

REDUCTIONS IN EXPRESSION OF GROWTH REGULATING GENES ARE OBSERVED
IN SKELETAL MUSCLE AND ORGANS WITH AGE

BY

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THESIS

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ABSTRACT

A set of growth regulating genes has been identified that decline in expression in organs with age. This downregulation is thought to coordinate organ growth cessation observed as humans and animals mature. The objective of this study was to observe the expression of 7 of these growth regulating genes (*Ezh2*, *Gpc3*, *Mdk*, *Mest*, *Mycn*, *Peg3*, and *Plagl1*) in male and female skeletal muscle, heart, and liver with age. It was hypothesized that the expression of these genes would differ not only between sexes, but also between tissues. Female and male C57BL/6J mice were sacrificed at 1, 7, 21, 35, and 49 days of age. Samples collected consisted of heart and liver from all mice, all muscles from both hind limbs of 1- and 7-day-old mice, and specific muscles (bicep femoris, gastrocnemius, tibialis anterior, and triceps brachii) from 21-, 35-, and 49-day-old mice. Expression was determined by quantitative real-time PCR, normalized to *Rn18s*, standardized to a single 1-day-old male liver sample present on each plate, and compared to the average of 1-day-old male expression for each tissue. Data were analyzed using the Proc Mixed procedure in SAS as repeated measures with significant differences ($P < 0.05$) determined by a Bonferroni-adjusted pdiff. Orthogonal contrasts were used to differentiate between linear, quadratic, and cubic patterns of expression among tissues. In general, expression declined from 1 or 7 days of age when compared to 49 days of age for all the genes in all the tissues except for expression of *Ezh2* and *Mycn*, which was not altered by age in liver. Some differences in expression were observed between sexes with the majority of the differences occurring at 49 days. Expression was usually increased in females compared with males at this time point. Occasionally, males had greater expression than females; this was observed in liver at 1 day of age for *Gpc3*, *Mest*, and *Plagl1* and muscle at 35 days of age for *Mest*. There were some similarities in the patterns of gene expression between muscle and heart or liver, but, there was not a single gene that had the same pattern of expression in all 3 tissues. Overall, these data

suggest that the downregulation of these growth regulating genes with age might play a role in the coordinated cessation of muscle growth similar to organ growth with limited differences between sexes.

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TABLE OF CONTENTS

Chapter	Page
I. REVIEW OF LITERATURE	1
Introduction.....	1
Downregulation of Growth Regulating Genes in Organs with Age.....	1
Growth Regulating Gene Functions.....	4
Skeletal Muscle Growth.....	10
Objective.....	12
References.....	13
II. REDUCTIONS IN EXPRESSION OF GROWTH REGULATING GENES ARE OBSERVED IN SKELETAL MUSCLE AND ORGANS WITH AGE	21
Abstract.....	21
Introduction.....	22
Materials and Methods.....	24
Results.....	26
Discussion.....	30
References.....	35
Tables and Figures	39
APPENDIX	46

CHAPTER I

REVIEW OF LITERATURE

Introduction

As humans and animals age, growth slows and eventually stops. Organs grow until they reach mature size, however, little is known about how growth cessation is regulated and coordinated. An understanding of the regulation of growth decline may be useful to alleviate and treat diseases such as cancer and undergrowth syndromes. Previously, a set of genes was identified that may be involved in the coordinated cessation of organ growth (Finkielstain et al., 2009; Lui et al., 2008; Lui et al., 2010). Though these genes have been well characterized in organs, the expression patterns of these genes in growing muscle are unexplored. This discussion will focus on the previous research examining the downregulation of 7 of these genes (*Ezh2*, *Gpc3*, *Mdk*, *Mest*, *Mycn*, *Peg3*, and *Plagl1*) in organs with age and their overall functions along with specific functions identified in muscle. General muscle growth as well as a general comparison of muscle growth with that of organs will also be discussed.

Downregulation of Growth Regulating Genes in Organs with Age

A microarray analysis comparing 1 and 4 week old C57BL/6 male mouse lung, kidney, and heart tissue revealed over 1200 genes that were downregulated in all 3 organs (Finkielstain et al., 2009). Attention was focused on 5 of those genes (*Igf2*, *Igf2bp3*, *Mest*, *Peg3*, and *Sox4*) which had been previously identified as regulating growth (Curley et al., 2005; DeChiara et al., 1990; Lefebvre et al., 1998; Liao et al., 2005; Ya et al., 1998). *Igf2bp3* and *Sox4*, while involved in growth, have more often been implicated in embryonic growth than in postnatal growth. Real-time PCR of 1, 4, and 8 week old male mouse mRNA from kidney, heart, lung, and liver revealed that expression of all 5 genes declined with age. Using in situ hybridization, it was

determined that the decline of *Igf2*, *Mest*, and *Peg3* expression was due to an overall decrease of expression in each cell and not from a decrease in the number of cells expressing the genes.

Though compelling, it is hard to tease apart if the observed downregulated expression was due to age or growth. Therefore, male Sprague Dawley rats were growth retarded by inducing hypothyroidism from 1 day to 5 weeks of age and allowed to recover from 5 to 9 weeks (Finkelstein et al., 2009). Generally, there was a decline of *Igf2*, *Mest*, and *Peg3* expression from 3 to 9 weeks of age as expected in the kidney, liver, and heart of control rats. When compared at 7 and 9 weeks of age, the expression of *Igf2*, *Peg3*, and *Mest* was generally increased in the hypothyroid induced rats when compared to the controls. This pattern was not consistently observed between control and growth retarded mice for *Sox4* or *Igf2bp3*. Due the fact that these 2 genes are more involved in embryonic development and are not affected by growth delay, they are not likely to be involved in postnatal growth regulation. The decline of expression with age may be residual remnants from increased embryonic expression. Overall, a mechanism of growth decline involving the downregulation of a set of growth regulating genes as animals age was proposed. As expression of these genes declines, growth begins to slow and eventually stops. This decline is dependent upon growth itself such that after growth restriction, expression of these genes increases with an expected increase in growth rate.

Another investigation was conducted to find further evidence for a genetic program that regulates the cessation of growth (Lui et al., 2010). A microarray analysis was conducted comparing 1 and 5 week old male Sprague Dawley rat kidney and lung tissue. Using mouse data from a previous study (Finkelstein et al., 2009), 235 genes were downregulated with age in all organs and in both rodent species. To characterize the effects of certain genes more closely, 8 genes (*Ezh2*, *Gpc3*, *Mdk*, *Meis1*, *Mest*, *Mycn*, *Peg3*, and *Plagl1*) were chosen based upon their

probable function as regulatory genes. Real-time PCR was performed on these genes in the lung, kidney, liver, and heart of male C57BL/6 mice and Sprague Dawley rats through 8 weeks of age. All genes in all organs of both rodents declined in expression with age, though, expression of *Ezh2* and *Mycn* in mouse liver was downregulated more in embryonic stages than postnatally. Reduced expression thru siRNA transfection of most of these genes in hepatocytes from fetal mouse liver resulted in reduced proliferation. In contrast, *Gpc3* knockdown resulted in greater proliferation of hepatocytes. These data indicate that these 8 genes are involved in growth regulation and as growth progresses, these genes decline in expression possibly causing the slowing and eventually ceasing of growth. However, the question of whether this downregulation is dependent upon age or growth still remains. To examine this, newborn male Sprague Dawley rats were growth delayed by feeding dams tryptophan deficient diets until 4 weeks of age when an adequate diet was resumed (Lui et al., 2010). Expression of the 8 previously identified genes was generally increased at 5 and 6 weeks in growth delayed rats when compared to controls. This strongly suggests that these genes are dependent on growth and not age.

This coordinated decline in expression might be orchestrated by epigenetic histone modifications in the promoter regions of these genes (Lui et al., 2010). The most consistently observed modification was decreased trimethyl-H3K4 (H3K4me3) from 1 to 4 weeks of age observed in the promoter regions of *Mdk*, *Peg3*, and *Plagl1* in the kidney, liver, and lung of male mice. In a larger set of age related genes (11 downregulated and 5 upregulated), 7 downregulated genes had a decline of H3K4me3 in all 3 organs and 2 downregulated genes had a decline in 2 of the 3 organs. Only 2 upregulated genes showed a change in H3K4me3 with increased levels in at least 2 of the 3 organs. H3K4me3 is a signature of actively transcribing genes and permissive chromatin (Koch et al., 2007; Pokholok et al., 2005; Santos-Rosa et al., 2002; Yan and Boyd,

2006). Permissive chromatins have a more open structure while non-permissive chromatins are more compact (as reviewed by Lund and Zaina, 2009) . Genes within the permissive chromatin are potentially active while genes within the non-permissive chromatin are generally inactive (as reviewed by Berger, 2007). H3K3me3 recruits multiple effector molecules, some of which have activation functions, and, therefore, the decline of H3K4me3 with age might be indicative of decreased recruitment of activation transcription factors and, thus, less activation of these genes leading to the observed downregulation of expression.

These studies demonstrate that a large set of genes is downregulated in organs with age. Though this downregulation helps to slow postnatal growth, the expression of these genes is also dependent upon growth. The downregulation occurs through an overall decrease of expression in each cell and might be regulated by an H3K4me3 histone modification in the promoter region of these genes. This is a proposed mechanism of growth decline in organs, however, at this point, there has been limited data about these genes in growing muscle. To further understand the function of 7 of these genes (*Ezh2*, *Gpc3*, *Mdk*, *Mest*, *Mycn*, *Peg3*, and *Plagl1*), their general and specific functions in skeletal muscle will be discussed in more detail.

