codons indicates all start sites of transcription of FABP4 in 5 mammals.
Figure B. 3
Figure B. 4. Sequences and muti-alignment of the 3rd distal conserved FABP4 promoters of 5 mammals, and putative or reported transcription factor binding sites on their FABP4 promoters. The abbreviation under dot empty boxes indicates the protein binding sites of nuclear factor-conserved lympokine element 0a, NF-CLE0a; heat shock factor, HSF; pituitary-specific transcription factor 1a, POU1F1a (Pit-1a); C/EBPα. Direct repeat 1 (DR1)-type peroxisome proliferator-activated receptor (PPAR) binding element (PPRE). The black arrow under DR1-PPRE indicates a binding direction of their binding proteins.
Figure B. 5. Sequences and muti-alignment of the 5th distal conserved FABP4 promoters of five mammals, and putative or reported transcription binding sites on their FABP4 promoters. The abbreviation under dot empty boxes indicates the transcription factor binding sites of a T (thymus) cell-specific factor-2 alpha, TCF-2a (Ho et al., 1990) and globin transcription factor 1, GATA-1. A fat-specific enhancer bearing 2 response elements was shown as ARE6, ARE2, and ARE7. The black arrow under ARE sites indicates a binding direction of their binding proteins.
Figure B. 6. The gene expression patterns of peroxisome proliferator-activated receptor gamma (PPARγ) in various tissues of mice (FVB), cattle (Angus), and pigs (Landrace). (A) Predominant mRNA expression of PPARγ in mouse adipose tissue (n = 4). (B) Abundant mRNA expression of PPARγ in bovine adipose, spleen, and lung tissues (n = 4). (C) Abundant mRNA expression of PPARγ in porcine adipose, spleen, and lung tissues (n = 4). Quantitative real-time PCR was performed to show the relative gene expression. Cyclophilin was used as a reference for PPARγ gene expression in mice, cattle, and pigs. The bar indicates relative values of gene expression, representing mean and SEM. A letter above each bar indicates statistical significance of the gene expression among various tissues using one-way ANOVA. White adipose tissue, WF; Heart, Hrt; Muscle, Mus; Spleen, Spl; Lung, Lu; Liver, Li; Kidney, Kid.