Growth Regulating Gene Functions

Ezh2 encodes a Polycomb group protein methyltransferase, a subunit of the Polycomb-repressive complex 2 (PRC2), which acts as a transcriptional repressor by trimethylating lysine 27 of histone H3 (H3K27me3) (as reviewed by Sauvageau and Sauvageau, 2010). This methylation causes the repression of genes, such as the *Hox*, *Pax*, and *Six* genes, that are needed for the determination of cell lineage and differentiation of embryonic stem cells (Boyer et al., 2006). Inactivation of this gene is lethal in mice as embryos ceased development between embryonic days 5-10 with incomplete gastrulation (O'Carroll et al., 2001). In muscle, *Ezh2* is

needed for stem cells to commit to the muscle lineage and inactivation of the gene in satellite cells results in diminished postnatal muscle growth (Juan et al., 2011). Mutant mice with ablation of *Ezh2* in satellite cells have reduced muscle mass and smaller fiber size than controls with less ability for muscle regeneration and satellite cell proliferation. There is unexpected expression of non-muscle lineage genes in the muscle tissue of these mutant mice suggesting that *Ezh2* might normally repress non-myogenic cell lineages. In comparison, overexpression of *Ezh2* represses certain muscle genes, such as myosin heavy chain and, in turn, reduces differentiation (Carette et al., 2004). This might have implications in cancer as *Ezh2* is also overexpressed in rhabdomyosarcoma, a cancer of skeletal muscle (Ciarapica et al., 2009). Overall, it is clear that *Ezh2* is important not only for embryonic development and viability, but also for postnatal muscle growth. As muscle matures, it is expected that the expression of *Ezh2* would decrease because there is less need for a large amount of satellite cell proliferation and differentiation into new muscle cells because rapid muscle growth has ceased.

Gpc3 encodes a heparan sulfate proteoglycan that is membrane bound to the cell surface by a glycosylphosphatidylinositol (as reviewed by Filmus and Selleck, 2001). The exact mechanism of *Gpc3* function is not known; however, it is directly involved with growth. Loss of function mutations in *Gpc3* lead to the Simpson-Golabi-Behmel overgrowth syndrome (Pilia et al., 1996). This disease is characterized by skeletal and visceral abnormalities that include polydactyly, cleft palate, enlarged and cystic kidneys, enlarged head, supernumerary nipples, and hernias (Golabi and Rosen, 1984). *Gpc3* deficient mice exhibit abnormalities similar to the Simpson-Golabi-Behmel overgrowth syndrome. These mice demonstrate pre and postnatal overgrowth symptoms including larger birth weights, placentas, and lungs than wild-types (Cano-Gauci et al., 1999; Chiao et al., 2002). *Gpc3* deficient mice also have a delay in

endochondral ossification, decreased osteoclast differentiation, heart defects, and reduced viability (Cano-Gauci et al., 1999; Chiao et al., 2002; Ng et al., 2009; Viviano et al., 2005). No published research indicates muscle abnormalities in humans or mice deficient in *Gpc3*, though overexpression of the *Gpc3* protein is noted in rhabdomyosarcoma indicating that it could have functions within muscle growth (Thway et al., 2011). *Gpc3* is involved in hepatocyte growth and liver regeneration (Liu et al., 2009). In hepatocytes, a reduction in the expression of *Gpc3* in culture results in increased proliferation. A partial hepatectomy in rats results in *Gpc3* expression which peaks during the early and middle stages of regeneration. It is thought that *Gpc3* is a negative regulator of growth such that increased expression causes a reduction in proliferation. With age, it is expected that *Gpc3* expression will decline in organs and muscle because there is less need to negatively regulate proliferation when growth has ceased. Overall, *Gpc3* is clearly involved with normal heart, liver, and bone growth as well as the development of rhabdomyosarcoma; therefore, it might also have implications in the normal development of muscle.

Mdk encodes a heparin binding protein that has various effects on growth (Tomomura et al., 1990). In the nervous system, *Mdk* promotes neurite out-growth and migration (Kaneda et al., 1996). Postnatally, *Mdk* knockout mice exhibit delayed brain development as well as a decrease in memory at 4 weeks of age (Nakamura et al., 1998). In humans, *Mdk* is thought to be involved with Alzheimer's disease because ectopic *Mdk* protein has been found in the brain of Alzheimer's patients located in senile plaques (Yasuhara et al., 1993). Additionally, when injury was induced to the sciatic nerve near the soleus, *Mdk* null mice had delayed nerve degeneration and regeneration properties which resolved by 5 weeks post-injury (Sakakima et al., 2009). In muscle, *Mdk* is expressed during the proliferation and differentiation of myoblasts, and levels are

increased in the early stages of muscle regeneration (Hu et al., 2002; Sakakima et al., 2006). Elevated levels are also prevalent following skin burns, gastric ulcers, bone fractures, heart infarctions, and brain infarctions indicating that *Mdk* may be involved in the repair of many different tissues (Fukui et al., 2008; Iwashita et al., 1999; Maekawa et al., 1999; Ohta et al., 1999; Yoshida et al., 1995). Furthermore, *Mdk* is overexpressed in rhabdomyosarcoma cells demonstrating that *Mdk* has functions in both normal and abnormal muscle development (Jin et al., 2008). Overall, *Mdk* functions within various tissues all over the body and is involved in the muscle growth process. With age, *Mdk* expression is expected to decrease in organs and muscle because the proliferative characteristics of this gene are needed less as growth slows.

Mycn (also known as N-*myc*) encodes a nuclear phosphoprotein with helix-loop-helix and leucine zipper regions that exhibit DNA binding (Landschulz et al., 1988; Murre et al., 1989; Prendergast and Ziff, 1989; Ramsay et al., 1986; Slamon et al., 1986). Loss of *Mycn* function leads to lethality at embryonic day 11, growth retardation, and several tissue defects in mouse models (Charron et al., 1992; Sawai et al., 1993; Stanton et al., 1992). The majority of these defects arise from the underdevelopment of the limb buds, lungs, heart, liver, gut, and urogenital tract. *Mycn* also plays a large role in the nervous system as it functions to promote the proliferation of neural progenitor cells and inhibit neural differentiation (Knoepfler et al., 2002). Though evident in many different tissues, *Mycn* has not been well characterized in skeletal muscle; however, this gene is overly expressed in rhabdomyosarcoma (Toffolatti et al., 2002; Williamson et al., 2005). *Mycn* may have the same functions it shows in neural progenitors, increased proliferation and decreased differentiation, in muscle progenitors to help this cancer advance. Overall, *Mycn* is essential for normal organogenesis, neurogenesis, and growth and might play a role in the normal development of skeletal muscle. With age, *Mycn* expression

would be expected to decline in organs and muscle as there is less need for the proliferative properties of this gene as growth slows.

Mest (also known as *Peg1*) and *Peg3* (also known as *Pw1*) are similar in that both are paternally expressed imprinted genes that help regulate behavior (Lefebvre et al., 1998; Li et al., 1999). The exact function of these two genes is still unknown. *Mest* encodes a protein that is similar in structure to an α/β hydrolase fold enzyme that has 8 β -sheets connected by α -helices (as reviewed by Leckman and Herman, 2002; Ollis et al., 1992). *Peg3* encodes a C₂H₂ zinc finger protein which is involved with DNA binding (Kuroiwa et al., 1996; Relaix et al., 1996). When either gene is inactivated, it produces female mice with abnormal maternal behavior (Lefebvre et al., 1998; Li et al., 1999). Mutant females produce fewer offspring that live past weaning age, and the pups that do survive are small and growth stunted. *Peg3* inactivation leads to pups that, after weaning, could catch up in growth to their wild-type counterparts while *Mest* inactivation leads to pups that are permanently growth retarded. For both genes, this reduction in growth results from abnormal maternal behaviors such as decreased nest building, pup retrieval, and nursing.

Mest and *Peg3* are also involved in regulating adipose deposition and adipocyte size. Obese mice have an increased expression of *Mest* in white adipocytes (Nikonova et al., 2008; Takahashi et al., 2005). Transgenic mice with an overexpression of *Mest* exhibit increased adipocyte size and increased expression of key adipogenic genes, such as leptin and GLUT4. When adipocyte size is reduced, *Mest* expression decreases. *Mest* knockout mice also have reduced epididymal and inguinal fat depot weights when compared to controls. Consequently, *Peg3* has the opposite effect on fat accumulation. *Peg3* inactivation leads to adult mice with increased white adipose tissue and leptin levels when compared to their wild-type counterparts

(Curley et al., 2005). These mice also have reduced food intake with a lower metabolic rate and body temperature. They fail to exhibit an increased body temperature when exposed to cold temperatures, which might explain why they have an increased accumulation of fat.

Similar to *Mest* and *Peg3*, *Plagl1* (also known as *Zac1* and *Lot1*) is a paternally expressed imprinted gene that encodes a zinc finger protein that exhibits DNA binding and transactivation activity and might act as a transcription factor (Kamiya et al., 2000; Spengler et al., 1997; Varrault et al., 1998). It also has some apoptotic and growth inhibition properties in tumors, though it is tumor specific because this is not observed in all cancers (Rezvani et al., 2012). In addition to cancer growth, *Plagl1* is involved with normal growth. *Plagl1* deficient mice exhibit smaller birth, hind limb, lung, and liver weights than wild-type controls with decreased ossification of the caudal vertebrae and ankle bones and reduced survivability (Varrault et al., 2006). In the heart, *Plagl1* is necessary for normal cardiac formation. *Plagl1* null mice display heart malformations and increased apoptosis of cardiac cells which might play a role in the observed reduction in viability (Yuasa et al., 2010).

Mest, *Peg3*, and *Plagl1* are all important in muscle growth such that they are involved in muscle regeneration. Expression of these 3 genes was observed in the tibialis anterior during muscle regeneration after injury (Yan et al., 2003). In comparison, before injury, the muscle was irradiated with gamma radiation to inhibit satellite cell proliferation, and thus, block muscle regeneration. This resulted in no regeneration and no expression of *Mest*, *Peg3*, and *Plagl1*. This suggests that all 3 of these genes are associated with muscle regeneration and might have functions within satellite cell activation, proliferation, or differentiation. Additionally, *Peg3* inactivation in mice results in smaller hind limb muscle mass, a 60% reduction in fiber size as compared to wild-type mice at 6 days of age, and increased expression of *Atrogin-1*, a marker of

muscle atrophy (Curley et al., 2005; Nicolas et al., 2005). Furthermore, overexpression of these 3 genes is evident in rhabdomyosarcoma while inhibition leads to decreased rhabdomyosarcoma cell proliferation (Rezvani et al., 2012). Overall, *Mest*, *Peg3*, and *Plagl1* have important roles in the normal growth and development of several tissues as well as possible functions in muscle regeneration. With age, it is expected that the expression of these 3 genes in organs and muscle would decrease because as growth slows, there is less need for proliferation and less need for the proliferative functions that these genes supply.

In summary, it is evident that these 7 genes are important for normal growth in many different tissues. Deficiencies lead to reduced viability as well as many growth defects and abnormalities. Several of these genes have also been associated with muscle growth. Inactivation mutations lead to reduced muscle mass and less ability for muscle regeneration. In addition, overexpression of all of these genes is observed in rhabdomyosarcoma, a mesodermal cancer that arises in skeletal muscle, further indicating a possible role in muscle development. With age, the expression of these genes is expected to decline as the need for their growth associated functions decreases. In order to better understand how these genes might play a role in muscle development, the regulation of normal skeletal muscle growth will be discussed in more detail.

Skeletal Muscle Growth

Skeletal muscle growth can occur in two ways: hyperplasia, increase in cell number, and hypertrophy, increase in cell size. Muscle growth by hyperplasia occurs mainly during prenatal muscle growth, termed myogenesis, while postnatal growth is primarily the result of hypertrophy (Enesco and Puddy, 1964). The steps of myogenesis are controlled by many different muscle regulatory factors including MyoD, Myf5, myogenin, and MRF4 (as reviewed by Chong et al., 2009). The first step during myogenesis is commitment of mesenchymal cells to the muscle

lineage, which is marked by the expression of *MyoD* and *Myf5*. Now termed myoblasts, these cells continue to proliferate until the upregulation of myogenin and *MRF4* signal terminal differentiation. It should be noted that *MRF4*, while a terminal differentiator, also might have functions in commitment as well. The next step in prenatal muscle growth is fusion. This is when the myoblasts fuse together in a biphasic nature to form primary and secondary myotubes and results in a multi-nucleated muscle cell. The last step of myogenesis is myofibrillogenesis. This process involves the assembling of proteins into the structural units of muscle known as sarcomeres to produce mature, functional muscle.

Postnatally, muscle grows primarily by hypertrophy until it reaches its mature size. The muscle nuclei are unable to divide after fusion, so to acquire further nuclei and increase size, satellite cells are activated and fuse with the muscle fibers (Moss and Leblond, 1971; Stockdale and Holtzer, 1961). Satellite cells are myogenic stem cells that have a single, centrally located nucleus and are located between the sarcolemma and basal lamina of the muscle tissue (as reviewed by Shefer and Yablonka-Reuveni, 2007). They are quiescent until activation by growth stimuli or muscle injury to produce myoblasts that fuse with existing muscle. When examining the function of the previous 7 genes in muscle growth, 5 of them have a large effect on muscle regeneration indicating that their function might be affecting satellite cell activation, proliferation, or differentiation.

Postnatal muscle growth differs from the postnatal growth of heart and liver. Similar to myocytes, cardiomyocytes lose their ability for hyperplastic growth shortly after birth (Li et al., 1996). However, cardiomyocytes undergo approximately 2 weeks of binucleation without cytokinesis once hyperplastic growth has ceased. After binucleation, the cardiomyocytes are only capable of hypertrophic growth. In contrast, hepatocytes proliferate rapidly up to approximately

2 weeks of age when proliferation begins to decline (Chang et al., 2008). Even though declining, hepatocyte proliferation can extend up to at least 6 weeks of age. Additionally, hepatocytes are capable of hyperplastic and hypertrophic growth throughout the lifetime of humans and animals (as reviewed by Michalopoulos, 1990). Due to these growth differences, it is possible that the patterns of expression of these 7 growth dependent genes might differ between tissues.

Objective

The objective of this study was to examine the expression patterns of 7 genes (*Ezh2*, *Gpc3*, *Mdk*, *Mest*, *Mycn*, *Peg3*, and *Plagl1*) in the heart, liver, and muscle of male and female mice over time. We expected the expression profiles to differ between males and females and differ between tissues because the expression of these genes is governed by growth. Males attain heavier body weights than females, so the expectation was that increased expression of these genes would be maintained for longer in males when compared to females. As mentioned earlier, postnatal growth differs between muscle, heart, and liver. These differences in growth indicate that there might be differences in the patterns of expression of these growth regulating genes between tissues. Over time, we expected that all of the genes in all of the tissues would decline as the mice age. These genes all have growth regulating functions and as the animal reaches mature size, it is necessary for these functions to be reduced. It is theorized that the decline of these genes coincides with reduced proliferation and the eventual cessation of growth.

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CHAPTER II

REDUCTIONS IN EXPRESSION OF GROWTH REGULATING GENES ARE OBSERVED IN SKELETAL MUSCLE AND ORGANS WITH AGE

Abstract

A set of growth regulating genes has been identified that decline in expression in organs with age. This downregulation is thought to coordinate organ growth cessation observed as humans and animals mature. The objective of this study was to observe the expression of 7 of these growth regulating genes (*Ezh2*, *Gpc3*, *Mdk*, *Mest*, *Mycn*, *Peg3*, and *Plagl1*) in male and female skeletal muscle, heart, and liver with age. It was hypothesized that the expression of these genes would differ not only between sexes, but also between tissues. Female and male C57BL/6J mice were sacrificed at 1, 7, 21, 35, and 49 days of age. Samples collected consisted of heart and liver from all mice, all muscles from both hind limbs of 1- and 7-day-old mice, and specific muscles (bicep femoris, gastrocnemius, tibialis anterior, and triceps brachii) from 21-, 35-, and 49-day-old mice. Expression was determined by quantitative real-time PCR, normalized to *Rn18s*, standardized to a single 1-day-old male liver sample present on each plate, and compared to the average of 1-day-old male expression for each tissue. Data were analyzed using the Proc Mixed procedure in SAS as repeated measures with significant differences ($P < 0.05$) determined by a Bonferroni-adjusted pdiff. Orthogonal contrasts were used to differentiate between linear, quadratic, and cubic patterns of expression among tissues. In general, expression declined from 1 or 7 days of age when compared to 49 days of age for all the genes in all the tissues except for expression of *Ezh2* and *Mycn*, which was not altered by age in liver. Some differences in expression were observed between sexes with the majority of the differences occurring at 49 days. Expression was usually increased in females compared with males at this time point.

Occasionally, males had greater expression than females; this was observed in liver at 1 day of age for *Gpc3*, *Mest*, and *Plagl1* and muscle at 35 days of age for *Mest*. There were some similarities in the patterns of gene expression between muscle and heart or liver, but, there was not a single gene that had the same pattern of expression in all 3 tissues. Overall, these data suggest that the downregulation of these growth regulating genes with age might play a role in the coordinated cessation of muscle growth similar to organ growth with limited differences between sexes.

Introduction

As humans and animals age, growth slows and eventually stops. Organs grow until they reach mature size; however, little is known about how growth cessation is regulated and coordinated in the body. A set of growth regulating genes, which include *Ezh2*, *Gpc3*, *Mdk*, *Mest*, *Mycn*, *Peg3*, and *Plagl1*, has been identified that is dependent upon growth and whose expression declines with age in organs (Finkelstein et al., 2009; Lui et al., 2010). In general, when expression of these genes decreases, proliferation decreases leading to a reduction in growth. Mutations of these genes also result in reduced viability, growth abnormalities, and diseases such as rhabdomyosarcoma, a skeletal muscle cancer, and Simpson-Golabi-Behmel syndrome (Charron et al., 1992; Chiao et al., 2002; Ciarapica et al., 2009; Jin et al., 2008; Lefebvre et al., 1998; Li et al., 1999; Nakamura et al., 1998; O'Carroll et al., 2001; Pilia et al., 1996; Rezvani et al., 2012; Sakakima et al., 2009; Sawai et al., 1993; Stanton et al., 1992; Toffolatti et al., 2002; Varrault et al., 2006; Williamson et al., 2005).

Though well characterized in organs, these genes have not been well examined in growing muscle. It is expected that expression of these genes would differ between muscle, heart, and liver because of the differences in how these tissues grow. Postnatally, muscle grows

by hypertrophy, an increase in cell size, and not hyperplasia, an increase in cell number (Enesco and Puddy, 1964). After prenatal muscle fiber fusion and maturation, muscle nuclei are unable to divide. Satellite cells are activated and fuse with existing muscle fibers to increase muscle growth (Moss and Leblond, 1971; Stockdale and Holtzer, 1961). Without the activation of satellite cells, postnatal muscle growth and regeneration is severely inhibited. Similar to muscle, heart loses its ability for hyperplastic growth shortly after birth; however, cardiomyocytes undergo approximately 2 weeks of binucleation without cytokinesis once hyperplastic growth has ceased (Li et al., 1996). After binucleation, the cardiomyocytes are only capable of hypertrophic growth. In contrast, hepatocytes proliferate rapidly up to approximately 2 weeks of age when proliferation begins to decline (Chang et al., 2008). Even though declining, hepatocyte proliferation can extend up to at least 6 weeks of age. Additionally, hepatocytes are capable of hyperplastic and hypertrophic growth throughout the lifetime of humans and animals (as reviewed by Michalopoulos, 1990). Due to these growth differences, it is possible that the patterns of expression of these 7 growth dependent genes might differ between tissues.

In muscle, it is known that the expression of *Ezh2*, *Mdk*, *Mest*, *Peg3*, and *Plagl1* is increased during regeneration while regeneration is reduced when these genes are inactivated (Hu et al., 2002; Juan et al., 2011; Sakakima et al., 2006; Yan et al., 2003). In contrast, *Mycn* and *Gpc3* have not been characterized in muscle. *Mycn*, however, increases proliferation of neural progenitor cells while *Gpc3* is upregulated in the early and middle stages of liver regeneration (Knoepfler et al., 2002; Liu et al., 2009). Because 5 of 7 of these genes are known to be upregulated with muscle regeneration, it is possible that they are affecting muscle growth by increasing activation, proliferation, or differentiation of satellite cells. With age, the expression of all of these genes is expected to decline in both organs and muscle because the growth

regulating functions of these genes would be less necessary once growth has ceased. The objectives of this study were to confirm the downregulation of these 7 genes in heart and liver with age, determine if these genes were downregulated in muscle with age, and compare the gene expression patterns between tissues along with observing any differences between males and females.

Materials and Methods

Animal procedures and sample collection

All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee. Mice from an internal C57BL/6J colony were allowed ad libitum access to pelleted rodent chow (Teklad F6 rodent diet, Harlan, Indianapolis, IN, USA) and tap water. Room temperature was maintained at 22°C with a 12 hour light/dark cycle. Male and female mice were bred in monogamous pairs and litters were weaned at 21 days of age. Weaned mice were group housed with littermates of the same sex. At 1, 7, 21 (+/- 1) days of age, and 35 and 49 (+/- 2) days of age, mice were weighed and then euthanized by CO₂ asphyxiation and decapitation. Mice were selected based upon the mean live body weight for each sex and age time point for a total of 54 male and 51 female mice. For the 1 day time point, 32 male and 23 female mice were used. At 7 days, 10 male and 14 female mice were used. At 21, 35, and 49 days, 4-5 male and female mice were used. Samples collected consisted of heart and liver from all mice, all muscles from both hind limbs of 1- and 7-day-old mice, and specific muscles (bicep femoris, gastrocnemius, tibialis anterior, and triceps brachii) from 21-, 35-, and 49-day-old mice. Tissues were weighed and stored at -80°C for further analysis. Tissues weighing less than 50 mg were pooled within age and sex to create samples of at least 50 mg (1 and 7 days n = 5 pooled samples per sex per time point; 21, 35, and 49 days n = 4 pooled samples per sex per time point).

Because hearts were the smallest of the tissues at 1 and 7 days, mice were sacrificed until an adequate number of heart samples were collected to create 5 pooled 50 mg samples per sex; however, this resulted in an excess of muscle and liver samples. Therefore, tissues from mice that were closest to the mean live body weight were used for further analysis (n = 5 pooled samples per sex per time point). All data presented is reflective of only mice analyzed for gene expression.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using TRI-Reagent (Sigma-Aldrich, St. Louis, MO, USA) and BCP phase separation reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturers' protocols. RNA purity and concentration were measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All samples had a 260/280 nm ratio between 1.9 and 2.1. For cDNA synthesis, RNA was diluted to 500 ng/ μ l using RNase-free water and 1 μ g of RNA was reversed transcribed using the Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA).

Quantitative real-time PCR

Validation experiments were conducted according to the Applied Biosystems quantitative PCR manual (Applied Biosystems, 2004) to determine the appropriate reference gene (*Rn18S*) and cDNA dilution (1:32 for muscle and 1:64 for heart and liver) for this study. Eighteen μ l of diluted cDNA, 2 μ l of primer/probe, and 20 μ l of Taqman Universal Master Mix (Roche, Branchburg, NJ, USA) were mixed together and plated in 10 μ l triplicates into a 384-well plate. Expression of *Ezh2*, *Gpc3*, *Mdk*, *Mest*, *Mycn*, *Peg3*, and *Plagl1* (Table 1) was determined by quantitative real-time PCR using an ABI Model 7900HT Fast Real-Time PCR System thermal cycler (Applied Biosystems, Foster City, CA, USA). The thermal cycler performed 40 cycles of

2 minutes at 50°C, 10 minutes and 15 seconds at 95°C, and 1 minute at 60°C. Expression was normalized to *Rn18s* (ΔCt), standardized to a single 1-day-old male liver sample present on each plate, and compared to the average of 1-day-old male expression for each tissue ($\Delta\Delta\text{Ct}$). Values were expressed as a fold change ($2^{-\Delta\Delta\text{Ct}}$ = fold change) compared to the 1-day-old male expression for each tissue.

Statistical analysis

Data were analyzed using the Proc Mixed procedure in SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) as repeated measures with either an unstructured, autoregressive, or compound symmetry variance/covariance structure based on the lowest Akaike scores. The model included the fixed effects of age, sex, and their interaction. Samples were considered outliers if Proc Univariate identified them as an extreme outlier in 5 or more genes. The entire sample was removed from the expression and weight analyses for that tissue. Data are presented as least squares means \pm s.e.m. with significant differences ($P < 0.05$) determined by a Bonferroni-adjusted pdiff. Orthogonal contrasts in Proc Mixed were used to differentiate between patterns of expression among tissues. Proc IML was used to determine the contrast coefficient matrix for unequal spacing of time.

Results

Body, muscle, heart, and liver weights

Body, muscle, heart, and liver weights were compared between males and females at each age time point to illustrate differences between sexes. For body and liver weights, there was an age and sex interaction ($P < 0.01$). Body and liver weights were similar between males and females at 1, 7, and 21 days of age, but males were heavier ($P < 0.05$) than females at 35 and 49 days (Figures 1A and 1D). For heart weight, there was also an interaction of age and sex ($P <$

0.01) such that weights were similar between males and females through 35 days of age, but males were heavier ($P < 0.05$) at 49 days (Figure 1C). For muscle weight, there was an age and sex interaction ($P < 0.01$). Males and females had similar hind limb muscle weights at 1 and 7 days of age and similar specific muscle weights at 21 and 35 days, but at 49 days of age, males had heavier ($P < 0.05$) gastrocnemius and trended toward heavier ($P = 0.06$) triceps brachii muscles than females (Figure 1B).

Expression of growth regulating genes in heart and liver

The first objective of this study was to confirm the downregulation of expression of these growth regulating genes in heart and liver and to determine if sex affected expression. The expression of these genes in heart was affected by age ($P < 0.01$) such that expression at 1 day of age was greater ($P < 0.05$) than expression at 49 days of age (Figure 2). Expression of *Gpc3* and *Mest* were affected by an age and sex interaction ($P < 0.05$); however, means between sexes at each age time point for *Mest* were not different ($P > 0.60$) while females had greater ($P < 0.05$) *Gpc3* expression than males only at 49 days of age. Overall, there was not an effect ($P > 0.10$) of sex on expression of these genes in heart.

In liver, the expression of *Gpc3*, *Mdk*, *Mest*, *Peg3*, and *Plagl1* was affected by age ($P < 0.01$) such that expression at 1 day was greater ($P < 0.05$) than expression at 49 days (Figure 3). However, the expression of *Ezh2* and *Mycn* were not altered ($P > 0.25$) by age in this study. Expression of *Gpc3*, *Mest*, and *Plagl1* were affected by an age and sex interaction ($P < 0.01$) such that expression in males was greater ($P < 0.05$) than expression in females at 1 day, but sexes were similar at 7, 21, 35, and 49 days. Interestingly, *Peg3* expression was also affected by an age and sex interaction ($P < 0.01$); however, this interaction differs from the others in that sexes are similar in expression at 1, 7, 21, and 35 days of age and females are greater ($P < 0.05$)

than males at 49 days. Though unaffected by age, expression of *Mycn* was affected ($P < 0.05$) by sex such that males had greater ($P < 0.05$) expression than females. There was an age and sex interaction ($P < 0.05$) for *Ezh2* expression, but means between sexes at each age time point were not different ($P > 0.20$).

Expression of growth regulating genes in muscle

The second objective was to determine if these genes were downregulated with age in muscle and if expression was different between males and females. All muscles from both hind limbs were collected at 1 and 7 days of age because of the small size of individual specific muscles. From 21 days of age and on, specific muscles were collected and expression was measured in each muscle. Therefore, it was first appropriate to determine the effect of specific muscle on gene expression. All expression was compared to 1-day-old male hind limb expression allowing for direct comparison of fold change. The main effect of specific muscle on gene expression revealed that there were inconsistent patterns of differences between specific muscles and the magnitude of these differences was small (Table 2). However, expression of *Ezh2* and *Mycn* were affected by a three-way age, sex, and specific muscle interaction ($P < 0.10$), but no clear pattern of differences between muscles was detected (Figure A.1 and Figure A.2). For each sex, means between muscles were not different ($P > 0.30$) within each age time point. *Peg3* and *Gpc3* also had a two-way interaction of age and specific muscle ($P < 0.10$). Means within age for *Gpc3* were not different ($P > 0.60$, Figure A.3). For *Peg3*, the biceps femoris and tibialis anterior had greater ($P < 0.05$) expression than the gastrocnemius and triceps brachii at 35 days and the tibialis anterior had greater ($P < 0.05$) expression than the other muscles at 49 days, but expression at these age time points only ranged from 0.15 to 0.35 (Figure A.4). Overall, analysis of the effect of specific muscle on gene expression revealed few additional interactions

of specific muscle with age or sex ($P > 0.20$); therefore, data presented in Figure 4 are pooled between all muscles within sex.

Expression of *Mdk* was similar between males and females over time (Figure 4, age and sex interaction $P = 0.83$). For all other genes, there was an interaction of age and sex ($P < 0.01$). Differences between males and females occurred exclusively at 35 and 49 days of age. Females had greater ($P < 0.05$) expression of *Ezh2*, *Gpc3*, *Mycn*, *Peg3*, and *Plagl1* than males at 49 days. Males had greater ($P < 0.05$) expression of *Mest* than females at 35 days. All genes were affected ($P < 0.01$) by age with an overall decline ($P < 0.05$) from either 1 or 7 day expression levels as age increased.

Comparison of expression patterns between tissues

The third objective was to compare the patterns of expression of each gene between tissues. As each tissue was normalized to the 1-day-old male expression of that tissue, direct comparisons of fold changes are not appropriate to compare absolute amount of expression between tissues. Therefore, expression between males and females was pooled and linear, quadratic, and cubic contrasts were performed to determine the best fit to describe the expression patterns of each gene in each tissue (Figure 5). In muscle, the downregulation of these genes was primarily a cubic pattern of expression with the exception being *Peg3* which had a quadratic pattern. In heart, the patterns of expression decline were split between quadratic and cubic. *Ezh2*, *Mdk*, and *Mycn* had quadratic patterns while *Gpc3*, *Mest*, *Peg3*, and *Plagl1* had cubic patterns. *Mest* and *Plagl1* had linear patterns of decline in liver while *Gpc3* and *Peg3* had quadratic patterns and *Mdk* had a cubic pattern. *Ezh2* and *Mycn* expression did fit into the pattern of expression categories because they did change with age in liver. There were no genes where the pattern of expression decline was the same for all tissues. Between muscle and heart, there were

3 genes (*Gpc3*, *Mest*, and *Plagl1*) that had similar patterns of expression and between muscle and liver, there were 2 genes (*Mdk* and *Peg3*) that had similar patterns of expression. There were no genes between heart and liver with similar patterns of downregulation.

Discussion

Previous research had identified a large set of growth regulating genes whose expression was dependent upon growth and was downregulated in organs with age (Finkelstein et al., 2009; Lui et al., 2010). In general, this downregulation decreased proliferation and, in turn, may be involved in growth cessation. *Ezh2*, *Gpc3*, *Mdk*, *Mest*, *Mycn*, *Peg3*, and *Plagl1* are part of this group of genes. These genes are downregulated with age in the heart, liver, lung, and kidney of mice and rats (Finkelstein et al., 2009; Lui et al., 2010). We investigated the expression of these genes in the muscle, heart, and liver of male and female mice over time to examine differences between sexes and tissues.

Our first objective was to confirm the downregulation of these genes in heart and liver and determine if there were any differences in expression between sexes. Though, there has been little comparison of expression of these genes between males and females, we expected differences because males achieve larger body, heart, and liver weights than females. In our results, males achieved larger body and liver weights at 35 and 49 days when compared to females and males attained larger heart weights than females at 49 days. Under this assumption, males were predicted to have increased expression levels maintained longer than females. To that end, *Gpc3*, *Mest*, and *Plagl1* expression was greater in males than females in liver at 1 day of age. However, surprisingly, there were few other differences between sexes in heart and liver. Females had greater *Gpc3* and *Peg3* expression than males at 49 days of age; however, expression at this time point is low and the expression differences are small, therefore, they

might not have a large effect on growth. In general, the expression of most of these genes declined from 1 day expression levels when compared to 49 day levels in heart and liver. This is not surprising considering that these genes have growth regulating functions, mostly proliferative, that are less needed as growth ceases. However, contrary to previous data, *Ezh2* and *Mycn* expression did not decline with age in liver. This may be attributed to the lack of prenatal time points. In addition to looking at postnatal ages, previous research had examined embryonic time points as well. In particular, *Ezh2* and *Mycn* are highly expressed in the prenatal liver and show smaller declines of expression after birth (Lui et al., 2010). With only postnatal time points, any downregulation which may have occurred since embryonic development was undetected. Overall, there are few expression differences between sexes and in general, we confirmed the downregulation of most of these genes in heart and liver with age.

Our second objective was to examine the expression of these genes in muscle and determine if there were any differences between sexes in this tissue. We expected males to have increased expression of these genes maintained for longer when compared to females because males attain larger body and muscle weights (Gall and Kyle, 1968; Rowe and Goldspink, 1969). In our results, however, not all muscle weights differed between sexes. Males had heavier gastrocnemius weights and trended towards heavier triceps brachii weights when compared to females at 49 days of age but, there were no other differences in muscle weight. There were also no large consistent differences in expression of these genes between specific muscles. Nevertheless, there were expression differences between males and females. Unexpectedly, the majority of those differences resulted from females having increased, not reduced, expression when compared to males at 49 days of age. In general, expression at these time points was low and the expression differences were small indicating that they might not have a large effect on

growth. The only exception is *Ezh2* and *Mycn* where the differences between sexes were larger, but still biased with expression increased in females compared to males and not the reverse as predicted.

As age increased, all of the genes were downregulated from 1 or 7 day expression levels in muscle. *Ezh2*, *Mdk*, *Mest*, *Peg3*, and *Plagl1* are known to be involved in muscle regeneration and all of the genes are overexpressed in rhabdomyosarcoma, a skeletal muscle cancer (Ciarapica et al., 2009; Jin et al., 2008; Juan et al., 2011; Rezvani et al., 2012; Sakakima et al., 2006; Thway et al., 2011; Toffolatti et al., 2002; Williamson et al., 2005; Yan et al., 2003). It is possible that these genes are aiding in postnatal muscle growth by activation, proliferation, or differentiation of satellite cells. It is already known that *Ezh2* is involved in satellite cell activation (Juan et al., 2011). *Mdk*, *Mest*, *Peg3*, and *Plagl1* might have similar functions because they are also involved in muscle regeneration (Sakakima et al., 2006; Yan et al., 2003). *Mycn* has not been well characterized in muscle, but it promotes the proliferation of neural progenitor cells and inhibits neural differentiation and, therefore, might exhibit similar functions in muscle with regard to satellite cells (Knoepfler et al., 2002). It is not surprising that these genes would decrease over time as there is less activation, proliferation, and differentiation of satellite cells and, thus, less need for the growth regulatory functions of these genes as growth slows. In contrast, *Gpc3* is thought to be a negative regulator of growth such that increased expression results in reduced proliferation (Liu et al., 2009). *Gpc3* functions in muscle have not been identified; however, *Gpc3* is involved in liver regeneration such that expression peaks during the early and middle stages of the process (Liu et al., 2009). This leads to the conclusion that *Gpc3* expression might increase early in postnatal development to negatively regulate growth and proliferation, so

overgrowth of organs and muscle does not occur, and decrease as growth slows because proliferation has decreased leading to less need for its regulatory functions.

Our third objective was to compare the patterns of expression of each gene between the 3 tissues. We expected the patterns of expression to differ between tissues because the postnatal growth of these tissues differs. Though there were no genes where the pattern of expression was the same for all 3 of the tissues, our hypothesis was not completely validated. For 5 of the genes, the patterns of expression were the same when compared between muscle and one of the organs. It is thought that the regulation of these genes in organs might occur by an epigenetic histone modification, trimethylated lysine 4 of histone H3 (H3K4me3), in the promoter region of these genes as the levels of H3K4me3 decreased with increased age (Lui et al., 2010). H3K4me3 is a signature of actively transcribing genes and permissive chromatin (Koch et al., 2007; Pokholok et al., 2005; Santos-Rosa et al., 2002; Yan and Boyd, 2006). Permissive chromatin has a more open structure while non-permissive chromatin is more compact (as reviewed by Lund and Zaina, 2009). Genes within the permissive chromatin are potentially active while genes within the non-permissive chromatin are generally inactive (as reviewed by Berger, 2007). H3K4me3 recruits multiple effector molecules, some of which have activation functions, and, therefore, the decline of H3K4me3 with age might be indicative of decreased recruitment of activation transcription factors and, thus, less activation of these genes leading to the observed downregulation of expression. There are some similarities between the expression patterns in organs and muscle indicating that the same potential regulation by H3K4me3 might be present in all 3 tissues.

In summary, this study examined the expression decline of 7 growth regulating genes over time in the muscle, heart, and liver of male and female mice. Overall, most of the genes

were downregulated with age in all 3 tissues. There were some differences between males and females, but the significance of these differences has yet to be determined. There are also some similarities in the patterns of expression of these genes between tissues indicating that these genes might be regulated similarly in multiple tissues, possibly by an H3K4me3 histone modification. Further research needs to be conducted to examine the regulation of these genes in muscle and satellite cells and confirm that an H3K4me3 histone modification is involved with the regulation of these genes. Knockdown assays in cultured satellite cells would be helpful to understand the effect these genes have on the proliferation and differentiation of postnatal muscle progenitor cells. In conclusion, these genes are known to be important in the cessation of organ growth. Now, this study has found that these genes might also help regulate the cessation of postnatal muscle growth potentially by regulating the activation, proliferation, and differentiation of satellite cells.

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Tables and Figures

Table 1. ABI primer probes.

Gene Symbol	Gene Name	Reference Sequence	ABI Assay ID
<i>Ezh2</i>	Enhancer of zeste homolog 2	NM_007971.2 NM_001146689.1	Mm00468464_m1
<i>Gpc3</i>	Glypican 3	NM_016697.3	Mm00516722_m1
<i>Mdk</i>	Midkine	NM_010784.4	Mm00440279_m1
<i>Mest</i>	Mesoderm specific transcript	NM_008590.1	Mm00484993_m1
<i>Mycn</i>	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived	NM_008709.3	Mm00476449_m1
<i>Peg3</i>	Paternally expressed 3	NM_008817.2	Mm01337379_m1
<i>Plagl1</i>	Pleiomorphic adenoma gene-like 1	NM_009538.2	Mm00494250_m1
<i>Rn18s</i>	18S ribosomal RNA	NR_003278.1	Mm03928990_g1

Table 2. Fold change of specific muscles in each gene.¹

Gene	Biceps Femoris	Gastrocnemius	Tibialis Anterior	Triceps Brachii	SEM
<i>Ezh2</i>	1.456	1.609	1.506	1.489	0.075
<i>Gpc3</i>	0.117 ^b	0.139 ^{ab}	0.112 ^b	0.149 ^a	0.008
<i>Mdk</i>	0.160	0.257	0.243	0.157	0.033
<i>Mest</i>	0.029 ^b	0.049 ^a	0.049 ^a	0.034 ^b	0.003
<i>Mycn</i>	0.462 ^b	0.763 ^a	0.755 ^a	0.672 ^a	0.043
<i>Peg3</i>	0.251 ^b	0.203 ^{bc}	0.311 ^a	0.194 ^c	0.013
<i>Plagl1</i>	0.044 ^{ab}	0.049 ^a	0.036 ^b	0.041 ^{ab}	0.003

¹Values are least squares means. Expression was pooled between sexes and time points for analysis. Fold change is compared to the average male 1-day-old muscle expression.

^{ab}Means within a row without a common superscript are different ($P < 0.05$).

Figure 1. Increasing body weight (A), muscle weight (B), heart weight (C), and liver weight (D) with age. (B) From 1-7 days, muscle weight includes all muscles from one hind limb, and from 21-49 days, muscle weight is depicted as specific muscles. (A-D) Plotted values are least squares means displayed with standard error bars. Asterisks indicate a difference ($P < 0.05$) between sexes within age group.

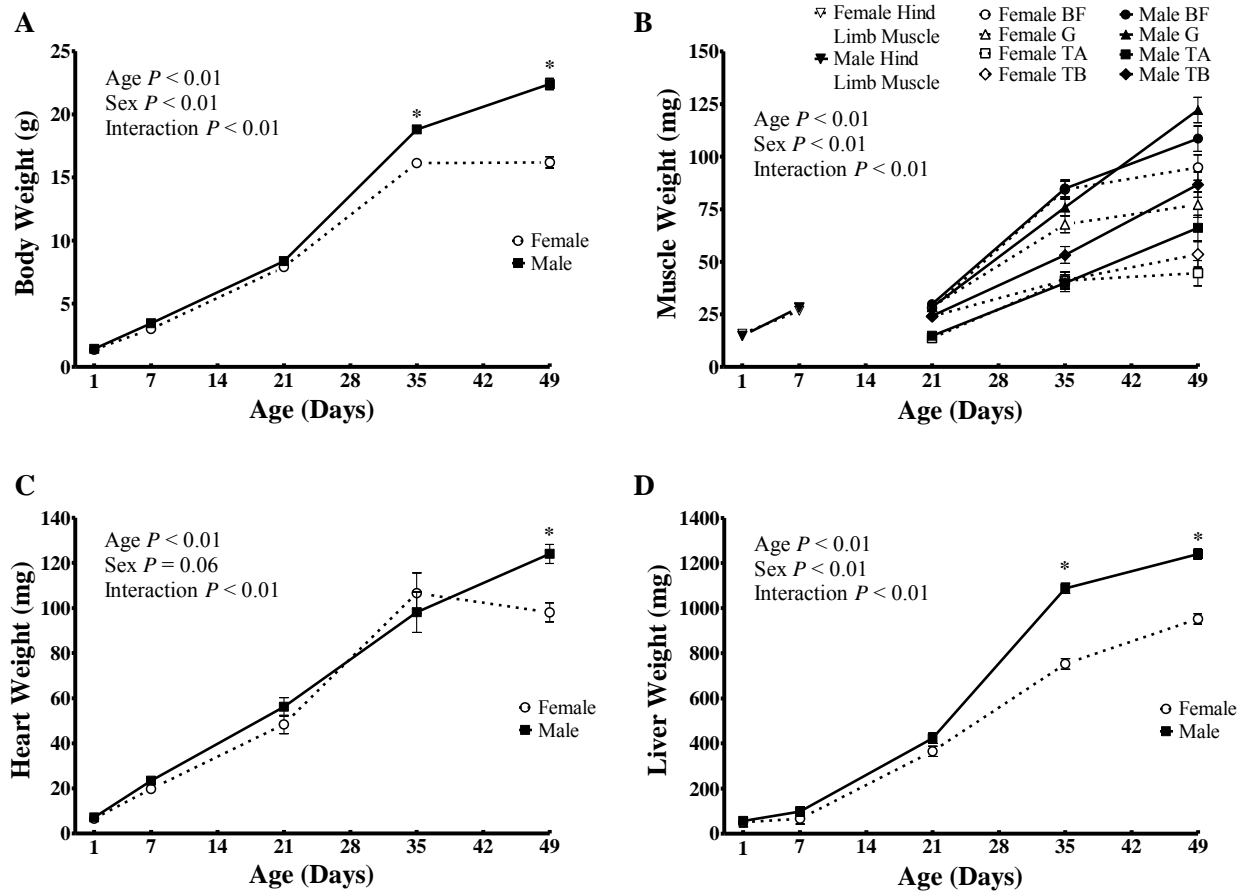


Figure 2. Expression of growth regulating genes in heart with age. Plotted values are least squares means displayed with standard error bars. Asterisks indicate a difference ($P < 0.05$) between sexes within age group. Fold change is compared to the average male 1-day-old heart expression.

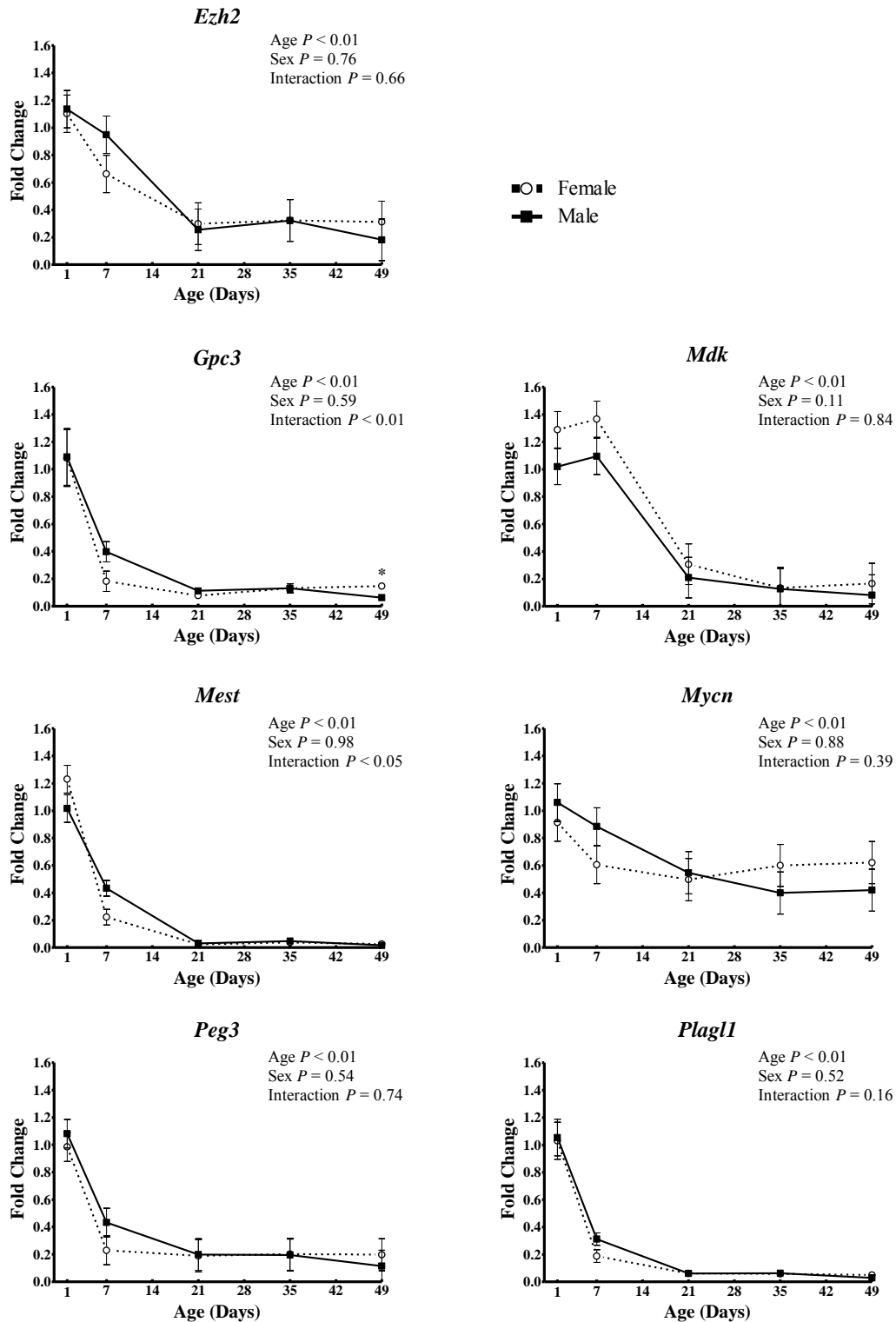


Figure 3. Expression of growth regulating genes in liver with age. Plotted values are least squares means displayed with standard error bars. Asterisks indicate a difference ($P < 0.05$) between sexes within age group. Fold change is compared to the average male 1-day-old liver expression.

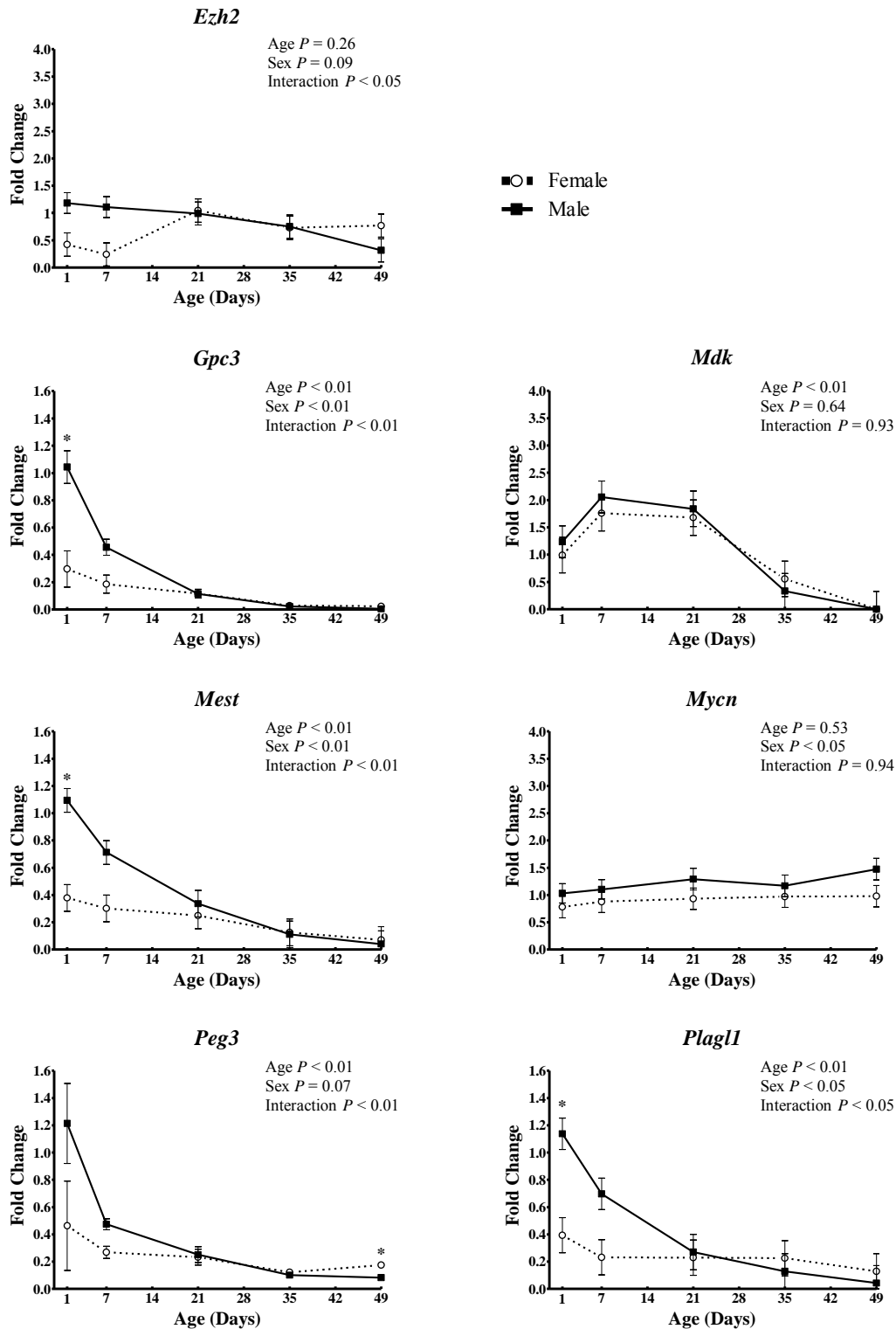


Figure 4. Expression of growth regulating genes in muscle with age. Plotted values are least squares means displayed with standard error bars. Expression of specific muscles for 21-49 days was pooled for analysis purposes. Asterisks indicate a difference ($P < 0.05$) between sexes within age group. Fold change is compared to the average male 1-day-old muscle expression.

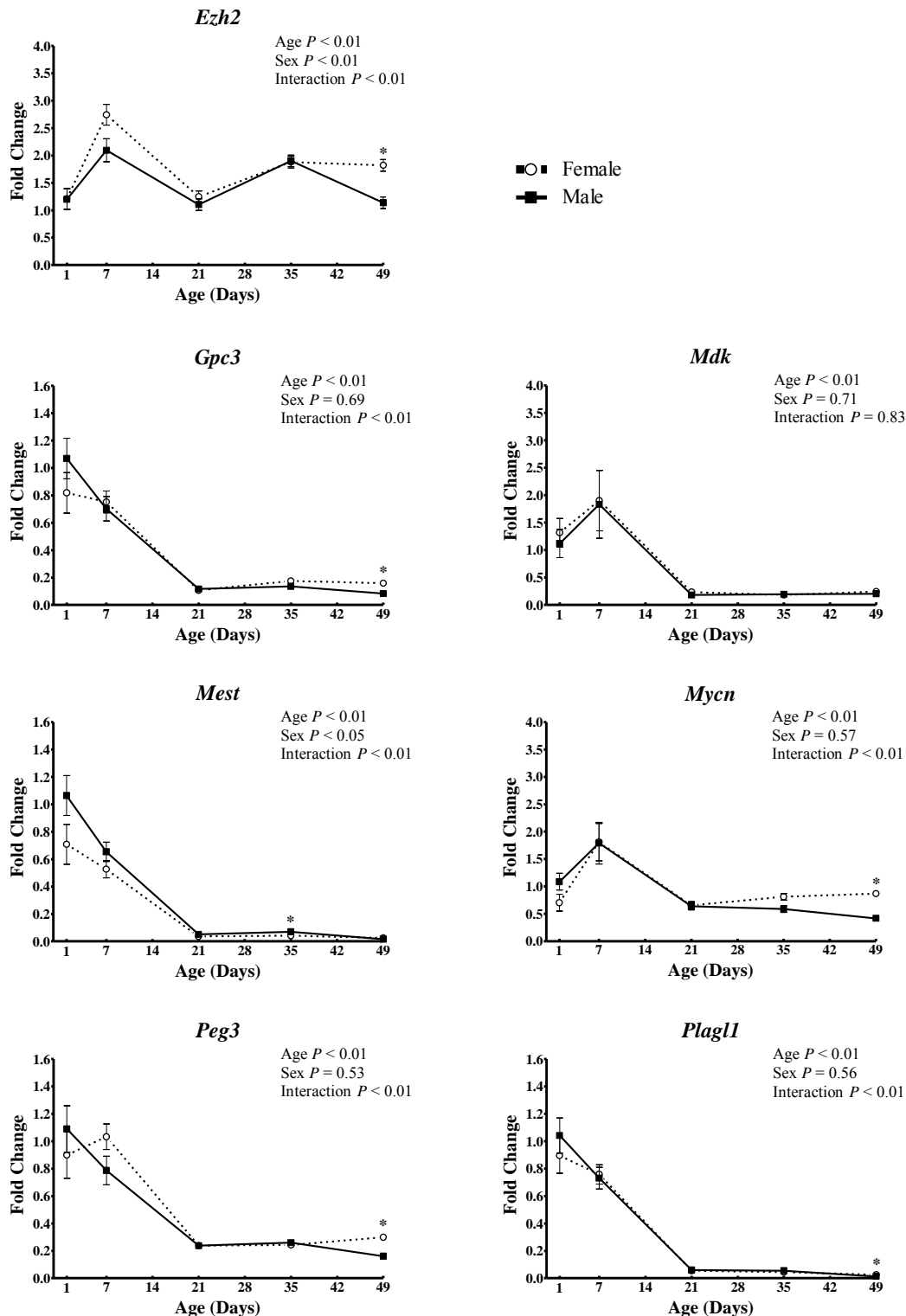
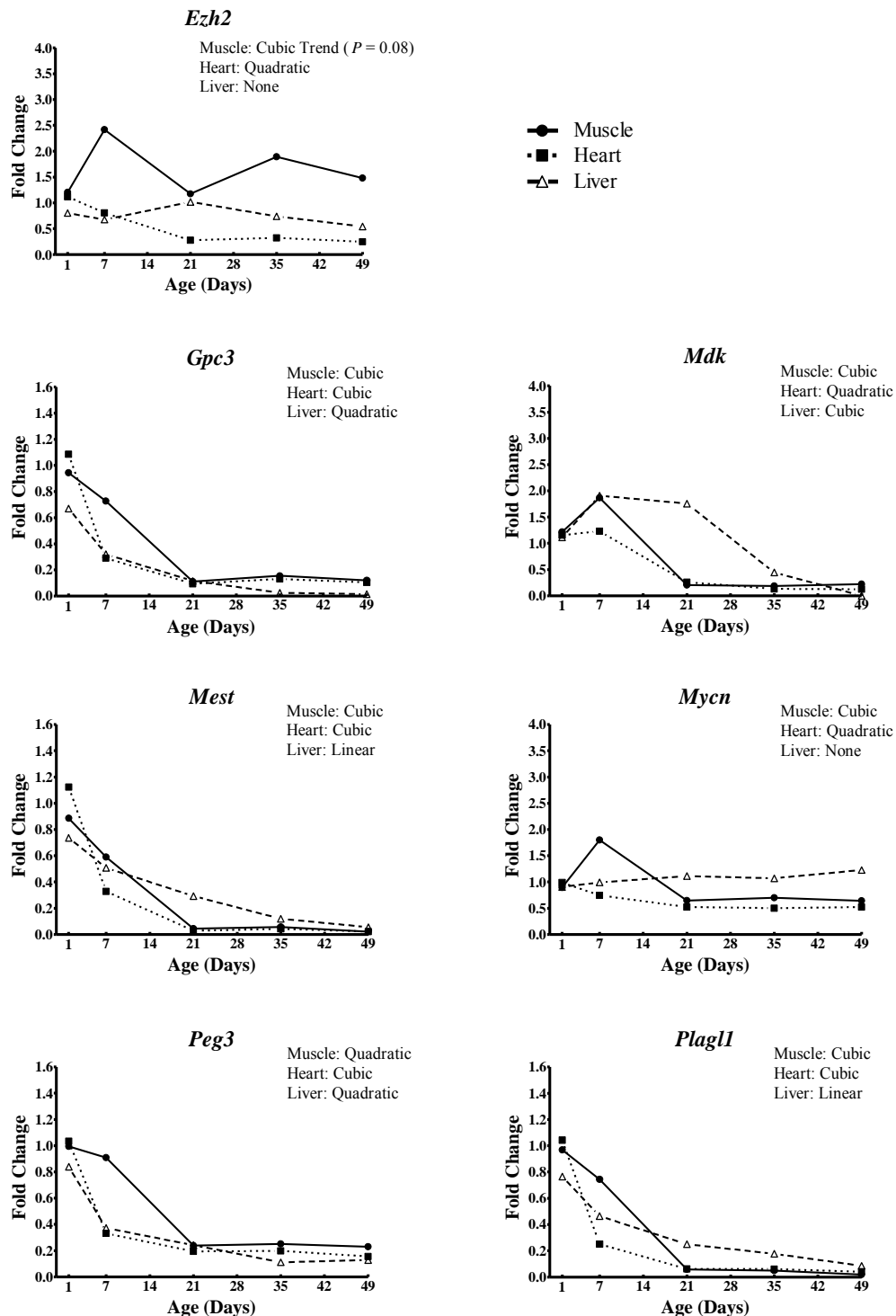


Figure 5. Comparison of expression between muscle, heart, and liver with age. Plotted values are least squares means. The pattern of expression is indicated by a linear, quadratic, or cubic decline. Male and female expression and expression of specific muscles for 21-49 days were pooled for analysis purposes. Fold change is compared to the average male 1-day-old expression for each tissue.



APPENDIX

Figure A.1. Age and specific muscle interaction for *Ezh2* expression. Plotted values are least squares means displayed with standard error bars. Fold change is compared to the average male 1-day-old muscle expression.

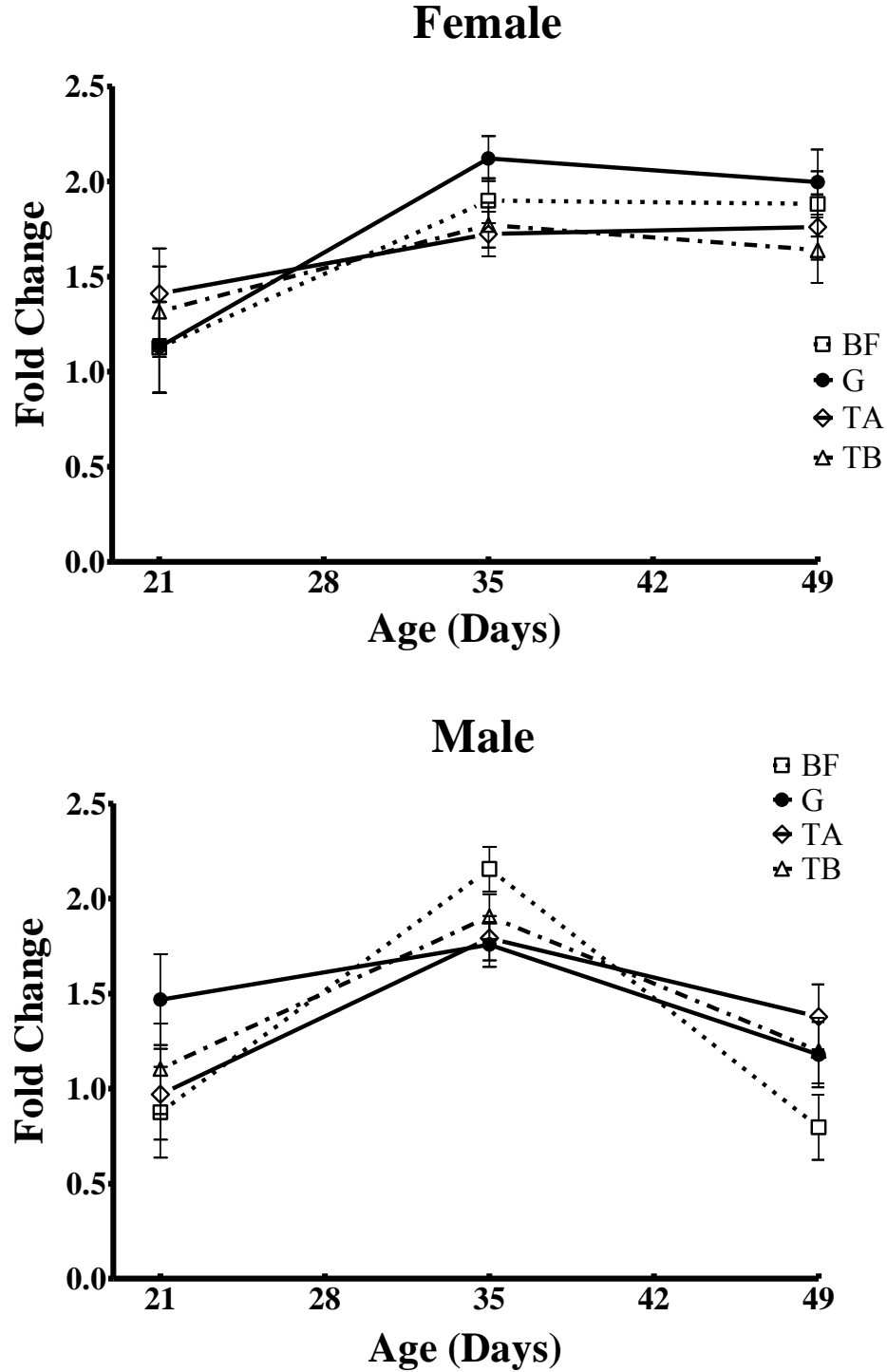


Figure A.2. Age and specific muscle interaction for *Mycn* expression. Plotted values are least squares means displayed with standard error bars. Fold change is compared to the average male 1-day-old muscle expression.

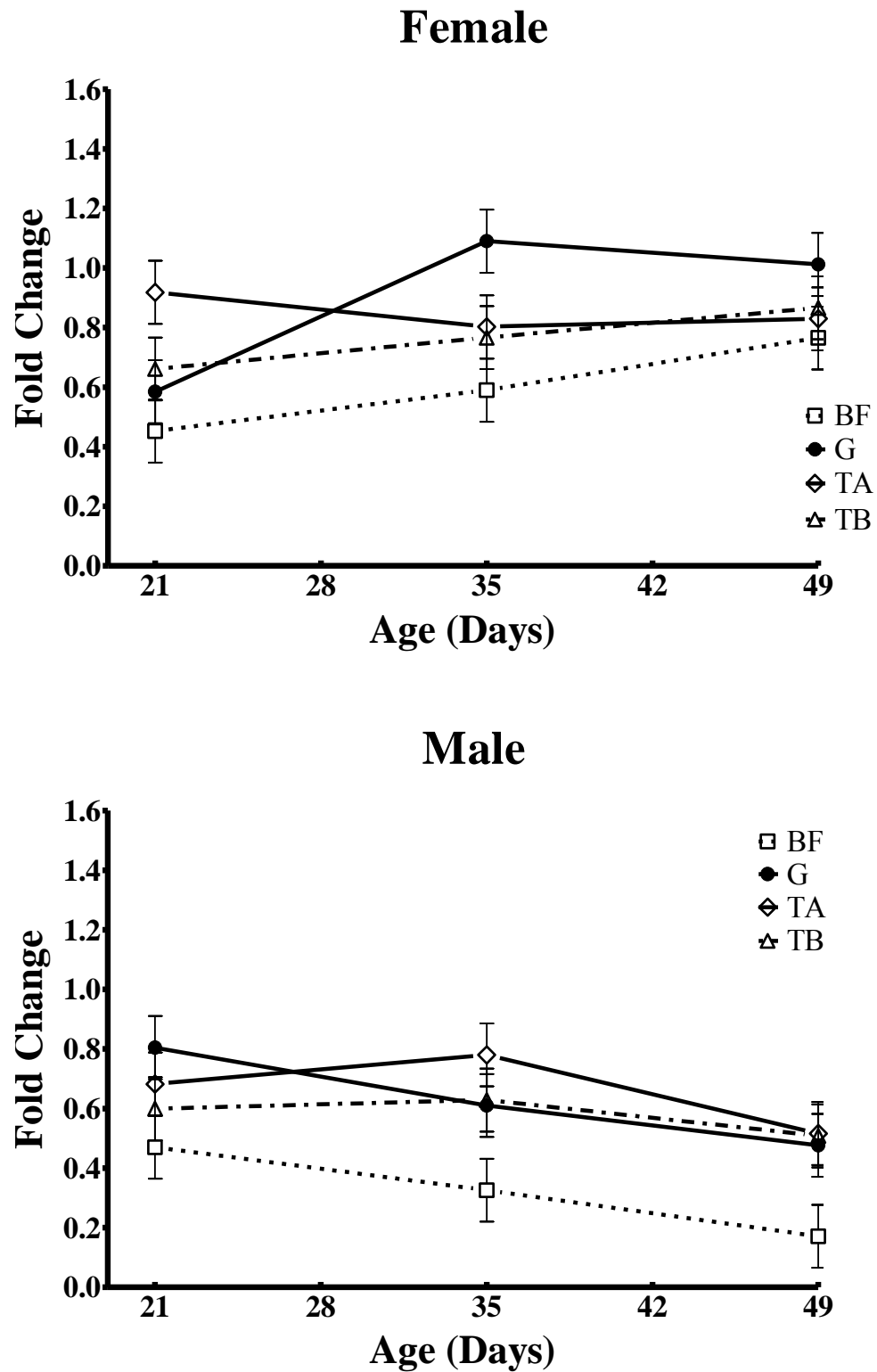


Figure A.3. Age and specific muscle interaction for *Gpc3* expression. Plotted values are least squares means displayed with standard error bars. Expression between sexes was pooled for analysis purposes. Fold change is compared to the average male 1-day-old muscle expression.

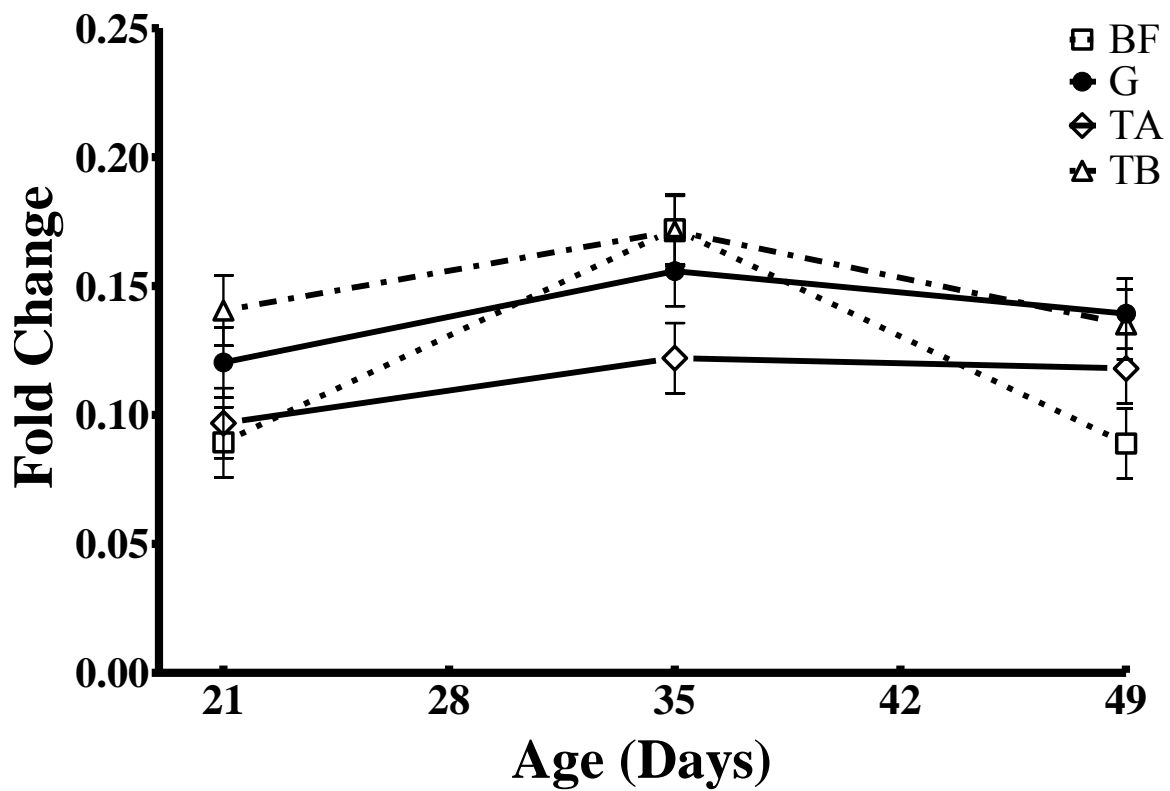


Figure A.4. Age and specific muscle interaction for *Peg3* expression. Plotted values are least squares means displayed with standard error bars. Expression between sexes was pooled for analysis purposes. Means at 35 and 49 days without a common letter differ ($P < 0.05$). Fold change is compared to the average male 1-day-old muscle expression.

